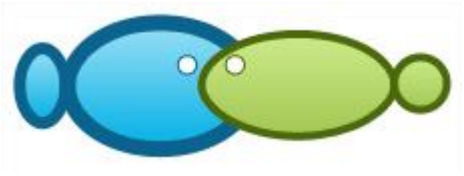

Efficacy of whole-cell and lipopolysaccharide vaccine of *Aeromonas hydrophila* on juvenile tilapia *Oreochromis niloticus* against motile aeromonad septicemia

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Abstract. *Aeromonas hydrophila* is a species of pathogenic bacteria that can infect all of the freshwater fish and causes motile aeromonad septicemia (MAS). The objective of this research was to determine the best vaccine substance among whole-cell, lipopolysaccharide (LPS), and the combination of those substances against MAS in juvenile tilapia *Oreochromis niloticus*. This research used tilapia with an average body length of 7.92±0.60 cm and average body weight of 7.49±1.63 g. The fish were then given an intraperitoneal injection with two vaccine substances and the combination of those substances, i.e., whole-cell, LPS, and the combination of whole-cell and LPS. Tilapia were further stocked and maintained in glass tanks at 60×30×30 cm^3 of size with an individual density of 10 per tank. Three weeks after vaccination, the juvenile tilapias were intramuscularly injected with 0.1 mL of *A. hydrophila* 10^5 CFU mL^{-1}. The hematology parameters were observed before stocking, after vaccination, and after a challenge test. The best vaccine substance was a combination of whole-cell and LPS with relative percent survival of 86.36%.

Key Words: vaccination, blood constituents, antibody, immunity, fish.

Introduction. One of the diseases that can attack tilapia is *motile aeromonad septicemia* (MAS) caused by *Aeromonas hydrophila* bacteria (Fernandez et al 2014). *A. hydrophila* is a species of Gram-negative bacteria and lives as normal flora in freshwater (Dehghani et al 2012). This species is non-specific to any hosts; thus it may attack all freshwater fish species. The impact starts from hemorrhage to death (Fu et al 2014).

Prevention of MAS is commonly done by applying probiotic, phytomedicine, and vaccine. The advantages of vaccine compared to probiotic and phytomedicine includes the ability to form and stimulate specific and non-specific immune system as well as creating long-term memory against pathogen attack (Longyant et al 2007; Barman et al 2013; Aaby et al 2014). Effect of vaccine protection will also occur even though vaccine substance in the fish body is no longer present.

Whole-cell and lipopolysaccharide (LPS) vaccine are widely used to prevent the attack of *A. hydrophila* (Dehghani et al 2012). The whole-cell vaccine is made by killing bacteria using 3% neutral buffered formalin. This vaccine can increase immunity by exposing the antigen of *A. hydrophila* whole-cell thus the fish immune system will form a specific antibody to this bacterium (Sugiani et al 2013). Lipopolysaccharide is a component of the outer membrane of Gram-negative bacteria causing bacteria to become virulent (Nya & Austin 2010). This molecule is amphiphilic and consists of lipid A, oligosaccharide, polysaccharide, and O-antigen (Merino et al 2015). Lipid A in lipopolysaccharide is endotoxin which can provide an immunomodulatory effect to host (Dehghani et al 2012).

A previous study showed that whole-cell and LPS vaccine of *A. hydrophila* were able to increase survival and immune response of various species of fish. Mulia (2007)

mentioned that gourami fish administered with whole-cell vaccine obtained a survival rate of 58%, while LPS resulted in the survival rate of 56%. Other study showed that LPS and whole-cell vaccine administration in rainbow trout resulted in survival of 60 and 80%, respectively, while control only reached a survival of 40% (Dehghani et al 2012). In addition to high survival rate, vaccination with whole-cell and LPS were also able to increase specific and nonspecific immune response in fish (Silva et al 2009; Bailone et al 2010; Dehghani et al 2012). This study was aimed to determine the most effective vaccine substance between whole-cell, LPS, and combination of those substances to prevent MAS disease in juvenile tilapia *Oreochromis niloticus*.

Material and Method

Time and place of experimental study. This study was conducted at Laboratory of Aquatic Organism Health, Department of Aquaculture, Bogor Agricultural University, Indonesia on March-July 2016.

