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Efficacy of *Streptococcus Agalactiae* Whole Cell Vaccine with *Artemia Sp.* Vector for the Prevention of Streptococcosis in Tilapia (*Oreochromis Niloticus*)

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Abstract
This study aims to examine the efficacy of S. agalactiae whole cell vaccine orally with Artemia sp as a vaccine vector for prevention against Streptococcosis in tilapia. Fish fry used in the study were 2-3 cm in size that did not carry S. agalactiae bacteria, kept in 3 l volume jars as many as ten heads / jars. Giving the vaccine to fish orally by first soaking the vaccine into Artemia sp.. The length of soaking time of the vaccine is 15, 30, 60, 90, 120 and 150 minutes. The parameters observed were relative percent survival (RPS), antibody titer value, total leukocytes, pagocytic index, and leukocyte differential. The results of the RPS value study showed that the treatment between vaccines was not significantly different, while the treatment of vaccines with controls was significantly different ($P<0.05$). The highest RPS value was obtained in vaccine immersion treatment in Artemia sp. for 30 minutes and 120 minutes with a value of 93.33%. Furthermore, RPS is 86.67% at 15 and 150 minutes soaking and RPS is 80% at 30 minutes immersion. This shows that the level of vaccine protection against streptococcosis in tilapia fry is very high

Keywords: Artemia Sp, Streptococcus Agalactiae, Soaking Time, Tilapia

Introduction

The disease that causes harm in tilapia culture today is streptococcosis caused by Streptococcus bacteria. Streptococcal bacterial infection causes tilapia mortality by about 50% from the first month and increases almost to 80% until the end of rearing in caramba in the Philippines (Clark et al. 2000), infection causes death up to 60% in tilapia farming in South Sumatra (Yuasa et al. 2008). On a large scale continuous streptococcal outbreaks occurred with high mortality (30-80%) in 2009 - 2011 (Chen et al. 2012). Cases of disease in tilapia farming in several regions in West Java, Central Java, East Java, North Sulawesi and West Papua, obtained a type of Streptococcus bacteria that causes Streptococcosis in tilapia. Where 80% of streptococcosis is caused by Streptococcus agalactiae and 20% is caused by Streptococcus iniae (Taukhid and Purwaningsih 2009). Some researchers have also reported spread and infection of S. agalactiae bacteria in tilapia in Indonesia (Lusiastuti et al. 2009; Anshary et al. 2014).

One of the preventive efforts that can be done to overcome the problem of streptococcosis is by growing immunity in the body of fish, including by vaccination. Vaccination is an effective way to overcome fish diseases (Ellis 1988). Vaccination can increase immunity in the body of fish so that it is resistant to certain diseases for some time, so that mortality can be suppressed as small as possible. Vaccination in fish will stimulate the formation of antibodies that will protect against certain diseases. Hardi et al. (2013) suggested that the administration of S. agalactiae whole cell vaccine by injection method provides protection for tilapia with an RPS value of 70%.

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There are several ways to apply vaccines to fish, namely :(1). Application of vaccines by immersion (2). Application of vaccines through feed (3). Application of vaccines by injection. Each vaccination method has advantages and disadvantages in its application. Vaccination of small fish (fry) is more effective using soaking methods and oral methods. The method of giving vaccines orally by mixing feed with vaccines has been widely done, and provides a fairly good level of protection. Both in fry and in fish that have been raised in aquaculture ponds. Artemia sp apart from being a natural feed, can also be used as a carrier (vector) of vaccines, some essential nutrients, antibiotics, pigments, drugs, and immunostimulants (Isnansetyo and Kurniastuti 1995). The advantages of vaccinating through Artemia sp. according to Lin et al. (2007) is Artemia sp. is a natural feed starter for fish larvae so it is expected that vaccines in the body of Artemia sp. quickly gets into the body of the fish. Some uses of Artemia sp. as vectors are immustimulants (Hurriyani 2011), hormone vectors (Dewi 2010), essential nutrient vectors (Santoso 2006), vaccine DNA vectors (Hadibowo 2011). This study wanted to examine the efficacy of Streptococcus agalactiae whole cell vaccine given orally with the vector Artemia sp.

Research Method

Research Materials

The test animals used were tilapia fry with a size of 2-3 cm which had been verified as not carrying S. agalactiae. Before being used in the experiment, the test fish were first adapted to laboratory conditions in a temporary holding tank. Fish were given commercial feed at a dose of 3% of the weight of biomass, feed protein content of 33% and given twice a day, ie in the morning and evening. Water quality is maintained at optimal conditions for fish growth. Bacterial isolate S. agalactiae N14G was obtained from the Bogor BPPBAT collection. The research container used a jar with a volume of 3 l, which was filled with 10 tilapia fish/jar.

