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Efficacy of *Aeromonas hydrophila* formalin-killed cells and lipopolysaccharides vaccines in maternal immunity of tilapia broodstock and the offspring resistance

Efikasi vaksin formalin killed cells dan lipopolisakarida *Aeromonas hydrophila* pada imunitas maternal induk ikan nila dan ketahanan benih yang dihasilkannya

Rahma Mulyani[1], Sukenda[2], Sri Nuryati[2]

[1]Graduate School of IPB University, Study Program of Aquaculture Science
[2]Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, West Java, Indonesia 16680
*Corresponding author: sukenda@ipb.ac.id

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ABSTRACT

The study aimed to analyze the efficacy of *Aeromonas hydrophila* formalin-killed cells (FKC) and lipopolysaccharides (LPS) vaccines in maternal immunity of tilapia broodstock and the resistance of the offsprings. This study consisted of two consecutive studies. Firstly, the broodstocks were vaccinated with PBS (non-vaccinated), FKC, LPS, and combinations of FKC and LPS. Secondly, the offsprings from both the vaccinated and non-vaccinated broodstock were vaccinated with the same vaccine for broodstock by immersion method. After vaccination, the offsprings were challenged with pathogenic *A. hydrophila*. Antibody level and lysozyme were measured in the broodstock, egg, and offsprings. In the first experiment, the result showed that vaccinated broodstock and their offsprings had delivered a significant antibody level and lysozyme activity compared with the control. The highest relative percent survival (RPS) of offspring resulted from the combination of FKC and LPS vaccinated broodstock at 5, 10, and 15 days post-hatching at 85.00%, 75.23%, and 67.56%, respectively. The second experiment showed vaccinated offsprings produced from vaccinated broodstock had a higher RPS than the RPS of vaccinated offsprings which produced from non-vaccinated broodstock. In conclusion, vaccination that using a combination of FKC and LPS vaccine in broodstock potentially improved the maternal immunity and protect their offspring from *A. hydrophila* infection.

Keywords: *Aeromonas hydrophila*, antibody, maternal immunity, tilapia, vaccination

ABSTRAK

Penelitian ini bertujuan menganalisis efikasi vaksin formalin-killed cells (FKC) dan lipopolisakarida (LPS) *A. hydrophila* pada imunitas maternal induk ikan nila dan ketahanan benih yang dihasilkan. Dalam penelitian ini, terdapat dua penelitian. Pertama, induk ikan nila divaksin dengan PBS (non-vaksinasi), vaksin FKC, LPS, dan kombinasi vaksin FKC dan LPS. Benih selanjutnya diuji ditantang dengan menggunakan patogen *A. hydrophila* pada 5, 10, dan 15 hari pasca menetas. Kedua, benih yang dihasilkan oleh induk yang telah divaksinasi dan induk yang tidak divaksinasi tersebut divaksin dengan vaksin yang sama seperti pada induk dengan metode imersi. Setelah vaksinasi, keturunan diuji tantang dengan patogen *A. hydrophila*. Tingkat antibodi dan lisozim diukur pada induk, telur, dan benih. Pada percobaan pertama, hasilnya menunjukkan bahwa induk yang divaksinasi dan benih menunjukkan tingkat antibodi dan aktivitas lisozim yang signifikan dibandingkan dengan kontrol. Persentase kelangsungan hidup relatif (RPS) tertinggi benih yang dihasilkan dari induk yang divaksinasi FKC dan LPS masing-masing pada 5,10, dan 15 hari pascatetas pada 85,00%; 75,23%; dan 67,56%. Percobaan kedua menunjukkan bahwa benih yang divaksinasi dari induk yang divaksin menunjukkan RPS yang lebih baik daripada benih yang divaksinasi dari induk yang tidak divaksin. Vaksinasi menggunakan kombinasi FKC dan LPS pada induk berpotensi meningkatkan kekebalan induk dan melindungi benih dari infeksi *A. hydrophila*.

Kata kunci: *Aeromonas hydrophila*, antibodi, imunitas induk, nila, vaksinasi

INTRODUCTION

Motile aeromonad septicemia (MAS) is a bacterial disease caused by *Aeromonas hydrophila* bacteria that is capable of attacking fish from fingerling to broodstock phase. This disease generally affects many freshwater fish species in tropical waters, one of which is tilapia *Oreochromis niloticus* (Rahmaningsih, 2012; Li *et al*., 2017). According to Dehghani *et al*. (2012), *A. hydrophila* bacteria live in freshwater as normal flora. *A. hydrophila* bacterium is capable of causing tissue swelling, stomach oedema, reddish body, necrosis, ulceration, and haemorrhagic septicemia in fish (Citarasu *et al*., 2011; Hardi & Pebrianto, 2012; Fu *et al*., 2014).

