Efficacy of *Streptococcus agalactiae* vaccine strains N_3M and N_4M in fry tilapia infected by different strains of *S. agalactiae*

Efikasi vaksin *Streptococcus agalactiae* strain N_3M dan N_4M pada benih ikan nila yang diinfeksi strain *S. agalactiae* berbeda

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ABSTRACT

Streptococcus agalactiae is a major bacterial streptococcosis disease that infects tilapia. This study aimed to analyze a specific and nonspecific immune system in fry tilapia that has been given with *S. agalactiae* vaccine from N_3M and N_4M strain and examine the protective immunity against *S. agalactiae* N_3M, N_4M, $N_{17}O$, NK_1, and $N_{14}G$ strains infection. Fry tilapia used in this study has the weight of 7.086±0.948 g and length of 7.443±0.353 cm. The *S. agalactiae* strains that used were N_3M, N_4M, $N_{17}O$, NK_1, and $N_{14}G$. Fry tilapia was vaccinated through intraperitoneal injection method with 0.1 mL per fish of N_3M and N_4M vaccines. Fish reared in aquarium sizing of $60 \times 30 \times 50$ cm^3 with a density of 10 fishes/aquarium. Two weeks after vaccination, fry tilapia was tested with 0.1 mL/fish of each N_3M, N_4M, $N_{17}O$, NK_1, and $N_{14}G$ strains through intraperitoneal injection method. Antibody level measured with indirect enzym-linked immunosorbent assay (ELISA) method. The result of antibody level in vaccinated fish after vaccination of N_3M, N_4M vaccine, N_3M and N_4M control were 0.767; 0.743; 0.587; and 0.544, respectively. Relative percent survival in N_3M vaccinated fish after challenged with N_3M and N_4M was 87.50% dan 64.70%, respectively, otherwise in N_4M vaccinated fish was 62.50% dan 76.47%, respectively. N3M and N4M vaccine strain have better protection as only if it tested with similar bacteria strain.

Keywords: formalin-killed cell, tilapia, protection, *Streptococcus agalactiae*, strains

ABSTRAK

Streptococcus agalactiae merupakan bakteri utama penyakit streptococcosis yang menginfeksi ikan nila. Tujuan penelitian ini adalah menganalisis sistem imun spesifik dan nonspesifik pada benih ikan nila yang diberi vaksin *S. agalactiae* strain N_3M dan N_4M serta mengkaji imunitas protektif terhadap infeksi *S. agalactiae* strain N_3M, N_4M, $N_{17}O$, NK_1, dan $N_{14}G$. Benih ikan nila yang digunakan memiliki bobot 7,086 ± 0,948 g dan panjang 7,443 ± 0,353 cm. Bakteri yang digunakan adalah *S. agalactiae* strain N_3M, N_4M, $N_{17}O$, NK_1, dan $N_{14}G$. Benih ikan nila divaksinasi menggunakan metode injeksi pada bagian intraperitoneal sebanyak 0,1 mL/ekor vaksin N_3M dan N_4M. Pemeliharaan dilakukan pada akuarium berukuran $60 \times 30 \times 50$ cm^3 dengan kepadatan 10 ekor/akuarium. Dua minggu setelah vaksinasi benih ikan diuji tantang dengan strain bakteri N_3M, N_4M, $N_{17}O$, NK_1, dan $N_{14}G$ menggunakan metode injeksi pada bagian intraperitoneal dengan dosis 0,1 mL/ekor. Level antibodi diukur dengan metode *indirect enzym-linked immunosorbent assay* (ELISA). Hasil menunjukkan level antibodi ikan setelah vaksinasi pada perlakuan vaksin N_3M, N_4M, kontrol N_3M, dan kontrol N_4M berturut-turut 0,767; 0,743; 0,587; dan 0,544. Kelangsungan hidup relatif ikan yang divaksin N_3M setelah diuji tantang dengan strain N_3M dan N_4M berturut-turut 87,50% dan 64,70%, sedangkan pada ikan yang divaksin N_4M berturut-turut 62,50% dan 76,47%. Vaksin strain N_3M dan N_4M memiliki proteksi lebih baik jika diuji tantang dengan strain bakteri yang sama.

Kata kunci: formalin-killed cell, *Streptococcus agalactiae*, strain, proteksi, nila

INTRODUCTION

Development of intensive tilapia fish farming caused disease problems, such as the viral, bacterial, and parasitic disease which needed to get more attention. Disease outbreak in fish was caused by a low fish defense, endurance, feed quality, and water quality (Giordano *et al.*, 2010). One of the bacterial diseases which often attacked tilapia was streptococcosis (Sheehan *et al.*, 2009). Streptococcosis was a disease caused by bacterial pathogen *Streptococcus* sp. (Abuseliana *et al.*, 2011). Indonesia became one of the countries exposed mass mortality on tilapia farming as the result of streptococcosis outbreaks (Taukhid & Purwaningsih, 2011). Tilapia intensive culture had a high risk of great loss as streptococcosis disease caused more than 90% mortality from the population (Ye *et al.*, 2012).

Tilapia fish which was infected by *Streptococcus agalactiae* suffered exophthalmia, opacity, purulent, whirling, discoloration, abdominal abscess, and clear operculum (Hardi *et al.*, 2011). Isolation of *S. agalactiae* from some regions in Indonesia which were discovered from streptococcosis disease outbreak obtained some isolation strains, such as N_3M (eyes), N_4M (eyes), $N_{17}O$ (brain), $N_{14}G$ (kidney) came from Cirata and NK_1 (brain) came from Klaten (Hardi *et al.*, 2013). *S. agalactiae* was the main bacteria which attacked various freshwater, brackishwater, and marine fish in the tropical and subtropical region, infecting fry until adult stadia (Bowater *et al.*, 2012).

Some steps that have been made to control this disease outbreak were the use of antibiotics and other chemical products. However, these treatments had an impact on the environment and human health in a long period (Giordano *et al.*, 2010). One way to prevent a disease outbreak is vaccination (Chen *et al.*, 2012). This activity would help form specific and non-specific immune system. Vaccination also formed a long-term memory for the immune system, therefore it only took one or two times for the treatment of fish (Barman *et al.*, 2013).

Vaccination on tilapia fish had been tested to be capable to enhance specific antibody formation and to protect from *S. agalactiae* infection (Giordano *et al.,* 2010; Chen *et al.,* 2012; Hardi *et al.,* 2013). However, *S. agalactiae* from the isolation result contains too many strains, producing various type of vaccine. Every vaccine from *S. agalactiae*

strain has a different protection mechanism. Hardi *et al.* (2013) reported that fish which was given *S. agalactiae* $N_{14}G$ strain after challenged with $N_{14}G$ and NK_1 strain produced relative percent survival as much as 79% and 75% respectively, while fish vaccinated with NK_1 strain, then challenged with $N_{14}G$ and NK_1 strain produced relative percent survival as much as 62.5% and 75%, respectively.