Preparation of experimental animals. Fish used in this study were obtained from the Field Laboratory of Babakan, Faculty of Fisheries and Marine Science, Bogor Agricultural University. The fish was the output of tilapia strain Nirwana broodstock spawning from the Institute for Freshwater Fish Seed Development (BPBIAT) Wanayasa, West Java, Indonesia. Juvenile fish used had an average length of 7.92±0.60 cm and an average weight of 7.49±1.63 g.

Preparation of bacteria. An isolate of *A. hydrophila* (ATCC 49140) bacteria was obtained from the Laboratory of Aquatic Organism Health, Department of Aquaculture, Bogor Agricultural University. Bacteria were re-characterized using API 20E kit. The result of API 20E kit was read by using the online API WEB software. Characterization result showed that the bacteria identified were *Aeromonas hydrophila* bacteria.

Preparation of whole-cell vaccine. The production of the whole-cell vaccine was referred to Sugiani et al (2013) through a modification of the concentration of neutral buffered formalin (NBF). An isolate of bacteria produced from re-isolation was cultured in 50 mL of brain heart infusion broth (BHIB) media and further incubated for 24 hours at a temperature of 28-30ºC. Later, the total amount of bacteria on culture media was counted using the method of total plate count (TPC). The counting result (10^9 CFU mL^{-1}) was added with 38% NBF amounted to 3% of the volume of culture media then incubated for 24 hours. Moreover, cells were harvested by centrifugation at a speed of 5000 rpm for 30 minutes. The cell pellet was taken and washed twice using sterile phosphate buffered saline (PBS) through centrifugation after the first and second washing. The vaccine produced was further diluted with PBS to the initial volume. After vaccine preparation was completed, a viability test was performed by streaking vaccine candidate bacteria in brain heart infusion agar (BHIA) media. If bacteria did not grow, the vaccine was safe to administer.

Preparation of lipopolysaccharide (LPS) vaccine. Preparation of lipopolysaccharide vaccine was referred to the study of Fernandez et al (2014). An isolate of bacteria was cultured in BHIB media of 50 mL and incubated for 24 hours at a temperature of 28-30ºC. Cultured bacteria produced (10^9 CFU mL^{-1}) were further centrifuged at a speed of 1600 rpm for 20 minutes and diluted with PBS at a volume of 10 folds the volume of *A. hydrophila* cell. The cell of *A. hydrophila* was heated for 2 hours at a temperature of 100ºC then re-centrifuged at a speed of 1600 rpm for 20 minutes. Later, a pellet of *A. hydrophila* cell was diluted in 95% ethyl alcohol at a volume of 10 folds the pellet volume. The mix of *A. hydrophila* cell pellet and ethyl alcohol was incubated for four hours at a temperature of 37ºC. Pellet of *A. hydrophila* cell was washed using acetone and centrifuged, and then diluted with PBS to the initial volume.

Preparation of fish tank and maintenance. Fish tank in the form of 15 aquariums with the size of 60×30×30 cm was used in this study. Preparation of tank included aquarium cleaning, aeration setting, and disinfection of media. Aquariums were washed using tank water without detergent. After washing, the tank was filled with water to reach a height of 15 cm, and an aerator was put inside each aquarium. The water was further disinfected using chlorine ($Ca(ClO)_2$) of 30 mg L^{-1} for 24 hours. Neutralization of chlorine was done by adding sodium thiosulfate ($Na_2S_2O_3$) of 15 mg L^{-1}. Experimental fish were maintained in an aquarium at a stocking density of 10 fish/aquarium. Fish were fed three times a day to satiation with pellet contained protein of 32%. Water quality during the study was maintained with range: temperature of 27-28 °C, pH of 6.3-7.4, dissolved oxygen of 6.3-8.6 mg L^{-1}, and ammonia of 0.0001-0.0039 mg L^{-1}. Siphoning of aquarium and water exchange of 75% were conducted every three days.