Vaccine Preparation

The bacterial isolate of S. agalactiae in a petri dish was taken as much as 1 ose and put into 10 ml of liquid BHIB aseptically. Incubated at 28OC in the incubator for 24 hours. Then 1 ml of bacterial culture was taken and then put into each 9 ml of BHIB, and incubated at 28oC for 24 hours. 10 ml of the bacterial culture was then put into each 90 ml of BHIB and incubated for 72 hours assuming a concentration of 4 x 109 cfu/mL (Evans et al. 2006). Bacterial cultures with a volume of 100 ml were added with neutral buffer formaline 3% of the culture volume (Hardi et al. 2013; Amrullah 2014) and incubated again for 24 hours. Whole cell vaccine was prepared by inactivated bacterial culture and then centrifuged at 12,000 rpm for 30 minutes at 4oC. The supernatant solution and the pellet precipitate formed were then separated. The separated bacterial pellet precipitate was then washed by adding 100 ml of Phosphate Buffer Saline (PBS) and then centrifuged at 5,000 rpm for 15 minutes, washing bacterial cells was carried out three times with PBS, the pellet precipitate was then added with PBS again up to 100 ml and stored in refrigator for later use in fish vaccination.

Vaccination

Before vaccinating tilapia, enrichment of artemia with whole cell vaccine is done by immersing Artemia sp. in the vaccine solution at different times as follows:

- Treatment E: Soaking Artemia sp. with whole cell vaccine for 2 hours
- Treatment D: Immersion of Artemia sp. with whole cell vaccine for 1.5 hours
- Treatment C: Immersion of Artemia sp. with whole cell vaccine for 1 hour
- Treatment B: Immersion of Artemia sp. with whole cell vaccine for 0.5 hours

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- Treatment A: Immersion of Artemia sp. with whole cell vaccine for 0.25 hours
- Treatment F: Artemia sp. Without immersion vaccine (control)

Artemia sp. The brand used is Supreme Plus which is produced by Golden Mark®, USA, with a hatching rate of around 80–90%. System Artemia sp. incubated in an inverted mineral water bottle with a dark wall and equipped with an aeration system. Artemia sp. incubated with a salinity of 29 ppt for 18–24 hours. Artemia sp. Those that have hatched are separated using a filter with a size of 150 mesh and then weighed according to the dose to be given to the tilapia. One Artemia sp. able to eat up to 105 cfu/mL of bacteria (Lin et al., 2007), so 40 Artemia sp. for one tilapia to be vaccinated with a bacterial dose of 109 cfu/mL.

Challenge Test

Test challenge fish that have been vaccinated with S. agalactiae bacteria with doses according to the Lethal Dose 50% (LD50) test by suppressing fish for 30 minutes in water containing virulent S. agalactiae bacteria.

Fish mortality is recorded and the relative protection rate of the vaccine is calculated by the Relative Percent Survival (RPS) formula (Ellis, 1988):

$$\mathbf{RPS} = 1 - \left(\frac{\mathbf{Percent\ of\ immunized\ mortality}}{\mathbf{Percent\ of\ control\ mortality}}\right)\mathbf{x\ 100\%}$$

Antibody Titer Test

Antibody titers were calculated by taking fish blood on the 14th day. After the fish were vaccinated, the blood was centrifuged at 5,000 rpm for 5 minutes. After the serum was separated from the blood cells, the serum was transferred to Eppendorf and incubated at 44OC for 20 minutes to inactivate complement. The agglutination test was carried out in microplate titers by injecting 25 µl of PBS solution into each well, then 50 µl of serum was added to the first well. The serum and PBS solution were stirred to become homogeneous, then transferred to the second well as much as 25 µl and so on until serial dilution occurred up to the 11th well. As much as 25 µl of bacteria was inoculated into each well up to well 12, the microplate was shaken gently to homogenize the mixture in the well. Furthermore, the mixture of serum and bacteria was incubated at 37OC for 2 hours, then stored in the refrigerator at 4OC overnight until clots (fog) formed. The occurrence of clumps of small particles at the base of the microplate as an indicator of the presence of antibodies in the serum. Where the last well where there are lumps is the agglutination titre value. Table 1 shows the antibody titer values.

Table 1 Readings of antibody titer values

Observation hole number (n)	Serum dilution	Antibody titer (-log2)
1	1 : 4	2
2	1 : 8	3
:	:	:
:	:	:
11	1 : 4096	12
12		Control

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Calculation of haematological parameters

Observation of haematological parameters was carried out 3 times during the study, namely before the vaccine treatment, after the vaccine treatment, after the challenge test. This activity was carried out by taking blood samples from the test fish and then observing the number of leukocytes, phagocytic activity, and differential leukocytes. Blood collection using a sterile syringe that has been rinsed using sodium citrate (Na-citrate) 3.8% as an anticoagulant. Blood was taken from the caudal vein and then placed in a microtube which had also been rinsed with 3.8% Nasitrate for further observation. Haematological parameters measured include:

1) White blood cell count (Blaxhall and Daisley, 1973)

The blood sample was sucked using a white pipette to a scale of 0.5, then the Turk solution was sucked up to a scale of 5-11. The pipette is then shaken in a figure-eight shape for 3-5 minutes to homogenize the blood with Turk's solution. The first two drops from the pipette are discarded, the next drop is dropped on the hemacytometer to count the number of white blood cells. Observations were made using a microscope by counting the number of blood cells in five large hemacytometer boxes. Calculation of the number of white blood cells using the following formula:

$$\Sigma \text{ SDP} = \text{average of calculated cells x } \frac{1}{Large\ Box\ Volume} \text{ x Diluent factor}$$

2) Measurement of phagocytosis activity (Anderson and Siwicki, 1993).