Fish that had been vaccinated by the different strain of *S. agalactiae* showed crossed protection in the sampled tilapia (Sukenda *et al.*, 2014). Various vaccine type made the vaccine was difficult to choose for protecting the fish from *S. agalactiae* exposed strain. Information about the effectivity of a vaccine for protecting the fish from *S. agalactiae* strain infections is necessary to be discovered. This study aimed to analyze the specific and nonspecific immune system on the tilapia fry which was given *S. agalactiae* strain N_3M and N_4M, as well as investigating the protective immunity against *S. agalactiae* N_3M, N_4M, $N_{17}O$, NK_1, and $N_{14}G$ strain infection.

MATERIALS AND METHOD

Streptococcus agalactiae bacteria

Bacteria of *S. agalactiae* from N_3M, N_4M, $N_{17}O$, $N_{14}G$ isolates were obtained from the Center for Research and Development of Freshwater Aquaculture, Bogor, West Java, Indonesia. Five strains of *S. agalactiae* bacterial virulence was recovered with Koch's postulate test. Bacteria were cultured in brain heart infusion broth (BHIB) liquid media for 72 hours on 140 rpm shaker and injected on fish as much as 0.1 mL in the intraperitoneal body part. Bacteria were isolated from the fish suffered the clinical signs of *S. agalactiae*. Isolation result was purified and characterized with Kit API 20 Strep.

Vaccine production

Vaccine production from *S. agalactiae* N_3M and N_4M strain were cultured in the liquid media for 72 hours (10^9 CFU/mL bacterial density), added with 38% neutral formalin buffer (NBF) as much as 3% of the liquid culture volume, and incubated for 24 hours. Bacteria were harvested using 7000 rpm centrifugation for 30 minutes and washed with phosphate buffered saline (PBS) twice, then added with PBS based on the initial volume. The vaccine produced was tested its viability level on brain heart infusion agar (BHIA) media.

Tank preparation and experimental fish rearing

As many as 48 pieces of aquarium tanks sized $(60 \times 30 \times 50)$ cm^3 with set aeration on each tank were prepared. Aquarium was washed and filled with water reaching 25 cm height, then disinfected along with the media using 30 mg/L of chlorine $(Ca(ClO)_2)$ for 24 hours and neutralized with sodium thiosulfate $(Na_2S_2O_3)$ as much as 15 mg/L. As many as 10 fish on each aquarium was maintained and given commercial feed three times a day with satiation. Water media was replaced as much as 75% of the volume every three days. Water quality maintained during the rearing process was 25.8−26.6°C of temperature, 6.7−7.1 of pH, 5.26−6.23 of dissolved oxygen, and 0.0019−0.0038 of TAN.

Vaccination and challenge test

Fish was vaccinated with 0.1 mL dose of two vaccines of N$_3$M (vaccine A) and N$_4$M (vaccine B) strain for each fish on the intraperitoneal part. Challenge test was conducted after 14 days of vaccination using *S. agalactiae* N$_3$M (bacteria a), N$_4$M (bacteria b), N$_{17}$O (bacteria c), NK$_1$ (bacteria d), and N$_{14}$G (bacteria e) strains with 0.1 mL dose of 10^7, 10^7, 10^6, 10^6, 10^6 CFU/fish respectively (Based on LD50).

Design

This study used factorial complete randomized design method containing two factors, which were *S. agalactiae* vaccine strain (N$_3$M (vaccine A) and N$_4$M (vaccine B)) and challenge test (*S. agalactiae* N$_3$M (a), N$_4$M (b), N$_{17}$O (c), NK$_1$ (d), and N$_{14}$G (e) strain) types with three replications.

Parameter

Parameters observed in this study were mortality rate, relative percent survival, antibody level (ELISA), blood characterization (erythrocyte total, leucocyte total, hematocrit, hemoglobin, phagocytic activity, and respiratory burst), lysozyme activity, and clinical signs.

Blood characterization, antibody levels, and lysozyme activity parameter were observed before vaccination, two weeks after vaccination, and a week after the challenge test. Mortality range and relative percent survival were observed two weeks after challenge.

Relative percent survival

Relative percent survival (RPS) was calculated to measure the vaccine protection level against *S. agalactiae* infection on the fry sample. The relative percent survival was calculated on the following formula.

$$RPS\ (\%) = 1 - \left[\frac{\text{Vaccinated fry mortality rate}}{\text{Fry control mortality rate}} \right] \times 100$$

$$\text{Mortality rate}\ (\%) = \frac{\text{The total dead fish}}{\text{The total fish}} \times 100$$

Antibody level

Antibody level was measured using an indirect method of enzyme-linked immunosorbent assay (ELISA), based on Sumiati *et al*. (2015). *S. agalactiae* antigen was obtained from the sonication result. Protein concentration on antigen was measured with Bradford spectrophotometry method and diluted with carbonate-bicarbonate buffer (pH 9.6) until reaching 10 μg/mL concentration. As much as 100 μL diluted antigen was put into the microtiter plate wells and incubated for 12 hours at 4°C. The microtiter plate was washed with PBS-T (PBS, pH 7.4 + 0.05% Tween-20). Bovine serum albumin (BSA) 3% in aquabidest w/v was added 100 μL on the well and incubated at 25°C for an hour, then washed with PBS-T. Tilapia serum samples were diluted with 1:50 PBS-T, added to the microtiter plate wells in accordance with 100 μL antigen. The microtiter plate was incubated at 25°C for an hour and washed with PBS-T. A hundred μL long

Table 1. Experimental design

Vaccine	Challenge test				
	N$_3$M (a)	N$_4$M (b)	N$_{17}$O (c)	NK$_1$ (d)	N$_{14}$G (e)
K(-)	K-a	K-b	K-c	K-d	K-e
K(+)	K+a	K+b	K+c	K+d	K+e
N$_3$M (A)	Aa	Ab	Ac	Ad	Ae
N$_4$M (B)	Ba	Bb	Bc	Bd	Be

Note: Control without vaccination and challenge test (K-), with a challenge test (K+). Vaccination using N$_3$M (A) and N$_4$M (B) vaccine, challenged with N$_3$M (a), N$_4$M (b), N$_{17}$O (c), NK$_1$ (d), N$_{14}$G (e) strain.

chain immunoglobulin of anti-tilapia for a specific polyclonal antibody which had been diluted 1:200 (v/v) with PBS-T was added on the microtiter plate well and incubated 25°C for an hour, then washed with PBS-T. Peroxidase-conjugated goat anti-rabbit diluted with PBS-T 1:15,000 was added 100 μL onto the microtiter plate wells and incubated at 25°C for 1 hour. The microtiter plate was washed with PBS-T and added with 100 μL of One Step Ultra-TMB-ELISA (TMB 5 mg + 10 μL 38% H_2O_2 in 5 mL of acetate buffer pH 5), then incubated at 25°C for 15 minutes. ELISA reaction was stopped with the addition of 100 μL H_2SO_4 3 M into microtiter plate wells. Optical density on the microtiter plate was read using a Microplate Reader (Kayto RT-2100C) with 450 nm wavelength. The cut off value (CV) was determined as the antibody formation standard. The optical density with a higher value than CV would represent antibody existence. CV value was determined with the following formula:

$$CV = \text{Optical density average of negative control} + (2 \times \text{deviation standard})$$