Vaccination and challenge test. The study applied Completely Randomized Design and consisted of five treatments and three replications. Juvenile fish were vaccinated at the beginning of stocking with three different vaccine substances, namely whole-cell (FKC), lipopolysaccharide (LPS), and the combination of whole-cell and lipopolysaccharide (FKC+LPS) at a ratio of 50:50% (v/v). Treatment of control was divided into negative (K-) and positive (K+) control. Fish in control treatment were not vaccinated, yet they were injected with phosphate buffered saline (PBS). Vaccination was done through the method of intraperitoneal injection at a dose of 0.1 mL $fish^{-1}$ (Aly et al 2015). Three weeks after vaccination, challenge test was performed on treatments FKC, LPS, FKC+LPS, and K+ by intramuscularly injecting 0.1 mL of *A. hydrophila* 10^5 CFU mL^{-1} culture cell, while K- treatment was only injected by PBS. Juvenile mortality was further observed for one week after the challenge test and the relative percent survival was calculated (Amend 1981).

Fish hematology. Observation of fish blood constituents was conducted one-week post vaccination of the broodstock. The first step was taking blood samples from the caudal vein using a syringe. Assessments of the blood parameters were done for the total red blood cells and total white blood cells following the Blaxhall & Daisley (1973) procedure; hemoglobin content was assessed using the Sahli method with a haemometer (Wedemeyer & Yasutake 1977); hematocrit and phagocytic activity were assessed according to the method described by Anderson & Siwicki (1995); lysozyme activity (Hanif et al 2004); respiratory burst using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anions production as described by Anderson & Siwicki (1995); level of antibody was measured using the method of indirect enzyme-linked immunosorbent assay (ELISA) (Sumiati et al 2015).

Clinical sign. Observation of clinical sign in fish was done after a challenge test. The clinical sign was observed on the external fish organ by comparing fish infected with *A. hydrophila* and fish that were not infected by *A. hydrophila* (negative control). The clinical sign observed included the presence of inflammation and wound (Figure 1).

Data analysis. Data of parameter measurement result were tabulated using Microsoft Office Excel 2016. Data were further analyzed with ANOVA (analysis of variance) using the application of Minitab 16 at a confidence level of 95%. If ANOVA showed a significantly different result, Tukey post hoc test was performed. The parameter of relative percent survival, clinical sign, and water quality was descriptively analyzed.

Results

Mortality rate and relative percent survival. The mortality rates of the three vaccine treatments were significantly different from positive control ($p < 0.05$). The mortality rate of FKC+LPS treatment was significantly lower ($p < 0.05$) than that of LPS, yet it was not significantly different ($p > 0.05$) compared to FKC treatment. The highest survival

was obtained by treatment of combination of FKC+LPS. Relative percent survival of juvenile fish is presented in Table 1.

Table 1

Mortality rate and survival percent survival of juvenile tilapia one-week post challenge test

Treatment	*Mortality rate (%)*	*Relative percent survival*
K+	73.33 ± 11.55^{a}	0
K-	0.00 ± 0.00^{c}	-
LPS	40.00 ± 0.00^{b}	45.45
FKC	20.00 ± 10.00^{bc}	72.73
FKC+LPS	10.00 ± 0.00^{c}	86.36

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Level of antibody. Antibody level of FKC vaccine treatment and the combination of FKC+LPS significantly increased ($p < 0.05$) compared to the two control treatments one-week post vaccination. After the challenge test, the antibody level of FKC treatment and combination of FKC+LPS significantly increased ($p < 0.05$) along with the two control treatments. Antibody level of experimental fish is presented in Table 2.

Table 2

Antibody level of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	*Antibody level (optical density)*		
	Initial	*Post vaccination*	*Post challenge test*
K+	0.78 ± 0.05	0.78 ± 0.01^{bc}	0.88 ± 0.02^{cd}
K-	0.78 ± 0.05	0.76 ± 0.11^{c}	0.84 ± 0.02^{d}
FKC	0.78 ± 0.05	0.96 ± 0.03^{a}	0.99 ± 0.03^{ab}
LPS	0.78 ± 0.05	0.91 ± 0.06^{ab}	0.95 ± 0.04^{bc}
FKC+LPS	0.78 ± 0.05	0.96 ± 0.05^{a}	1.06 ± 0.04^{a}

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Total leukocyte. Total leukocyte in the treatment of FKC and FKC+LPS significantly increased ($p < 0.05$) compared to control and LPS one-week post vaccination. One week after the challenge test, total leukocyte in the treatment of combination of FKC+LPS also significantly increased ($p < 0.05$) compared to both control treatments and LPS. Total leukocyte of experimental fish is presented in Table 3.