The development of disease management in fish culture nowadays tends to use preventive principle, such as vaccines. Vaccines can induce specific and nonspecific immunity of fish, besides protecting fish from certain pathogens in the long term period. Vaccination combined with good cultural management system practices can prevent disease attacks (Barman *et al*., 2013; Sugiani *et al*., 2013; Aaby *et al*., 2014; Amrullah *et al*., 2014). Fingerling quality stocked becomes one aspect that affects the success of tilapia culture (Nisaa *et al*., 2016). Vaccination given to larvae in preventing disease has not given maximum results due to body defense limitation system. Larvae have not been able to respond to the vaccine. Larvae have to withstand direct exposure to pathogenic bacteria in aquatic environments until the adaptive immune system is formed (Swain & Nayak, 2009; Zhang *et al*., 2013). Therefore, building immunity through maternal immune transfer in fish should be done to provide larval protection against pathogenic attacks as early as possible (Mingming *et al*., 2014; Zhang *et al*., 2014). Maternal immunity transfer can establish broodstock specific and non−specific immunity as well as providing protection to the offspring produced against *A. hydrophila* bacterial infection (Sukenda *et al*., 2017).

Some types of vaccine candidates from *A. hydrophila* intact bacterial cell supplies, such as formalin−killed cell (FKC) and lipopolysaccharides (LPS) have been used to prevent *A. hydrophila* (Dehghani *et al*., 2012). FKC is a vaccine from *A. hydrophila* bacterial intact cell containing bacteria and proteins by bacterial inactivation using BNF 3%. The results reported that FKC vaccine applied to tilapia broodstock gave RPS value 78.26% on day five to the offspring after challenged with *A. hydrophila* (Sukenda *et al*., 2017), while lipopolysaccharides were a molecular constituent of Gram-negative bacterial outer membranes that are toxic to fish, making the bacteria become virulent. This molecule is amphiphilic and composed of oligosaccharides, polysaccharides, O−antigen, and lipid−A in the form of acetylglucosamine phosphate fatty acid (Merino *et al*., 2015). LPS in bacteria are able to provide an immunomodulatory effect on the host (Dehghani *et al*., 2012).

The general purpose of this study was to determine effective vaccine substance between LPS, FKC, and combined LPS and FKC vaccine from *A. hydrophila* for motile aeromonas septicemia (MAS) prevention on tilapia fingerling. The specific purposes were to testify the main immunogenicity of vaccinated broodstock and offspring produced, fingerling protection and immunogenicity condition from vaccinated broodstock, and fingerling protection from vaccinated and unvaccinated broodstock.

METHODS

Research design

The study design consisted of two consecutive studies. The first study was female broodstock vaccination and the second study was offspring vaccination on day 20 of rearing from broodstock spawning on the first study.

Research procedures

Vaccine supply test

A vaccine tested in this study was formalin-killed cells (FKC) and lipopolysaccharides (LPS) vaccine supply from *A. hydrophila* bacteria. FKC vaccine or whole-cell vaccine was created by *A. hydrophila* inactivation using buffered neutral formalin (BNF) 3%. The generated whole-cell vaccine of *A. hydrophila* referred to Sugiani *et al*. (2013). *A. hydrophila* bacteria was cultured in trypticase soy broth (TSB) medium and incubated with shaker for 24 hours at 28°C. Total of bacteria in the culture medium was calculated using total plate count (TPC) method. The result of bacterial culture (10^9 CFU/mL) was inactivated by adding BNF 3% of culture medium volume and incubated for 24 hours at 28°C. Inactivated bacterial cell was harvested by centrifugation method with

5000 rpm for 30 minutes. Supernatant created was subsequently taken and washed twice using sterile phosphate-buffered saline (PBS). Washed supernatant was resuspended to the initial volume using sterile PBS.

After vaccine produced, viability test was done by streaking the vaccine candidate into TSA medium. Vaccine candidate that showed no growth was safe to be used. LPS vaccine was made based on Fernandez *et al*. (2014). *A. hydrophila* isolate was cultured in TSB media as much as 50 mL and incubated for 24 hours at 28°C. The result of bacterial culture (10^9 CFU/mL) was centrifuged at 1600 rpm for 20 minutes and injected with PBS ten times of product volume generated. Bacterial cells were heated for two hours at 100°C. Bacterial cells were centrifuged and discarded the supernatant. Bacterial cells were suspended in ethyl alcohol 95% ten times of cell liquidity and incubated at 37°C for 4 hours. Bacterial cells were washed with acetone and resuspended to the initial volume with PBS. After the vaccine production is completed, the viability test was done by streaking the vaccine candidate solution to trypticase soy agar (TSA). Vaccine candidate that showed no growth was safe to be used.