Erythrocyte total

Blood which was taken from the fish sample was absorbed using red pulpy Sahli pipette until scale 0.5 and diluted using Hayem's solution until reaching scale 101. Blood solution was homogenized by shaking the pipette forming number eight for 3-5 minutes. One drop of blood was thrown to eliminate the unmixed blood part and closed with cover glass. Total erythrocyte was calculated using :

$$\frac{\Sigma \text{ erythrocyte}}{(\text{cell/mm}^3)} = \frac{\Sigma \text{ Counted}}{\text{cells}} \times \frac{1}{\text{Big square volume}} \times \frac{\text{dillution}}{\text{factor}}$$

Hemoglobin

Hemoglobin was measured based on Sahli method using haemometer. Hemoglobin tube was filled with 0.1 N HCl until reaching scale 10 on the red scale. Blood was taken using Sahli pipette until scale 20 and put into the hemoglobin tube, then steadied for 5 minutes. The solution was added with aquadest until the solution color was the same as the standard color. Hemoglobin level was read on the yellow scale (g/dL).

Hematocrit

Hematocrit was measured to determine the blood cell percentage existed in the fish blood. Fish blood was taken in the microhematocrit tube

until ¾ part of the tube, then the tube tip was closed using crytoceal. Tube filled with fish blood was centrifuged using 3000 rpm for 5 minutes. Hematocrit content was determined with the following formula:

$$\text{Hematocrit (\%)} = \frac{\text{Packed red cells}}{\text{Total blood volume}} \times 100$$

Leucocyte total

Blood was absorbed using white pulpy Sahli pipette until scale 0.5 and added with Turk's solution until scale 11, then homogenized for 3-5 minutes by shaking the pipette to form a number eight. One to two solution drops from the pipette was thrown and another one was dropped on the haemocytometer. Leucocyte total was calculated with :

$$\frac{\Sigma \text{ Leucocyte}}{(\text{cell/mm}^3)} = \frac{\Sigma \text{ Counted}}{\text{cells}} \times \frac{1}{\text{Big square volume}} \times \frac{\text{dillution}}{\text{factor}}$$

Phagocytic activity (PA)

Staphylococcus aureus bacteria with 10^7 CFU/mL was added on the blood in the sterile microtube with 1:1 ratio and incubated for 20 minutes at the room temperature. The blood sample was spread thinly on the object glass and dried at the room temperature. The blood sample was fixated using methanol 95% for 5 minutes. The blood sample was dried and colored with Giemsa 5% for 15 minutes, then cleaned with aquadest. The phagocytic activity on blood sample was observed using a microscope and counted 100 phagocytic cells (monocytes and neutrophils). Observation result was calculated with the following formula:

$$\text{Phagocytic activity (\%)} = \frac{\Sigma \text{ Phagocytizing cell}}{\Sigma \text{ Phagocytic cell}} \times 100$$

Respiratory burst (RB)

Respiratory burst measurement was based on the nitroblue tetrazolium (NBT) reduction existence as the anion superoxide. The blood sample was put into the microtiter plate as much as 50 μL and incubated at 37°C for 1 hour. Blood inside the microtiter plate was thrown and cleaned with PBS pH 7.4 three times. 100 μL NBT (0.2% in PBS pH 7.4) was added in the microtiter plate and incubated at 37°C for 1 hour, then cleaned using PBS. Methanol absolute was added as much as 100 μL and kept steady for 10 minutes, then thrown and cleaned with methanol 30% three times. A sample in the microtiter plate was

added with 60 μL KOH 1 M and 70 μL dimethyl sulfoxide (DMSO). Respiratory burst value was determined from the optical density read using Microplate Reader (Kayto RT-2100C) on 630 nm wavelength.

Lysozyme activity

A hundred μL blood serum was put in a microtiter plate and added with a liquid suspension of *Micrococcus lysodeikticus* (Sigma) bacteria as much as 100 μL (0.4 mg/mL in 0.1 M PBS pH 6.2) at 25°C. Absorbance was read using Microplate Reader (Kayto RT-2100C) with 450 nm wavelength for 30 seconds and 30 minutes mixing. Lysozyme activity measurement unit was based on the decreased 0.0001 per minute of optical density (OD) on the following formula:

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

Clinical signs

Clinical signs on the fry were observed after the challenge test conducted by comparing the external organ of the fry infected with and without *S. agalactiae*. Clinical signs observed were exopthalmus, dropsy, whirling, opacity, purulent, and discoloration.

Data analysis

Measured parameter data result was tabulated in Microsoft Office Excel 2007 and analyzed using analysis of variance (ANOVA) on Minitab 16 application with 95% confidence level (P<0.05), as well as continued Tukey test.

RESULTS AND DISCUSSION

Results

Mortality and relative percent survival

The mortality rate of fry vaccinated by vaccine A and B were lower significantly (P<0.05; Table 2), compared with the positive control, except vaccine B treatment with d challenge test. The best mortality range was observed at vaccine A on a challenge test with 6.67 ± 5.77%. The highest relative percent survival on every vaccine type treatment was at vaccine A on a challenge test (87.50 ± 10.82%) and B on b challenge test (76.47 ± 10.18%) that both were significantly different (P<0.05) with d challenge test.

Antibody level

Antibody level after vaccination increased significantly (P<0.05; Table 3) for two vaccine types compared with control treatment, however, showing no significant difference among vaccine

Table 2. Mortality rate (MR) dan relative percent survival (RPS) of fry tilapia on two weeks after the challenge test

Vaccine	Challenge test	MR (%)	RPS (%)
K-		0.00 ± 0.00[f]	-
	a	53.33 ± 5.77[ab]	-
	b	56.67 ± 5.77[a]	-
K+	c	56.67 ± 5.77[a]	-
	d	60.00 ± 10.00[a]	-
	e	56.67 ± 5.77[a]	-
	a	6.67 ± 5.77[ef]	87.50 ± 10.82[a]
	b	20.00 ± 10.00[cde]	64.70 ± 17.64[abc]
A	c	26.67 ± 5.77[cd]	52.94 ± 10.18[bc]
	d	33.33 ± 5.77[c]	44.44 ± 9.62[bc]
	e	26.67 ± 5.77[cd]	52.94 ± 10.18[bc]
	a	20.00 ± 10.00[cde]	62.5 ± 18.75[abc]
	b	13.33 ± 5.77[def]	76.47 ± 10.18[ab]
B	c	30.00 ± 0.00[cd]	47.05 ± 0.00[bc]
	d	36.67 ± 5.77[bc]	38.89 ± 9.62[c]
	e	26.67 ± 5.77[cd]	52.94 ± 10.18[bc]

Note: Different superscript letter in the same column shows a significant difference (P<0.05). Control without vaccination and challenge test (K-), with a challenge test (K+). Vaccination using N_3M (A) and N_4M (B) vaccine, challenged with N_3M (a), N_4M (b), $N_{17}O$ (c), NK_1 (d), $N_{14}G$ (e) strain.