Table 3

Total leukocyte of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	*Total leukocyte ($\times 10^5$ cell mm^{-3})*		
	Initial	*Post vaccination*	*Post challenge test*
K+	0.96 ± 0.09	1.14 ± 1.11^{b}	1.64 ± 0.15^{b}
K-	0.96 ± 0.09	1.13 ± 1.02^{b}	1.13 ± 0.05^{c}
FKC	0.96 ± 0.09	1.48 ± 1.14^{a}	1.90 ± 0.18^{ab}
LPS	0.96 ± 0.09	1.19 ± 0.03^{b}	1.65 ± 0.09^{b}
FKC+LPS	0.96 ± 0.09	1.61 ± 0.11^{a}	2.13 ± 0.12^{a}

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Phagocytic activity. Phagocytic activity of treatment of FKC+LPS combination experienced a significant increase ($p < 0.05$) compared to both control treatments and LPS on the first week after vaccination. One week after challenge test, phagocytic activity of the treatment of combination of FKC+LPS significantly increased ($p < 0.05$) compared to both control treatments and LPS, yet it was not significantly different ($p > 0.05$) from FKC vaccine treatment. Phagocytic activity of experimental fish is presented in Table 4.

Table 4

Phagocytic activity of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	Phagocytic activity (%)		
	Initial	Post vaccination	Post challenge test
K+	15.97±0.06	22.08±2.89^{b}	23.34±1.53^{bc}
K-	15.97±0.06	21.92±2.61^{b}	20.37±0.63^{c}
FKC	15.97±0.06	27.82±1.34^{ab}	29.25±1.64^{ab}
LPS	15.97±0.06	22.20±3.48^{b}	26.68±2.41^{bc}
FKC+LPS	15.97±0.06	31.37±1.34^{a}	33.97±4.76^{a}

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Respiratory burst. The value of respiratory burst did not show a significantly different effect ($p > 0.05$) between the treatment of vaccination and both control treatments one-week post vaccination. One week after the challenge test, a respiratory burst of treatment of FKC and combination of FKC+LPS significantly increased ($p < 0.05$) compared to both control treatments. Respiratory burst of experimental fish is presented in Table 5.

Table 5

Respiratory burst of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	Respiratory burst (Optical density)		
	Initial	Post vaccination	Post challenge test
K+	0.22±0.01	0.26±0.01^{a}	0.26±0.00^{b}
K-	0.22±0.01	0.26±0.01^{a}	0.21±0.01^{c}
FKC	0.22±0.01	0.28±0.02^{a}	0.29±0.01^{a}
LPS	0.22±0.01	0.27±0.00^{a}	0.27±0.01^{b}
FKC+LPS	0.22±0.01	0.28±0.05^{a}	0.30±0.00^{a}

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Lysozyme activity. Lysozyme activity in vaccination treatment was not significantly different ($p > 0.05$) compared to the two control treatments after vaccination. One week after the challenge test, lysozyme activity of vaccine treatment of FKC and combination of FKC+LPS experienced a significant increase ($p < 0.05$) compared to the two control treatments. Lysozyme activity of experimental fish is presented in Table 6.

Table 6

Lysozyme activity of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	Lysozyme activity (Unit mL^{-1})		
	Initial	Post vaccination	Post challenge test
K+	21.56±6.02	23.93±5.25^{a}	29.67±2.65^{b}
K-	21.56±6.02	24.52±6.67^{a}	27.44±1.17^{b}
FKC	21.56±6.02	31.04±7.39^{a}	42.67±3.46^{a}
LPS	21.56±6.02	25.93±2.86^{a}	34.89±3.08^{ab}
FKC+LPS	21.56±6.02	29.33±2.52^{a}	38.89±5.00^{a}

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Total erythrocytes. Total erythrocyte of vaccination treatment did not show a significant difference ($p > 0.05$) than that of the two control treatments one-week post vaccination. After the challenge test, total erythrocyte on treatments FKC, LPS, FKC+LPS and K- were significantly higher ($p < 0.05$) compared to the treatment of K+. Total erythrocyte of experimental fish is presented in Table 7.