First study

Tilapia broodstock vaccination and spawning

Female tilapia *nirwana* strain weighing 197 ± 14.9 g vaccination was done after one week of ovulation period or at gonad maturation level two (TKG2) (Nisaa *et al*., 2016). The vaccine was injected intraperitoneally with 10^9 CFU/mL concentration at 0.4 mL/kg fish doze. Treatments applied were the injection of PBS (control), FKC vaccine, LPS vaccine, and 50:50 (V/V) combination of FKC and LPS (GAB). Natural spawning ratio between male and female broodstock was 1:3 conducted in a concrete tank with 2.5×2.5×1 m^3 size.

Tilapia offspring challenge test

Tilapia offspring challenge test was done by soaking the offspring into the solution containing *A. hydrophila* at the age of 5, 10, and 15 days after hatching with 10^7 CFU/mL concentration for 30 minutes. The percentage of mortality and relative percent survival (RPS) on each treatment with three times replication were calculated on the seventh day after vaccination.

Serum preparation sample from broodstock, egg liquid, and offspring body

Serum samples in this study were obtained by taking blood from the broodstock through the tail-end veins. Serum was collected by centrifuged 5000 rpm for 10 minutes at 4°C. Serum was separated into the microtube and stored at -20°C for antibody and lysozyme test. Eggs were taken after broodstock spawning, while the offspring samples were taken on 5, 10, 15, and 20 days after hatching. Ten eggs and five offsprings were homogenized each in PBS-T (PBS + 0.05% Tween-20) solution with 1:4 (g/v) ratio. Egg and offspring sample was centrifuged in 5000 rpm for 10 minutes at 4°C. The supernatant was separated into microtube and stored at -20°C for antibody and lysozyme level test.

Second research

Fingerling vaccination

Second stage vaccination from the first study on fingerling aged 20 days after hatching with K+ (unvaccinated fingerling from unvaccinated broodstock), K+FKC (unvaccinated fingerling from FKC vaccinated broodstock), K+LPS (unvaccinated fingerling from LPS vaccinated broodstock), K+GAB (unvaccinated fingerling from GAB vaccinated broodstock), FKC (FKC vaccinated fingerling from FKC vaccinated broodstock), LPS (LPS vaccinated fingerling from LPS vaccinated broodstock), and GAB (GAB vaccinated fingerling from GAB vaccinated broodstock). Fingerling weighing 0.16 ± 0.021 g was soaked in vaccine solution with 10^7 CFU/mL for 30 minutes as much as 20 fingerlings on each treatment replicated three times.

Tilapia fingerling challenge test

Tilapia fingerling reared for 11 days after vaccination was challenged with *A. hydrophyla* (10^7 CFU/mL) by soaking the fingerlings into the media containing a bacterial solution for 30 minutes. Soaked fingerlings were reared in an aquarium sized 30×20×20 cm^3 with 20 fingerlings in each aquarium. The percentage of mortality and relative percent survival (RPS) on fingerlings were calculated on 7 days after the challenge test.

Fingerling body liquid preparation

Fingerling body liquid was obtained from fingerlings in each treatment collected on 11 days after vaccination. Five fingerlings were

homogenized in PBS-T (PBS + 0.05% Tween-20) solution at 1:4 (g/v) ratio. Fingerling samples obtained were centrifuged in 5000 rpm for 10 minutes at 4°C. The supernatant was separated into a microtube and stored at -20°C for antibody and lysozyme level test.

Parameter

Relative percent survival (RPS)

Relative percent survival (RPS) was calculated using the following formula:

Fingerling mortality rate

$$\text{RPS (\%)} = \frac{\text{Mortality of vaccinated fish}}{\text{Mortality of control fish}} \times 100$$

Fingerling mortality rate was calculated after challenged with *A. hydrophila* using the following formula:

Antibody

$$\text{Mortality (\%)} = \frac{\text{Total dead fish}}{\text{Total population}} \times 100$$

Broodstock, egg, and fingerling antibody level were measured using indirect enzyme-linked immunosorbent assay (ELISA) method (Sumiati *et al*., 2015). Antigen concentration was measured using Bradford spectrophotometry method. A total of 100 µL antigen that has been diluted 1:1 using PBS (pH 7.2) was inserted in microplates well added with 100 µL carbonate-bicarbonate buffer (pH 9.6) on each well. Antigen was incubated at 4°C for 12 hours. Well was washed using PBS-T (PBS pH 7.4 + 0.05% Tween-20) and added with 100 µL bovine serum albumin (BSA, Sigma) 3% (w/v) in H_2O, then incubated at 25°C. The mixture was incubated for an hour and washed using PBS-T solution.