Table 3. Antibody level of fry tilapia with N_3M and N_4M antigen, before and after vaccination, as well as after the challenge test

Treatment				
N_3M Antigen-antibody level (optical density)		N_4M Antigen-antibody level (optical density)		
Before vaccination				
Initial	0.492 ± 0.058		Initial	0.462 ± 0.014
After vaccination				
Control	0.587 ± 0.024^b		Control	0.544 ± 0.005^b
A	0.767 ± 0.026^a		B	0.743 ± 0.026^a
CV	0.635		CV	0.554
After challenge test				
K-	0.477 ± 0.026^d	K-		0.549 ± 0.057^{cd}
		a	0.487 ± 0.024^d	a	0.611 ± 0.029^c
		b	0.505 ± 0.028^{cd}	b	0.484 ± 0.029^d
K+	c	0.523 ± 0.010^{cd}	K+	c	0.563 ± 0.031^{cd}
		d	0.520 ± 0.018^{cd}	d	0.500 ± 0.026^{cd}
		e	0.501 ± 0.041^{cd}	e	0.593 ± 0.019^{cd}
		a	0.830 ± 0.014^{ab}	a	0.748 ± 0.014^b
		b	0.821 ± 0.015^{ab}	b	0.887 ± 0.023^a
A	c	0.766 ± 0.009^b	B	c	0.744 ± 0.024^b
		d	0.790 ± 0.013^{ab}	d	0.756 ± 0.028^b
		e	0.767 ± 0.030^b	e	0.762 ± 0.028^b
CV	0.530	CV		0.664

Note: Different superscript letter in the same column shows a significant difference (P<0.05). Control without vaccination and challenge test (K-), with a challenge test (K+). Vaccination using N_3M (A) and N_4M (B) vaccine, challenged with N_3M (a), N_4M (b), $N_{17}O$ (c), NK_1 (d), $N_{14}G$ (e) strain. CV is cut of value as an antibody formed standard.

treatments. Antibody level formed from vaccine treatment was higher than CV. OD value for vaccine A treatment was 0.767 ± 0.026 and vaccine B was 0.743 ± 0.026. Antibody level after challenge test was significantly different (P<0.05) on vaccine type treatments and their interactions, however, it showed no significant difference on the challenge test (P>0.05). Antibody level on vaccine A and B treatment increased significantly on the challenge test treatment (P<0.05; Table 3) compared with negative and positive control, however, there was no significant difference (P>0.05) compared among vaccine treatments on all challenge tests. OD value on vaccine treatment with different challenge test was higher than the cut of value. The highest OD value was observed at vaccine A treatment on a challenge test with 0.830 ± 0.014 and vaccine B treatment on b challenge test with 0.887 ± 0.023.

Blood characterization

Erythrocyte total after vaccination on vaccine B treatment was significantly different (P<0.05) compared with control treatment. Erythrocyte total after challenge test on vaccine A treatment with b, d challenge test and vaccine B treatment with a challenge test were significantly different (P<0.05) compared with the a, b, and d control challenge test. Leucocyte total on the vaccine A and B treatment were significantly higher (P<0.05) than control treatment after vaccination. Leucocyte total after challenge test on vaccine A treatment with all challenge tests and vaccine B with d challenge test exception differed significantly (P<0.05) compared with the control treatment on c challenge test. Hemoglobin level on vaccine A after a, c, e challenge test and vaccine B after a challenge test was significantly different (P<0.05) compared with control treatment on a challenge test. Hematocrit level after vaccination on all treatments had no significant difference (P>0.05).

Table 4. Blood figures (erythrocyte, leucocyte, hemoglobin (Hb), hematocrit (Hc), phagocytic activity (AF), respiratory burst (RB), and lysozyme activity on fish fry before vaccination, after vaccination, and after the challenge test.