Table 7

Total erythrocyte of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	Total erythrocyte ($\times 10^6$ cell mm^{-3})		
	Initial	Post vaccination	Post challenge test
K+	1.63±0.02	1.82±0.13[a]	1.16±0.12[b]
K-	1.63±0.02	1.75±0.17[a]	1.90±0.12[a]
FKC	1.63±0.02	1.93±0.10[a]	1.64±0.11[a]
LPS	1.63±0.02	1.93±0.26[a]	1.60±0.06[a]
FKC+LPS	1.63±0.02	2.05±0.13[a]	1.80±0.13[a]

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Hemoglobin level. Hemoglobin level one week after vaccination was found to be not significantly different ($p > 0.05$) in all treatments. One week after challenge test, on treatments FKC, LPS, FKC+LPS and K- obtained significantly higher hemoglobin level ($p < 0.05$) compared to treatment of K+, yet it was not significantly different ($p > 0.05$) from vaccine treatment of FKC, LPS, and the combination of both substances. Hemoglobin level of experimental fish is presented in Table 8.

Table 8

Hemoglobin level of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	Hemoglobin level (g dL^{-1})		
	Initial	Post vaccination	Post challenge test
K+	4.23±0.51	5.53±0.31[a]	3.07±0.20[b]
K-	4.23±0.51	5.13±0.42[a]	5.13±0.61[a]
FKC	4.23±0.51	5.80±0.20[a]	4.80±0.20[a]
LPS	4.23±0.51	5.67±0.70[a]	4.40±0.40[a]
FKC+LPS	4.23±0.51	6.20±0.72[a]	4.33±0.58[a]

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Hematocrit. The hematocrit of experimental fish did not show significantly different one week after vaccination ($p > 0.05$). One week after challenge test, treatment of combination of FKC+LPS had a significantly higher level of hematocrit ($p < 0.05$) compared to the positive control, yet it was not significantly different ($p > 0.05$) from the treatment of single vaccine of FKC, LPS, and negative control. The hematocrit of experimental fish is presented in Table 9.

Table 9

The hematocrit of experimental fish at the beginning of stocking, one-week post vaccination, and one-week post challenge test

Treatment	Hematocrit (%)		
	Initial	Post vaccination	Post challenge test
K+	25.49±2.22	33.71±2.67[a]	20.21±1.27[b]
K-	25.49±2.22	31.30±2.66[a]	29.92±4.43[a]
FKC	25.49±2.22	35.41±4.36[a]	26.82±2.87[ab]
LPS	25.49±2.22	36.45±5.81[a]	22.44±2.93[ab]
FKC+LPS	25.49±2.22	35.57±1.59[a]	30.71±4.61[a]

The different superscript letter within the same column shows a significantly different effect ($p < 0.05$).

Clinical sign. Clinical sign found in experimental fish was bleeding (hemorrhage). In general, the clinical sign was seen on the second day after a challenge test. A clinical sign of fish infected with *A. hydrophila* is presented in Figure 1.

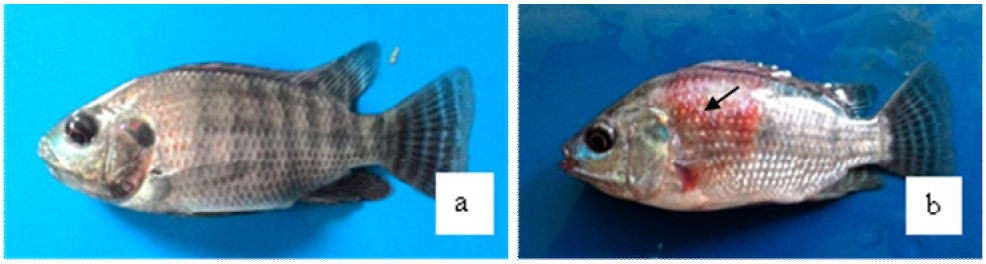

Figure 1. A clinical sign of tilapia infected with *A. hydrophila*. Fish of negative control (a), infected fish showed hemorrhagic (b).