Broodstock blood serum was diluted in PBS-T (pH 7.2) 1:50 (v/v) solution, while egg and body liquid were diluted in PBS-T (pH 7.2) solution 1:16, then inserted into the microplates as much as 100 µL with three replications and incubated for an hour at 25°C, then washed using PBS-T. Long-chain anti-tilapia immunoglobulin for polyclonal specific antibody was diluted in PBS-T solution 1:200 and added as much as 100 µL to each microplate, then incubated for an hour at 25°C and washed using PBS-T solution twice. Subsequently, peroxidase-conjugated rabbit anti-rabbit (Sigma) was diluted in PBS-T to 1:5000 as much as 100 µL was added to each microplate well. The microplate was incubated at 25°C for an hour and washed. 100 µL one step Ultra TMB-ELISA (*Sigma*) (TMB 5 mg + 10 µl H_2O_2 38% in 5 mL buffer acetate pH 5), while blanko was added 50 µL H_2SO_4 3 M into the first well of microplate and incubated for 20 minutes at 25°C. ELISA reaction was stopped by adding 50 µL H_2SO_4 3 M into the well. Optical density (OD) of microplate was read using microplate reader (Kayto RT-2100C) on 450 nm absorbance. Antibody level was expressed in OD value.

Lysozyme activity

Lysozyme level on broodstock, egg, and fingerling was determined using the method of Hanif *et al*. (2005). Egg and fingerling body liquid as much as 100 µl were added with the liquid suspension of *Micrococcus lysodeikticus* (*Sigma*) as much as 100 µl (0.4 mg/mL in PBS pH 6.2) at 25°C. Lysozyme level was read using microplate reader (Kayto RT-2100C) with 450 nm absorbance for 30 seconds and 30 minutes mixing. The lysozyme activity unit was measured by decreased OD value of 0.001 per minute. Lysozyme activity was formulated below:

$$\text{Lysozyme activity (unit/mL)} = \frac{\frac{(\text{initial OD} - \text{final OD}) \times 1000}{\text{final time measurement}}}{\text{sample volume}}$$

Data analysis

This study used a completely randomized design method. Data obtained was collected using Microsoft Office Excel 2010 and analyzed using one way ANOVA method with SPSS 16.0 software application.

RESULT AND DISCUSSION

Result

First study

Mortality rate and relative percent survival (RPS) value on tilapia offspring

The mortality rate of offspring in GAB vaccinated broodstock treatment was lower than the control treatment and LPS on the fifteenth day, but offspring from GAB treatment did not differ significantly (P>0.05) with FKC treatment on the fifteenth day. RPS value of GAB and FKC treatment on offspring also showed no significant difference (P>0.05), but showing a significant difference to the control treatment on the fifteenth day. Mortality rate and RPS value are presented in Table 1.

Table 1. Mortality rate and RPS value on offspring 5, 10, 15 days after hatching

Offspring age (day after hatching)	Treatment	Mortality (%)	RPS (%)
5	Control	70.00 ± 5.00^{c}	-
	FKC	15.00 ± 2.8^{8a}b	77.77 ± 5.87^{ab}
	LPS	25.00 ± 5.00^{b}	65.00 ± 5.00^{a}
	GAB	10.00 ± 2.88^{a}	85.00 ± 5.35^{b}
10	Control	73.33 ± 1.66^{c}	-
	FKC	20.00 ± 5.77^{a}	72.69 ± 7.72^{b}
	LPS	36.66 ± 1.66^{b}	50.00 ± 1.92^{a}
	GAB	18.33 ± 6.00^{a}	75.23 ± 7.79^{b}
15	Control	76.66 ± 3.33^{c}	-
	FKC	26.00 ± 3.33^{a}	65.47 ± 4.39^{b}
	LPS	41.66 ± 4.40^{b}	45.83 ± 4.16^{a}
	GAB	25.00 ± 2.88^{a}	67.56 ± 2.64^{b}

Note: Different superscript letter on the same column shows significant different treatment (Duncan test; $P<0.05$). K+ (unvaccinated fingerling from unvaccinated broodstock), K+FKC (unvaccinated fingerling from FKC vaccinated broodstock), K+LPS (unvaccinated fingerling from LPS vaccinated broodstock), K+GAB (unvaccinated fingerling from GAB vaccinated broodstock), FKC (FKC vaccinated fingerling from FKC vaccinated broodstock), LPS (LPS vaccinated fingerling from LPS vaccinated broodstock), and GAB (GAB vaccinated fingerling from GAB vaccinated broodstock).