Treatment		Erythrocyte ($\times 10^6$ cell/mm³)	Leucocyte ($\times 10^4$ cell/mm³)	Hb (g/dL)	Hc (%)	AF (%)	RB (OD on λ=630 nm)	Lysozyme (unit/mL)
Before vaccination								
Initial		2.23 ± 0.19	1.27 ± 0.11	5.8 ± 1.11	14.18 ± 0.16	22.22 ± 3.84	0.192 ± 0.02	18.88 ± 7.34
After vaccination								
Control		2.09 ± 0.10[a]	1.50 ± 0.10[c]	5.4 ± 0.61[b]	15.77 ± 0.79[a]	23.667 ± 1.52[b]	0.197 ± 0.007[b]	22.11 ± 1.01[b]
A		2.08 ± 0.03[ab]	2.23 ± 0.05[b]	7.0 ± 0.52[a]	17.47 ± 1.3[a]	32.33 ± 0.57[a]	0.201 ± 0.003[a]	33.11 ± 2.16[a]
B		2.02 ± 0.04[b]	2.47 ± 0.05[a]	5.5 ± 0.11[b]	17.90 ± 1.03[a]	34.00 ± 1.73[a]	0.204 ± 0.005[a]	32.22 ± 0.57[a]
After Challenge Test								
K-		1.79 ± 0.32[abcd]	2.73 ± 0.70[g]	5.6 ± 0.34[abcd]	16.57 ± 2.03[abc]	28.00 ± 1.00[d]	0.209 ± 0.002[bc]	26.22 ± 0.69[f]
K+	a	1.06 ± 0.07[de]	4.93 ± 0.25[efg]	4.2 ± 0.69[d]	11.21 ± 2.60[bc]	30.00 ± 1.00[d]	0.235 ± 0.002[abc]	27.78 ± 1.64[f]
	b	1.15 ± 0.18[e]	5.30 ± 0.60[ef]	5.4 ± 0.25[abcd]	11.73 ± 1.05[bc]	29.33 ± 0.57[d]	0.192 ± 0.001[c]	29.88 ± 1.26[ef]
	c	1.00 ± 0.16[cde]	4.60 ± 0.34[fg]	5.8 ± 0.69[abc]	10.91 ± 0.94[c]	34.67 ± 1.15[d]	0.202 ± 0.005[c]	30.55 ± 2.36[ef]
	d	0.93 ± 0.09[e]	5.60 ± 0.96[def]	6.0 ± 0.00[ab]	10.8 ± 0.84[c]	33.67 ± 1.52[d]	0.235 ± 0.004[abc]	32.22 ± 2.69[ef]
	e	1.18 ± 0.09[bcde]	5.27 ± 1.35[ef]	6.1 ± 0.23[a]	13.90 ± 1.04[abc]	33.67 ± 1.52[d]	0.198 ± 0.004[c]	28.00 ± 2.90[f]
A	a	1.37 ± 0.16[abcde]	7.93 ± 0.20[bcd]	6.3 ± 0.85[a]	18.57 ± 1.71[a]	49.00 ± 3.46[bc]	0.289 ± 0.006[ab]	79.44 ± 1.34[a]
	b	1.91 ± 0.47[ab]	8.40 ± 0.66[abc]	5.4 ± 0.34[abcd]	18.34 ± 1.47[a]	47.33 ± 3.05[c]	0.288 ± 0.003[ab]	40.89 ± 1.34[cd]
	c	1.64 ± 0.25[abcde]	8.86 ± 0.89[ab]	5.9 ± 0.55[ab]	16.19 ± 0.41[abc]	52.67 ± 4.61[abc]	0.297 ± 0.009[a]	47.33 ± 1.33[c]
	d	2.03 ± 0.09[a]	7.86 ± 0.32[bcd]	5.0 ± 0.34[abcd]	16.69 ± 0.80[abc]	54.67 ± 3.05[abc]	0.307 ± 0.002[a]	41.67 ± 1.52[bc]
	e	1.49 ± 0.18[abcde]	7.07 ± 0.67[bcde]	6.2 ± 0.56[a]	17.59 ± 0.81[ab]	55.33 ± 5.85[ab]	0.316 ± 0.003[a]	38.11 ± 0.83[de]
B	a	1.87 ± 0.12[abc]	8.76 ± 0.37[ab]	5.1 ± 0.11[abcd]	14.29 ± 0.51[abc]	49.67 ± 3.51[bc]	0.291 ± 0.002[ab]	37.22 ± 3.53[de]
	b	1.50 ± 0.15[abcde]	10.47 ± 0.70[a]	4.4 ± 0.60[cd]	15.51 ± 1.05[abc]	48.00 ± 1.73[bc]	0.290 ± 0.009[ab]	56.22 ± 4.53[b]
	c	1.51 ± 0.27[abcde]	7.03 ± 0.73[bcde]	5.1 ± 0.46[abcd]	17.02 ± 3.21[abc]	58.00 ± 1.73[a]	0.315 ± 0.004[a]	77.33 ± 0.88[a]
	d	1.18 ± 0.17[bcde]	6.33 ± 0.23[cdef]	4.6 ± 0.56[bcd]	12.67 ± 2.05[abc]	51.00 ± 1.73[abc]	0.297 ± 0.003[a]	42.33 ± 5.00[cd]
	e	1.42 ± 0.14[abcde]	7.93 ± 0.68[bcd]	5.8 ± 0.70[abc]	16.63 ± 1.65[abc]	55.33 ± 2.08[ab]	0.312 ± 0.009[a]	40.67 ± 6.88[cd]

Note: different superscript letter in the same column shows a significant difference (P<0.05). Control without vaccination and challenge test (K-), with a challenge test (K+). Vaccination using N_3M (A) and N_4M (B) vaccine, challenged with N_3M (a), N_4M (b), $N_{17}O$ (c), NK_1 (d), $N_{14}G$ (e) strain.
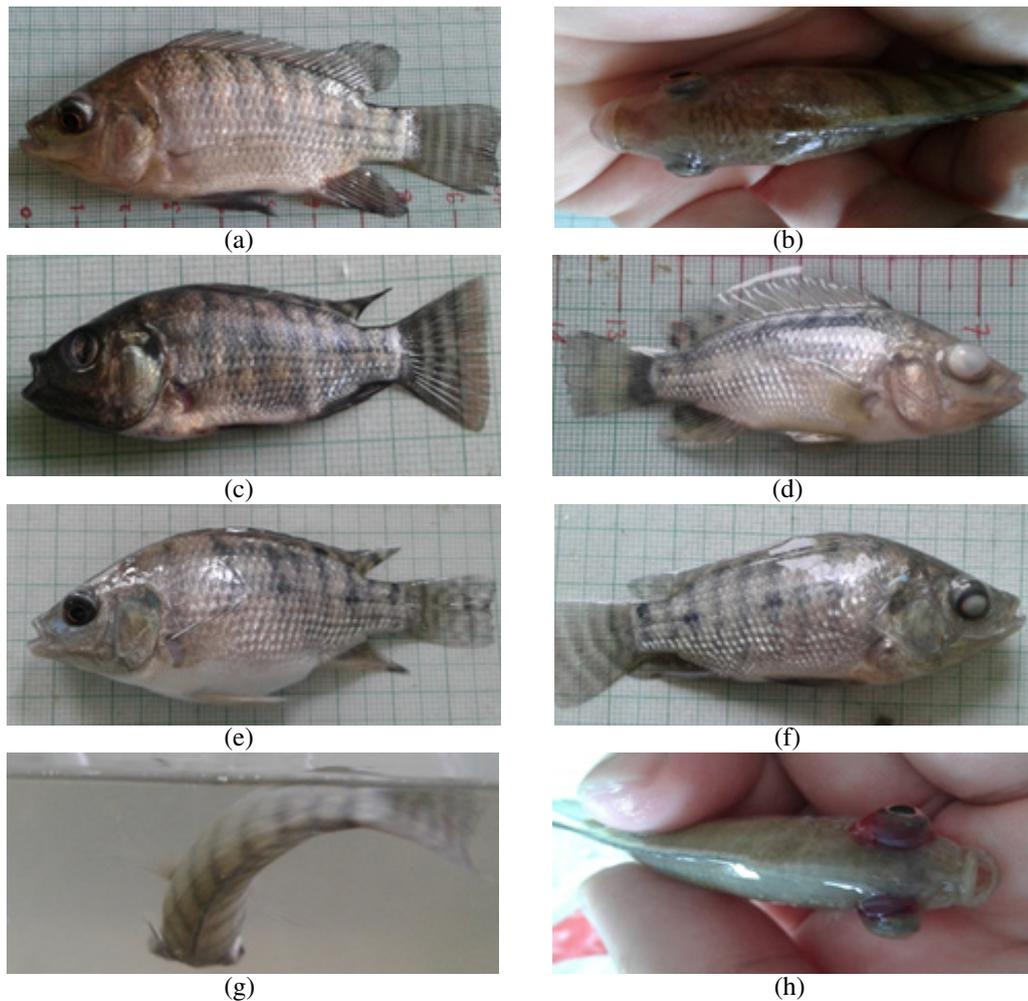
Figure 1. Clinical signs of tilapia fry infected *S. agalactiae*. Negative control (a), Negative control fry from the top (b), blackish body change (c), purulent (d), dropsy (e), opacity (f), whirling (g), exophthalmia (h).