Discussion. Vaccination with whole-cell and LPS of *A. hydrophila* was able to decrease the mortality rate of tilapia due to MAS disease as seen from the significantly low mortality rate of the three vaccine treatments ($p < 0.05$) compared to positive control. Of the three vaccination treatments, mortality rate resulted from a vaccine made of the combination of whole-cell and LPS (10.00±17.32) was significantly lower ($p < 0.05$) compared to the treatment of LPS vaccine (40.00±0.00), yet it was not significantly different from whole-cell treatment (20.00±10.00). This result was in line with Dehghani et al (2012) who mentioned that mortality rate of rainbow trout administered with a whole-cell vaccine of *A. hydrophila* and bivalent vaccine of *A. hydrophila* and *A. veronii* showed a mortality rate of 20%. The mortality rate of whole-cell and bivalent vaccine in that study was lower than that of LPS vaccine with a mortality rate of 40%. Ismail et al (2010) reported that vaccination with whole-cell in tilapia only resulted in the mortality rate of 8%.

Vaccination in this study was also able to increase fish protection level against MAS disease which was indicated by the relative percent survival. Of the three vaccine treatments, relative percent survival of vaccine produced from the combination of whole-cell and LPS (86.36%) was higher than that of the single vaccine of LPS (45.45%) and single vaccine of whole-cell (72.73%). This finding was by the study of Dehghani et al (2012) that relative percent survival of rainbow trout given whole-cell vaccine of *A. hydrophila* and bivalent vaccine through immersion reached 67%. This relative percent survival was higher if compared to the value obtained by the LPS vaccine of 34%. Other studies also showed that tilapia vaccination with LPS of *A. hydrophila* only resulted in relative percent survival of 27-58% (Fernandez et al 2014). Increase in relative percent survival of fish vaccinated was mainly due to the formation of nonspecific immune response followed by an increase in specific immunity (Sukenda et al 2015).

Antibody level is the main parameter to measure the specific immune response (Mashoof & Criscitiello 2016). At week-3 after vaccination, the three vaccine treatments showed an increase in specific antibody against *A. hydrophila* (Table 2). Treatment of whole-cell single vaccine and vaccine of a combination of whole-cell and LPS indicated a significant increase ($p < 0.05$) compared to positive and negative control. No significant difference ($p > 0.05$) was found between the three vaccine treatments. Sumiati et al (2015) reported that antibody level in tilapia administered with a whole-cell vaccine of *A. hydrophila* started to significantly increase compared to control at week-2 post vaccination. Among the three vaccine treatments, a combination of whole-cell and LPS (1.06±0.04) was found to have a significantly higher antibody level compared to LPS treatment (0.95±0.04). The result of a study conducted by Dehghani et al (2012) indicated that rainbow trout vaccinated with whole-cell had the optical density for antibody level of 1.10, while LPS treatment only produced an optical density of 0.92. The reason for high antibody level in vaccination with whole-cell and combination of whole-cell and LPS was the higher availability of antigen in whole-cell than in LPS. In addition to LPS, a component of *A. hydrophila* cell that has antigenic characteristic and may produce an immune response is protein (Viji et al 2013; Lacerda et al 2015).

One week after vaccination, there was a significant increase in total leukocyte ($p < 0.05$) in the treatment of whole-cell vaccine and the combination vaccine compared to control (Table 3). One week after the challenge test, a significant increase in total leukocyte ($p < 0.05$) was found in the treatment of combination vaccine compared to

positive control. Parameter strongly related to total leukocyte is phagocytic activity. A significant increase in phagocytic activity ($p < 0.05$) compared to control was observed in the treatment of the combination vaccine after vaccination and challenge test (Table 4). Phagocytic activity increase in vaccine made of the combination of whole-cell and LPS compared to control was caused by a significant increase in leucocyte level in this treatment compared to both controls. Treatment of whole-cell vaccine obtained a not-significantly different phagocytic activity from control since total leukocyte in this treatment was also not significantly different from control.

Respiratory burst is a production process of reactive oxygen species (ROS) resulted from phagocytic cell to kill pathogens that enter the body (Uribe et al 2011). The value of the respiratory burst of tilapia did not show a significant increase ($p > 0.05$) one week after vaccination (Table 5). A similar result was also reported by Aly et al (2015) that the value of respiratory burst in tilapia administered with a whole-cell vaccine of *A. hydrophila* had not shown a significant increase during the first week after vaccination. A significant increase in respiratory burst occurred at week-2 post vaccination. One week after the challenge test, respiratory burst in the treatment of whole-cell vaccine and the combination of whole-cell and LPS significantly increased ($p < 0.05$) compared to both controls. The result of a study carried out by Purwaningsih et al (2014) indicated that gourami fish given whole-cell vaccine of *A. hydrophila* obtained the higher value of respiratory burst compared to control after a challenge test. Value of respiratory burst in the treatment of combination vaccine was significantly higher compared to control since total leukocyte and phagocytic activity were also significantly higher than both controls.