Table 2. Mortality rate and RPS value of fingerling after vaccination

Treatment	Mortality (%)	RPS (%)
K-	75.00 ± 2.88^{c}	-
K+FKC	56.66 ± 4.40^{b}	24.50 ± 4.48^{a}
K+LPS	65.00 ± 2.88^{cd}	12.60 ± 6.84^{a}
K+GAB	55.00 ± 2.88^{a}	26.60 ± 3.39^{a}
FKC	33.33 ± 6.00^{a}	56.00 ± 6.24^{bc}
LPS	43.33 ± 3.33^{a}	41.95 ± 4.92^{b}
GAB	31.66 ± 1.66^{a}	57.79 ± 1.13^{c}

Note: Different superscript letter on the same column shows significant different treatment (Duncan test; $P<0.05$). K+ (unvaccinated fingerling from unvaccinated broodstock), K+FKC (unvaccinated fingerling from FKC vaccinated broodstock), K+LPS (unvaccinated fingerling from LPS vaccinated broodstock), K+GAB (unvaccinated fingerling from GAB vaccinated broodstock), FKC (FKC vaccinated fingerling from FKC vaccinated broodstock), LPS (LPS vaccinated fingerling from LPS vaccinated broodstock), and GAB (GAB vaccinated fingerling from GAB vaccinated broodstock).

Antibody and lysozyme level of tilapia egg and broodstock

Antibody level of the egg (A) from FKC (OD: 0.56) and GAB (OD: 0.58) vaccinated broodstock treatment were higher than control and LPS treatment. Lysozyme activity of eggs (B) from FKC (76.22 units/ml) and GAB (75.88 units/ml) vaccinated broodstock showed different level from control and LPS, but the lysozyme activity of egg from LPS (39.11 unit/ml) vaccinated broodstock was higher than control treatment (25.51 units/ml). Antibody level formed after broodstock vaccination affected the antibody level of offspring generated from fifth to the fifteenth day. Offspring from treated broodstock showed decreased antibody level from fifth to the twentieth day (Figure 1).

GAB treatment (OD: 0.36–0.52) had the highest antibody level of offspring compared to control (OD: 0.29–0.26) and other treatments on the twentieth day. Lysozyme activity of broodstock vaccinated with GAB (117.77 unit/ml) and FKC (122.32 unit/ml) was higher than other treatments. Lysozyme activity (B) on control and LPS treatment of broodstock showed no difference. Offspring from treated broodstock