Hematocrit level on vaccine A after a, b, and e challenge test was significantly different (P<0.05) compared with control treatment after c and d challenge test. Vaccine B treatment on all challenge tests showed no significant different (P>0.05) against two control treatments. Phagocytic activity after vaccination on vaccine A and B were significantly different (P<0.05) from control treatment. Phagocytic activity on vaccine A and B after all challenge test treatments were significantly different (P<0.05) with positive control. Respiratory burst (RB) level after vaccination had no significant difference in all treatments (P>0.05). RB level on vaccine A and B after all challenge test treatments had a significant difference (P<0.05) against positive control treatment after b, c, and e challenge test. Lysozyme activity after vaccination on vaccine A and B were significantly different (P<0.05) from control treatment. Lysozyme activity on vaccine A after e challenge test and vaccine B after a challenge test had no significant difference compared with control treatment after b, c, and d challenge test.

Clinical signs

Based on the observation result, the clinical signs occurred on fry tilapia were blackish body color, purulent, dropsy, opacity, whirling, and exophthalmia.

Discussion

Vaccination used formalin-killed cells vaccine of bacteria *S. agalactiae* strains N_3M (A) and N_4M (B) given was able to lower the mortality rate of tilapia fry from Streptococcosis. The mortality rate of fish fry vaccinated was significantly lower (P<0.05) compared with the positive control, except vaccine B vaccine on d challenge test treatment. The lowest mortality rates from vaccine treatments were vaccine A on a challenge test (6.67±5.77%) and vaccine B on b challenge test (13.33 ± 5.77%). The mortality rate of tilapia fry infected with the same bacteria as the vaccine

was lower than the different bacteria infection, which showed that the vaccine worked specifically against homologous bacteria. Giordano *et al.* (2010) reported that tilapia fish mortality rate which injected with inactive bacteria of *S. agalactiae* was 10.74%, based on the challenge test with homologous bacteria. This study reports that vaccine challenged with different bacteria had 20−36.67% mortality rate. Shoemaker *et al.* (2010) reported that fish mortality rate observed from the *S. iniae* vaccination using four strains were ranged from 0−7.5% after the challenge test. The mortality rate of tilapia vaccinated with one type of vaccine and tested using 10 combined strains of *S. agalactiae* was ranged 40−80% (Chen *et al.*, 2012). All treatments on d challenge test (NK₁) had the highest mortality rate from control treatment with 60%, vaccine A treatment with 33.33%, and vaccine B with 36.67%. According to Hardi *et al.* (2011), the bacteria strain NK₁ used to challenge test had virulence which was higher than the other. Vaccine A treatment had an averagely low mortality rate after challenged with different bacteria.

The increased protection level of tilapia against streptococcosis indicated the relative percent survival. The relative percent survival of both vaccines with different challenge tests showed different results. Tilapia relative percent survival on vaccine A after a, b, c, d and e challenge test were 62.50%, 76.47%, 47.05%, 38.89%, and 52.94%, respectively. Vaccine A was capable of protecting more than 50% infection of *S. agalactiae* strains a, b, c, and e, while vaccine B was capable of protecting from the bacterial infection from a, b, and e strains. The vaccine produced that was homologous with bacteria gives higher protection than the different bacteria after the challenge test. Homologous antigen comes from other individuals in one species. Meanwhile the heterologous antigen comes from other individuals in other species. The difference source of antigen will usually produce different immune response compared to other (Munang'andu *et al.*, 2016).

Evans *et al.* (2004) showed tilapia vaccinated and challenged with the heterologous bacteria generated 25% of relative percent survival, whereas with the bacterial homologous was 70%. Vaccine A and B for homologous bacterial protection also provided heterologous infected bacterial protection, called cross-protection. Chen *et al.* (2012) showed three among ten vaccine types were capable of protecting from different

strains and genotypes of *S. agalactiae* with relative percent survival ranged 53.57−100%. Vaccine protection from *S. iniae* infection in tilapia with four different strains showed different relative percent survival, ranging 79−100% (Shoemaker *et al.*, 2010). Based on the result obtained in this study, vaccine A gave a lot more protection from different *S. agalactiae* strains.

Parameters of the mortality and relative percent survival on vaccination treatment were influenced by the specific immune system followed with a non-immune system. Vaccination performed stimulated the formation of the specific immune system shown with increased antibody level in fish. Increased antibody level was supported by the activity of T cell (T helper cells, cytotoxic T cells, and memory T cells) and B cells (B cells and plasma B cell memory). The interaction between antigen and lymphocytes (B cells and T cells) caused an increased level of antibodies through vaccination. The vaccine contained antigens which induced the proliferation of B cells into memory B cells and plasma B cells. The role of memory B cells stored information characteristics of the antigen into the body, while the plasma B cells played a role in the production of specific antibodies against these antigens. Incoming antigen was phagocytized by macrophages, the process of antigen phagocytosis would transfer helper T cells via major histocompatibility complex (MHC) class II. Macrophage would activate helper T cells through cytokine molecules. Helper T cells produced cytokines that stimulated B cells, memory T cells, and cytotoxic T cells. The same antigen that penetrated the body for the second time would stimulate the role of memory T cells and memory B cells to conduct proliferation into the plasma cells as well as producing antibodies (Mashoof *et al.*, 2016).

Antibody levels after vaccination on both vaccine treatments happened to conduct an increased level of specific antibodies against *S. agalactiae* strain A and B. This was shown on the value of vaccine A (0.767 ± 0.026) and B (0.743 ± 0.026) optical density (OD) which were higher than any vaccine the cut of value (CV). Both vaccine treatments showed a significant increase (P<0.05) compared with the control treatment. Martins *et al.* (2011) also reported increased antibody level in tilapia serum compared with control treatment, after vaccinated with vaccines *S. iniae*. The antibody level after challenge test on vaccine A and B treatment was significantly higher (P<0.05) than positive and negative control treatment as

well as higher than CV. OD value on vaccine A after a,b,d challenge test treatment and vaccine B after all challenged tests were increased compared with OD value after vaccination. Increased OD value after challenge test also occurred on Sukenda *et al.* (2015), who reported that measurable antibodies were correlated with the relative percent survival. Increased OD treatment on vaccine A after all challenge tests were not significantly different (P>0.05) with vaccine B after a, c, d and e challenge test. Measurement of antibody level after challenge test using the same vaccine antigens, making the measured OD value was obtained from the antibodies formed from the same antigen and vaccines. This caused the OD value after challenge test was not much different to the value OD after vaccination. This result was on the contrary from Shomaker *et al.* (2010), who used the same antigen as a vaccine to testify the antibody level of fish vaccinated with *S. iniae* vaccine strain ARS-98-60 which was challenged with four different strains showed an increased antibody level. This was suspected to happen as of the protein profile of the five strains of *S. agalactiae* bacteria were used. Shomaker *et al.* (2010) stated that bacterial strains used had protein profile similarities inducing the antibody formation that allowed cross-protection. Based on antibody level measurement in this study, vaccine A had OD value that was higher after the challenge test treatments.