Lysozyme is a bacteriolytic enzyme produced in lysosome of the phagocytic cell (Uribe et al 2011). In this study, lysozyme activity in vaccine treatment did not show a significant increase ($p > 0.05$) compared to control one week after vaccination (Table 6). Silva et al (2009) reported that the activity of an antimicrobial substance in tilapia injected with *A. hydrophila* vaccine did not increase three weeks after vaccination. One week after the challenge test, lysozyme activity in vaccine treatment of whole-cell and combination of whole-cell and LPS experienced a significant increase ($p < 0.05$) compared to control (Table 6). Lysozyme increase in treatment of combination vaccine was caused by a significant increase in total leukocyte after a challenge test. Increasing lysozyme activity in whole-cell treatment occurred because total leukocyte in whole-cell treatment after the challenge test was not significantly different from the combination vaccine.

One week after vaccination, the value of total erythrocyte did not show a significant difference ($p > 0.05$) between treatments of vaccine and both controls (Table 7). Sugiani et al (2013) mentioned that the total erythrocyte of tilapia given *A. hydrophila* vaccine was not significantly different from control one week after vaccination. Total erythrocyte in all vaccine treatments was significantly higher than that in positive control one week after a challenge test. Total erythrocyte in positive control significantly decreased because *A. hydrophila* had hemolysin that can cause erythrocyte lysis (Mangunwudoyo et al 2009). Treatment of vaccination had a significantly higher count of total erythrocyte compared to positive control for its high level of antibody. According to Reece et al (2014), the antibody may bind to antigen on the bacterial cell surface, thus blocking the bacterial recognition site to its host and facilitates phagocytosis process. These three vaccine treatments did not show a significant difference since the protection effect produced by specific and nonspecific immune system between the three treatments was similar even though vaccine of whole-cell and combination had several higher immune parameter compared to LPS vaccine. Normal count of total erythrocyte in tilapia ranges of $1.7 \pm 0.45 \times 10^6$ cell mm^{-3} (Lourenço et al 2012).

Hemoglobin is a pigment in erythrocyte and has a function to bind oxygen to be further distributed to all over the body (Reece et al 2014). Level of hemoglobin (Table 8) and hematocrit (Table 9) in vaccination treatment were not significantly different ($p > 0.05$) from both controls after vaccination was performed. Aly et al (2015) and Sukenda et al (2017) reported that the levels of hemoglobin and hematocrit of tilapia broodstock one week after the administration of vaccine were not significantly different from control. One week after the challenge test, hemoglobin level of vaccine treatment was

significantly higher ($p < 0.05$) compared to positive control. This was due to the low protective effect of the immune system in negative control which led *A. hydrophila* to actively release erythrocyte and further declined hemoglobin level in blood. The three vaccine treatments showed protection of specific and nonspecific immune system, resulted in a low intensity of *A. hydrophil*a that led to erythrocyte lysis, caused the hemoglobin level remained high. Hemoglobin level of the three vaccine treatments were not significantly different since total erythrocyte of the three treatments were also not significantly different. Normal hemoglobin level of fish according to Lourenço et al (2012) is 5.8±1.04 g dL^{-1}, while hematocrit level is 26.3±4.3%.

Clinical sign caused by *A. hydrophila* in this study was in the form of bleeding (hemorrhage) at day-2 post-challenge test. This finding was in line with the study conducted by Hardi et al (2014) that clinical sign found in tilapia was a hemorrhage. This sign commonly appears 48 hours after intramuscular injection of *A. hydrophila* bacteria.

Conclusions. A single vaccination with whole-cell and lipopolysaccharide (LPS) of *A. hydrophila* was able to protect juvenile tilapia against MAS disease. The highest protection effect was found in the treatment of combination of *A. hydrophila* whole-cell and LPS, resulted in the relative survival rate of 86.36%.

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