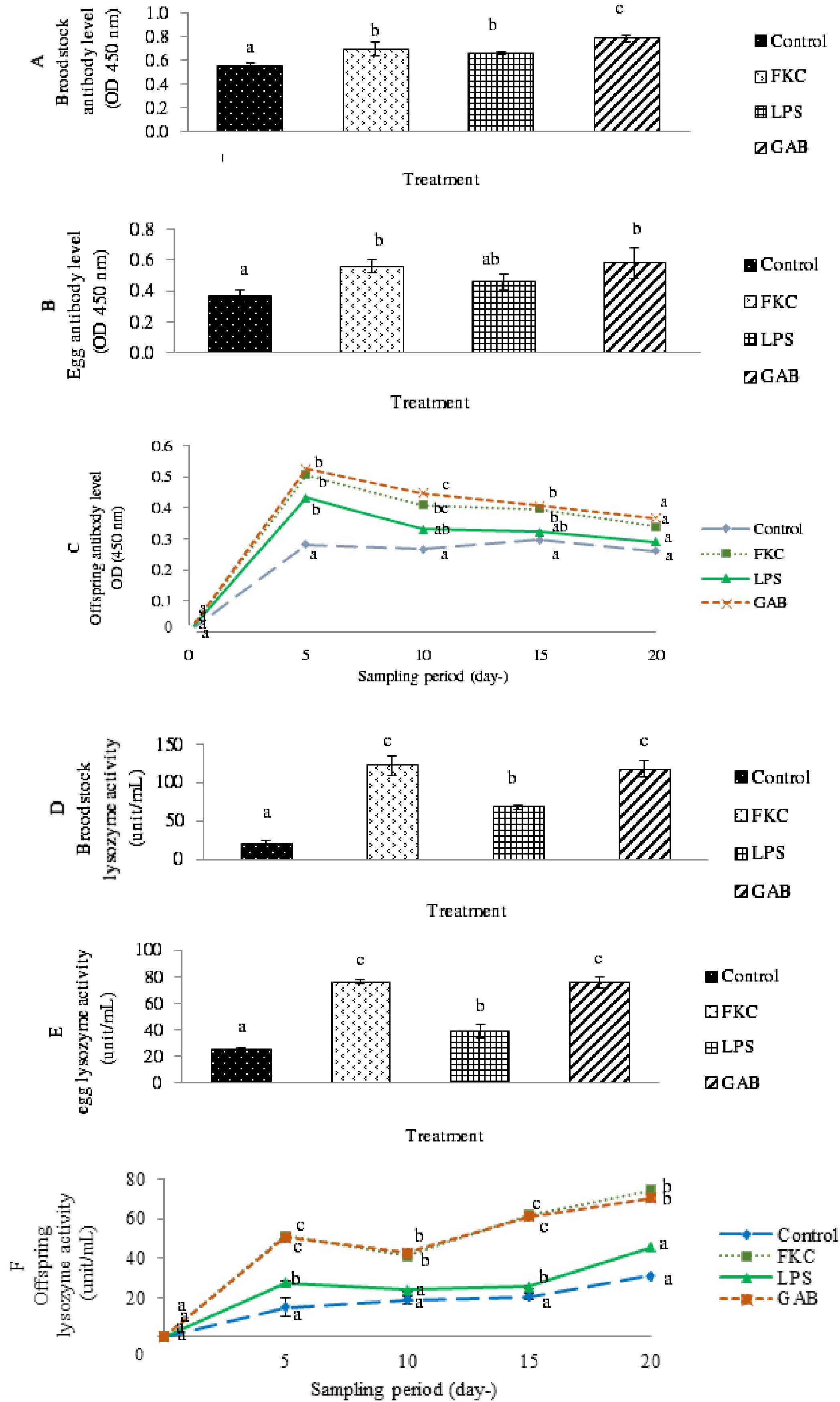

Figure 1. Antibody level of broodstock (A), egg (B), and offspring (C), lysozyme activity of egg (D), broodstock (E), and offspring (F) on each treatment

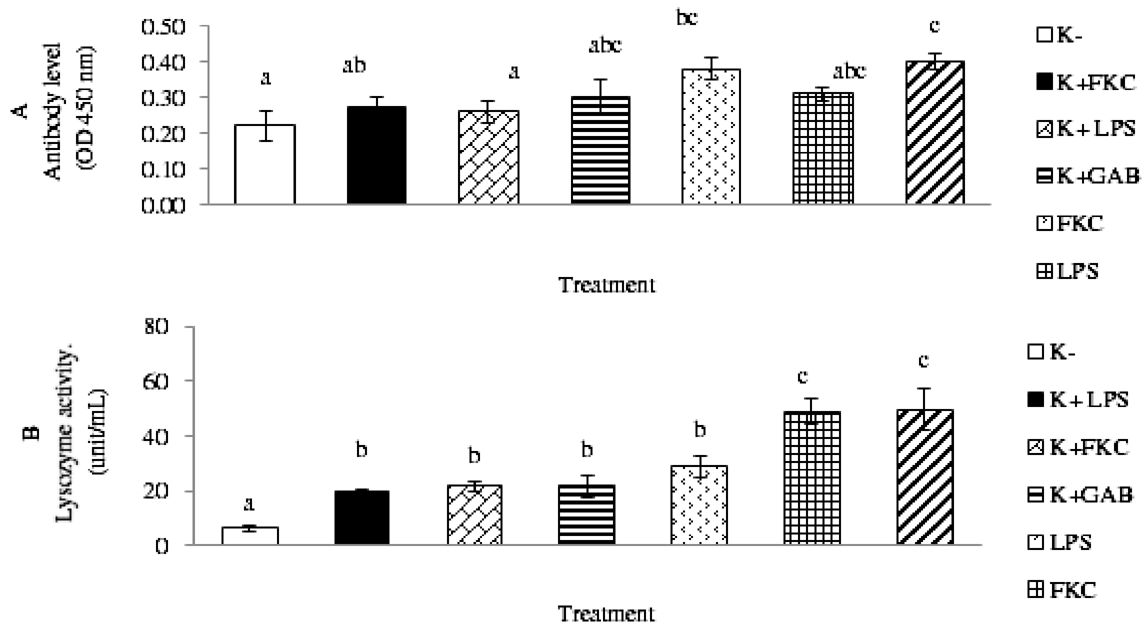

Figure 2. Antibody level (A) and lysozyme activity (B) of fingerling on each treatment. K+ (unvaccinated fingerling from unvaccinated broodstock), K+FKC (unvaccinated fingerling from FKC vaccinated broodstock), K+LPS (unvaccinated fingerling from LPS vaccinated broodstock), K+GAB (unvaccinated fingerling from GAB vaccinated broodstock), FKC (FKC vaccinated fingerling from FKC vaccinated broodstock), LPS (LPS vaccinated fingerling from LPS vaccinated broodstock), and GAB (GAB vaccinated fingerling from GAB vaccinated broodstock).

had decreased antibody level on the fifth to the twentieth day. Lysozyme activity of offspring from GAB treatment (70.22 units/ml) had the highest lysozyme activity compared to other treatments on fifth to twentieth day. Lysozyme activity of offspring from FKC and LPS treatment had significant different value compared to LPS and control (Figure 1).