The nonspecific immune system also had a role in mortality and relative percent survival which could be shown from hereditary immune system and hematology parameter (Sukenda *et al.*, 2017). Leucocyte total is one of the hematology parameters on the nonspecific immune system. Leucocyte total measured on vaccine A and B $(2.23-2.47 \times 10^4$ cell mm$^{-3})$ treatment after vaccination were significantly higher (P<0.05) than the control treatment, nevertheless showing no significant difference (P>0.05) on the vaccination treatments. Suanyuk and Itsaro (2011) also reported that the leucocyte total which had no significant difference between vaccination treatments. Leucocyte total on vaccine A and B treatment after all challenge tests $(6.33-10.47 \times 10^4$ cell/mm$^3)$ were significantly higher (P<0.05) than the negative control. The vaccine was suggested as an antigen penetrating the fish body which was responded by producing leucocyte and forming an antibody, though it was less effective during the initial infection, or in this case was vaccination period (Sukenda *et al.*, 2014). Increased leucocyte

total after the challenge test showed that the fish body still defended against the infection. The highest leucocyte total was observed at vaccine A on c challenge test and vaccine B on b challenge test treatment. According to Hardi *et al.* (2013), vaccination was not yet completely capable of helping eliminate the pathogen existed in the body, which was marked as high leucocyte total after the challenge test.

Leucocyte total is related to the phagocytic activity. Phagocytic activity on vaccine treatment A and B (32.33% and 34%) was significantly higher (P<0.05) than the control treatment after vaccination. Sukenda *et al.* (2018) showed that tilapia fish which were given FKC *S. agalactiae* vaccine differed significantly against the phagocytic activity. Improvement of phagocytic activity was in line with the significant increased of total leucocytes compared with control treatment. Phagocytic activity experienced an increased immune system, showing bacterial infection resistance after the challenge test. This was also supported by the leucocyte total and antibody level which were quite high after the challenge test. Formed antibodies after vaccination would accelerate the antigen elimination under opsonization process. Opsonized antigen will be more recognizable for macrophages as well as more effective to be destructed (Hardi *et al.*, 2013).

The activity of phagocyte in destroying pathogen was indicated with the respiratory burst parameter. According to Uribe *et al.* (2011), the respiratory burst was produced by neutrophils and macrophages in the phagocytosis process to attack pathogens by producing reactive oxygen species (ROS). Phagocyte cells that successfully ingested pathogens would expose ROS as well as enzymes for pathogen digestion (Holmstrom & Finkel, 2014). The number of phagocyte cells exposed ROS was measured by NBT reduction method to become formazan in the form of optical density (OD). In this study, the value of the respiratory burst after vaccination vaccine treatment A and B did not differ significantly (P>0.05) with the control. Aly *et al.* (2015) also reported similar results with FKC vaccination on tilapia with *Aeromonas hydrophila* on respiratory burst parameter after vaccination. The value of the respiratory burst was significantly higher (P<0.05) on the vaccine treatment A and B after all challenge test treatments test challenge than control treatment after on b, c and e challenge test treatments. Pridgeon and Klesius (2013) showed

an increased production of ROS by macrophages of fish vaccinated against different bacteria after two and five minutes incubation compared with controls measured using the chemiluminescence assay method. The value of respiratory burst increased along with the phagocytic activity on the same treatment.

Nonspecific immune response associated with the lysozyme activity is leucocytes. Lysozyme is an important component in the immune system that separates a peptidoglycan layer cell wall of Gram-positive bacteria for preventing from infection. The measurement of lysozyme was commonly used as an indicator for fish vaccination evaluation (Yi *et al.*, 2014). This study discovered increased lysozyme activity after vaccinations on treatment A and B, which were significantly different (P < 0.05) with control treatment. Pasaribu *et al.* (2018) reported an increased lysozyme activity in the tilapia broodstock after vaccination was followed by its descendants. The lysozyme activity on vaccine A and B treatment was higher than the positive and negative control treatment. The increased activity of lysozyme would enhance the antibacterial ability in tilapia (Huang *et al.*, 2013).

Nonspecific immune response parameter that affected the fish health status is the erythrocyte total. Measured erythrocyte total after vaccination showed a significantly lower decline on vaccine B treatment (P<0.05) than control treatment, while vaccine A treatment was not significant difference compared with the control treatment (P>0.05). Martins *et al.* (2011) reported that the erythrocyte total of tilapia fish after vaccinated using *S. iniae* vaccine had declined result with no significant difference against the control treatment. Total erythrocyte decline was suspected because the vaccine given was suggested as an antigen, making the fish become more stressful along with the declined erythrocyte production and increased leucocyte production. Every treatment showed higher erythrocyte total compared with the control treatment. Different bacteria used for challenge test did not influence the erythrocyte total. According to Evans *et al.* (2005), vaccination was able to minimalize fish stress infected by the bacteria, making the energy needs to protect from *S. agalactiae* was less than the vaccinated fish.

Hemoglobin parameter was related with the erythrocyte total which functioned as oxygen binder that was able to bring in all body parts. Hemoglobin level on vaccine B treatment had no significant difference (P>0.05) with

control treatment after vaccination. Martins *et al.* (2011) reported that the hemoglobin level was not significantly differencing with the control treatment, which had a correlation with erythrocyte total and hematocrit. Hemoglobin level on vaccine A treatment after a,c,e challenge test and vaccine B treatment after e challenge test were significantly different (P<0.05) with the control treatment after a challenge test. Normal hemoglobin level of fish was 5.8±1.04% (Lourenco *et al.*, 2012).

Fish health status parameter associated with erythrocytes is hematocrit. Hematocrit shows the percentage of the total volume of blood cells in fish. Hematocrit on vaccine A and B after vaccination did not differ significantly (P<0.05) with the control. Suanyuk and Itsaro (2011) reported that hematocrit level on tilapia vaccinated with *S. iniae* vaccine was not significantly different with the control treatment. Vaccine treatment had higher hematocrit level compared with positive control. Martin *et al.* (2011) also reported that the vaccinated fish showed higher hematocrit level during 18 days after challenge test compared with the control treatment.

Clinical signs due to the infection of *S. agalactiae* strains N_3M, N_4M, $N_{17}O$, NK_1, and $N_{14}G$ were black skin color change, opacity, dropsy, purulent, whirling and exophthalmia (Figure 1). Clinical signs generally appeared on the fifth day after the challenge test. Based on the research of Hardi *et al.* (2011), clinical signs appeared after infection were shrunk pupil, opacity, purulent, exophthalmia, followed with bleeding, blackened discoloration, stomach ulcer, abscess, whirling and gasping. These signs appeared after 24 hours after the challenge test.

CONCLUSION

Based on the result obtained, it concluded that tilapia fry after vaccinated with *S. agalactiae* N_3M dan N_4M strain showed the increased level of antibodies, leucocyte total, phagocytic activity, respiratory burst, and lysozyme activity. Vaccine *S. agalactiae* N_3M strain was capable of protecting the fry from N_3M, N_4M, $N_{17}O$, and $N_{14}G$ strain, while N_4M strain vaccine was only capable of protecting the fry from N_3M, N_4M, and $N_{14}G$ strain.