Second study

Mortality rate and relative percent survival value of tilapia fingerling after vaccination

Mortality rate of GAB vaccinated fingerling was the lowest value among other treatments. Mortality rate of fingerling on negative control and unvaccinated treatments (K+GAB, K+FKC, and K+LPS) significantly showed the highest value (P<0.05) (Table 2). RPS value of positive control fingerling on FKC, LPS, and GAB vaccinated broodstock treatment did not significantly differ (P>0.05), however, differed significantly (P<0.05) against FKC, LPS, and GAB vaccination on fingerling treatment. LPS and FKC vaccination on fingerling did not show a significant difference, while GAB treatment had a significant difference in RPS value (P<0.05) with LPS.

Antibody level of fingerling after vaccination

GAB treatment showed a significant difference (P<0.05) against K-, K+LPS, and K+FKC treatment. Fingerling on the negative control (unvaccinated fingerling from unvaccinated broodstock), K+LPS, K+FKC, K+GAB (unvaccinated fingerling from LPS, FKC, and GAB vaccicated broodstock), and LPS treatment did not show the significantly different result (P>0.05) (Figure 2). Lysozyme activity of fingerling after vaccination on each treatment differed significantly with K− treatment. Lysozyme activity after FKC (48.66 units/ml) and GAB (49.66 units/ml) vaccination had no significant difference, but higher than other treatments. Positive control treatment on each vaccination treatment also showed significant difference results (P>0.05) ranging 19.55−21.77 unit/ml (Figure 2).

Discussion

Results showed that vaccinations on tilapia broodstock using formalin-killed cells (FKC) and lipopolysaccharides (LPS) vaccine gave a significantly better effect on the maternal immunity transfer from broodstock to offspring. Study on broodstock vaccination was able to

increase specific and nonspecific immunity, besides decreasing the mortality rate of fish offspring after challenge test (Swain *et al*., 2006; Mingming *et al*., 2014; Nisaa *et al*., 2016; Sukenda *et al*., 2017). Mortality percentage of offspring on 5,10, and 15 days after hatching obtained the highest rate at the control treatment, which was significantly different from offspring produced by vaccinated broodstock (P<0.05). This suggests that the vaccination treatment is able to provide protection as a passive immunity to the offspring better than other treatments, while mortality rate and RPS values of offspring from vaccinated broodstock showed that FKC and GAB treatment was better than the control treatment. This suggests that offspring produced from vaccinated broodstock is able to increase the immunity system, thus making them more resistant to *A. hydrophila* bacterial infection. In addition, vaccination on the broodstock before spawning gives an advantage to the offspring produced as it was able to suppress the mortality rate after challenged with *A. hydrophila* bacteria compared to offspring from unvaccinated broodstock. The same study result was reported by Sukenda *et al*. (2017), who stated that *A. hydrophila* whole-cell vaccine given to tilapia broodstock was able to suppress mortality rate on the offspring with 78.26% RPS value on 5 days after hatching. Another study showed that vaccinated offspring using FKC and GAB showed a good value of RPS (Sukenda, 2018). According to Swain and Nayak (2009), sustaining fish broodstock immunity at high level during vitellogenesis and oogenesis processes was important to suppress mortality rate on the larval phase or post-larvae through the maternal immunity transfer. Higher RPS value in GAB treatment than control treatment was also supported by antibody level and lysozyme activity data.

Results on the antibody level of broodstock after vaccination differed significantly compared to the control treatment (P<0.05). This suggests that vaccination to the broodstock will induce antibody level compared to the unvaccinated broodstock. Antibody level was a parameter for determining the specific immune response formed. Antibody level of vaccinated broodstock will be formed in the body one week after vaccination (Sukenda *et al*., 2018). The antibody in high concentration could be obtained from fish serum (Magnadottir, 2010). Antibody level in egg and offspring after vaccination were higher than control or unvaccinated treatment. The presence of antibody found in the egg was obtained from the maternal immunity transfer of the vaccinated parent (Nisaa *et al*., 2016). The presence of antibody in offspring plays an important role in protecting the larvae against environmental and pathogenic influences due to the limited capacity of offspring immune system (Vadstein *et al*., 2013). The cause of high-level antibodies in egg and offspring vaccination using FKC and GAB was the availability of more antigen in intact cells compared to LPS. In addition to LPS vaccine, *A. hydrophila* cell component is antigenic bacteria that can elicit an immune response as a protein (Lacerda *et al*., 2015).