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REFERENCES

Abuseliana AF, Daud HHM, Aziz SA, Bejo SK, Alsaid M. 2011. Pathogenicity of *Streptococcus agalactiae* Isolated from a fish farm in Selangor to juvenile red tilapia *Oreochromis* sp. Journal of Animal and Veterinary Advance*s* 10: 914−919.

Aly SM, Albutti AS, Rahmani AH, Atti NMA. 2015. The response of new-season Nile tilapia to *Aeromonas hydrophila* vaccine. International journal of clinical and experimental medicine 8: 4508−4514.

Bowater RO, Faulkner JF, Anderson IG, Condon K, Robinson B, Kong F, Gilbert GL, Reynolds A, Hyland S, McPherson G, O'Brien J, Byle D. 2012. Natural outbreak of *Streptococcus agalactiae* (GBS) infection in wild giant Queensland grouper, *Epinephelus lanceolatus* (Bloch), and other wild fish in northern Queensland, Australia. Journal of Fish Diseases 35: 173−196.

Chen M, Wang R, Li LP, Liang WW, Li J, Huang Y, Lei AY, Huang WY, Gan X. 2012. Screening vaccine candidate strains against *Streptococcus agalactiae* of tilapia based on PFGE genotype. Vaccine 30: 6088−6092.

Evans JJ, Klesisu PH, Shoemaker CA. 2004. Efficacy of *Streptococcus agalactiae* (group B) vaccine in tilapia *Oreochromis niloticus* by intraperitoneal and bath immersion administration. Vaccine 22: 3769−3773.

Evans JJ, Klesius PH, Shoemaker CA, Fitzpatrick BT. 2005. *Streptococcus agalactiae* vaccination and infection stress in Nile tilapia *Oreochromis niloticus*. Journal of Applied Aquaculture 16: 105−115.

Giordano LGP, Miller EE, Klesius P, Silva VG da. 2010. Efficacy of an experimentally inactivated *Streptococcus agalactiae* vaccine in Nile tilapia *Oreochromis niloticus* reared in Brazil. Aquaculture Research 41: 1539−1544.

Hardi EH, Sukenda, Harris E, Lusiastuti AM. 2011. Characterization and phatogenicity of β and non haemolytic *Streptococcus agalactiae* in cultured nila tilapia. Jurnal Veteriner 12: 152−164.

Hardi EH, Sukenda, Harris E, Lusiastuti AM. 2013. Potential vaccine candidate of *Streptococcus agalactiae* for prevent strepcocococis on nila tilapia (*Oreochromis niloticus*). Jurnal Veteriner 14: 408−416.

Holmstrom KM, Finkel T. 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nature Reviews 15: 411−421.

Huang BF, Zou LL, Xie JG, Huang ZC, Li YW, Li AX. 2013. Immune responses of different species of tilapia infected with *Streptococcus agalactiae*. Journal of Fish Diseases 36: 747−752.

Martins ML, Shoemaker CA, Xu D, Klesius PH. 2011. Effect of parasitism on efficacy against *Streptococcus iniae* in Nile tilapia. Aquaculture. 314: 18−23.

Mashoof S, Criscitiello MF. 2016. Fish immunoglobulins. Biology 5: 45.

Munang'andu HM, Paul J, Evensen Ø. 2016. An overview of vaccination strategies and antigen delivery systems for *Streptococcus agalactiae* vaccines in Nile Tilapia *Oreochromis niloticus*. Vaccines 4: 48.

Pasaribu W, Sukenda S, Nuryati S. 2018. The Efficacy of Nile tilapia *Oreochromis niloticus* broodstock and larval immunization against *Streptococcus agalactiae* and *Aeromonas hydrophila*. Fishes 3: 16.

Pridgeon JW, Klesius PH. 2013. Development of live attenuated *Streptococcus agalactiae* as potentioal vaccines by selecting for resistance to sparfloxacin. Vaccine 31: 2705−2712.

Shoemaker CA, LaFrentz BR, Klesius PH, Evans JJ. 2010. Protection against heterologous *Streptococcus iniae* isolates using a modified bacterin vaccine in Nile tilapia, *Oreochromis niloticus* (L.). Journal of Fish Diseases 33: 537−544.

Suanyuk N, Itsaro A. 2011. Efficacy of inactivated *Streptococcus iniae* vaccine and protective effect of β-(1.3/1.6)–glucan on the effectiveness of vaccine in red tilapia *Oreochromis niloticus* x *O. Mossambicus*. Songklanakarin Journal of Science and Technology 33: 143−149.

Sugiani D, Sukenda S, Harris E, Lusiastuti AM. 2013. Vaccination of tilapia (*O. niloticus*) using monovalent and bivalent vaccines for motile aeromonas septicemia and streptococcosis disease. Jurnal Riset Akuakultur 8: 230−239.

Sukenda, Febriansyah TR, Nuryati S. 2014. Whole-cell vaccine of *Streptococcus*

agalactiae in Oreochromis sp. with immersion method. Jurnal Akuakultur Indonesia 13: 83−93.

Sukenda, Rusly, Nuryati S, Hidayatullah D. 2015. The protective duration of *Streptococcus agalactiae* vaccine in Nile Tilapia for the prevention of streptococcosis. Jurnal Akuakultur Indonesia 14: 192−201.

Sukenda S, Carman O, Rahman R, Hidayatullah D, Yumaidawati NS. 2017. Vaccination in Nile tilapia broodstock with whole cell vaccine and disease resistance in its fry against *Aeromonas hydrophila*. Jurnal Akuakultur Indonesia 16: 268−276.

Sukenda S, Rahman R, Nisaa K, Hidayatullah D, Vinasyiam A. 2018. The efficacy of Streptococcus agalactiae vaccine preparations, administered to tilapia broodstock, in preventing streptococcosis in their offspring, via transfer of maternal immunity. Aquaculture International 00: 1−14.

Sumiati T, Sukenda, Nuryati S, Lusiastuti AM. 2015. Development of ELISA method to detect specific immune response in Nile tilapia *O. niloticus* vaccinated against *A. hydrophila* and *S. agalactiae*. Jurnal Riset Akuakultur 10: 243–250.

Taukhid, Purwaningsih U. 2011. Screening of *Streptococcus* spp. isolates as an antigen candidate in vaccine development, and its efficacy to prevent streptococcosis on tilapia, *Oreochromis niloticus*. Jurnal Riset Akuakultur 6: 103−118.

Uribe C, Folch H, Enriquez R, Moran G. 2011. Innate and adaptive immunity in teleost fish: a review. Veterinarni Medicina 56: 486−503.

Ye X, Li J, Lu M, Deng G, Jiang X, Tian Y, Quan Y, Jian Q. 2011. Identification and molecular typing of *Streptococcus agalactiae* isolated from pond-cultured tilapia in China. Fisheries Science 77: 623−632.