Antibody level observed from vaccinated broodstock serum showed higher results compared to unvaccinated broodstock. The same result was reported by Sukenda *et al*. (2017), who stated that vaccinated broodstock with *A. hydrophila* a whole-cell vaccine was able to significantly increase the antibody level. Nisaa *et al*. (2016) and Pasaribu *et al*. (2018) also reported that the antibody level of broodstock after vaccination using *Streptococcus agalactiae* or bivalent vaccine of *S. agalactiae* and *A. hydrophila* increased compared to control treatment. The antibody was also found in the egg from vaccinated broodstock as allegedly because of the immunoglobulin transfer by broodstock naturally that was metabolized together with the yolk (Zhang *et al*., 2013).

In the gonad maturation process, environmental signals are received by the central nervous system and transmitted to the hypothalamus. Hypothalamus will release gonadotropin-releasing hormone (GnRH) which works in the pituitary gland. Pituitary releases FSH that works on theca cell and synthesizes testosterone. Granulosic layer that contains aromatase enzyme will convert testosterone into 17β-estradiol which stimulates the liver to synthesize vitellogenin (prospective yolk). Vitellogenin is carried by the bloodstream and absorbed by the follicle oocytes enlarged to the maximum size for vitellogenesis process. The pathway of this vitellogenesis process has the specific (antibody) and nonspecific (lysozyme) immunity to enter into the yolk to be transferred to the offspring (Swain & Nayak, 2009; Wang *et al*., 2011).

Nisaa *et al*. (2017) reported that tilapia fingerling antibody level originated from vaccinated broodstock decreased slowly until 28 days after hatching. The mechanism of antibody

formation after vaccination is caused by the contact between the antigen and lymphocytes (B cell and T cell). the antigen in the vaccine can directly induce the proliferation of B cell into the B memory cell and plasma cell. B memory cell will store the information about antigen characteristics entering the body, while the plasma cells will produce a specific antibody for antigen. An antigen that enters the body can also be phagocytized by macrophages. After phagocytized, the antigen will be exposed to T-helper cells through major histocompatibility complex (MHC) protein class II. T helper cells are activated by macrophages via cytokine molecules. Activated T-helper cells will produce cytokines that stimulate B cell, T memory cells, and cytotoxic T cells. When the same antigen exposure occurs for the second time, the T memory cell will stimulate B memory cell to proliferate into plasma cells and produce antibody (Reece *et al*., 2014).

The non−specific immune parameter is the lysozyme activity. Lysozyme activity is one of the bacteriolytic enzymes produced in lysosomes as phagocytes of fish body natural defense response during infection (Akbary *et al*., 2015; Wang & Zhang, 2010). Lysozyme activity of broodstock vaccinated with GAB (117.77 units/ml) and FKC (122.32 units/ml) increased significantly and were higher (P<0.05) than other treatments.

Swain and Nayak (2009) explained that vaccination improves non-specific and specific immune responses. Lysozyme is usually found in serum, homogenic seed, and mucous liquid as well as part of the fish body undergoing infection due to pathogenic attacks, such as skin and gills. Lysozyme acts in opsonization process that demonstrates the main inflammatory response due to the activation of the complement system and phagocytosis (Sugiani *et al*., 2012). The lysozyme activity of eggs and offspring also highly increased in the spawning process of vaccinated broodstock compared to egg and offspring produced from unvaccinated broodstock. Lysozyme activity has been found in egg and offspring because basically lysozyme appeared before, briefly, and after hatching to give immunity to fish fingerling (Magnadottir, 2006). Lysozyme activity on offspring from FKC, LPS, and GAB decreased on the tenth day and increased on the fifteenth day along with the offspring age. This was different from the lysozyme activity of offspring from broodstock control vaccine treatment that continued to increase from fifth to the twentieth day. Furthermore, lysozyme activity of fingerling after vaccination on positive control and vaccinated broodstock treatment did not differ significantly. However, FKC and GAB vaccination differed significantly with other treatments. Increased lysozyme activity of eggs and fingerlings was associated with the maternal immunity transfer from broodstock and induced the development of lymphoid organs such as kidneys, thymus, and spleen as increased immune transfer factor derived from broodstock (Swain & Nayak, 2009).

CONCLUSION

Vaccination using combined formalin-killed cells (FKC) and lipopolysaccharides (LPS) vaccine of *A. hydrophila* is able to provide induced maternal immunity to the offspring produced. Offspring immunity at the growth beginning was found in the offspring produced from vaccinated broodstock using combined FKC and LPS vaccine. Fingerling vaccination from vaccinated broodstock using combined FKC and LPS vaccine of *A. hydrophila* showed the best RPS value with 57.79%.

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