Blood samples were taken as much as 50 µL and placed in sterile microtubes. The blood was then mixed with Staphylococcus aureus bacteria with a density of 108cfu/mL of 50 µL and homogenized. The mixture is then incubated for 20 minutes. After that, the mixture is taken 5 µL and dripped on the preparation glass to be used as a review preparation. After drying, the preparation is soaked in methanol for 5-10 minutes and then dried. After drying, the preparation is soaked in Giemsa solution for 10-15 minutes and then dried again. After drying, the preparation can be observed using a microscope and calculated the percentage of cells that actively carry out the phagocytosis process from 100 phagocytic cells observed.

Determination of the value of phagocytosis activity using the following formula:

$$\text{AF (\%)} = \frac{\Sigma\, Actively\ Phagocytic\ Cells}{\Sigma\, Phagocytic\ cells} \text{ x } 100\%$$

3) Leukocyte differential (Blaxhall and Daisley, 1973)

Leukocyte differential observations were carried out before fish were vaccinated and after fish were vaccinated and after fish were tested challenged. Observation of leukocyte differential begins with the preparation of a review. Review preparations are made by dripping fish blood on the object's glass, then air-drying. Next, the review preparation is fixed in a methanol solution for 5 minutes, after which it is soaked in giemsa solution for 15 minutes, then the review preparation is rinsed under running water and dried then covered with a glass cover. Leukocyte differential is observed under a microscope, the percentage of leukocyte cells is calculated by observing 10 fields of view and and each counted leukocyte cell is grouped and percentaged according to type.

Data Analysis

Data on Relative Percent Survival (RPS) and fish mortality, analyzed by fingerprint analysis to determine the effect of the treatment tried, if the results of the analysis obtained a real difference ($P<0.05$) then continued with the Duncan test. Data on total total leukocytes,

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phagocytic activity, leukocyte differential and antibody titer values were analyzed descriptively.

Mortality and Relative Per cent Survival (RPS)

The death of vaccinated and unvaccinated fish occurred 24 hours after the challenge test. The mortality of fish treated with the vaccine was significantly different from the control (fish that were not vaccinated) ($P<0.05$), while the treatment between each vaccine treatment was not significantly different ($P>0.05$) (Table 2). The mortality rate of vaccinated fish was lower than that of control fish, where the mortality rate of vaccinated fish was highest in treatment C, which was 10%. While the average mortality of control treatment fish was 50%, this indicated that the vaccine treatment was able to increase the body's immunity in fish. The RPS value is used to determine the effectiveness of the vaccine to protect fish after being challenged with virulent bacteria that cause disease.

Table 2. Post-vaccination fish mortality and SRP values

Treatment	Mortality (%)	RPS (%)
A	6,67[a]	86,67 [a]
B	3,33 [a]	93,33 [a]
C	10 [a]	80 [a]
D	3,33 [a]	93,33 [a]
E	6,67 [a]	86.67 [a]
K	50[b]	0 [b]

The results showed that the vaccine treatment had no real effect, while the treatment of vaccinated fish with control (without vaccine administration) had a real effect. The five types of vaccine treatment are able to provide protection against test fish from virulent bacterial infection after the challenge test with RPS values of 80-93%, this is characterized by a low mortality rate of vaccinated fish compared to the mortality of unvaccinated fish (control).

Vaccine treatment in fish can stimulate the ignorance of the fish body against virulent bacterial infections that induce it so that the mortality rate of vaccinated fish is lower. Vaccines are antigenic ingredients used to produce active immunity against a disease so as to prevent or reduce the influence of infection (Alifuddin, 2002).

Overview of Fish Blood

Fish leukocytes are also one of the non-specific defense systems. Leukocytes are divided into two groups, namely; Agranulocytes are lymphocytes and monocytes as well as polymorphonuclear granulocytes, depending on the absence or presence of fine granules in the cytoplasm (Alifuddin 1996). The success of vaccination can be seen from the resulting RPS value. However, to answer why there is success or failure of vaccination can be seen from several supporting parameters such as total leukocytes, leukocyte differential, phagocytic index, and antibody titer (Table 3).

Table 3 Some post-vaccination tilapia blood parameters

Treatment	Total Leukocyte (10^5 sel/mm)	Phagocytic Index (%)	Neutrofil (%)	Limposit (%)	Monosit (%)	Antibody Titer Value (-log2)
Control	71	42,86	23	45	32	3
A	82,4	52	25	50	20	6
B	76,8	60,7	29	46	35	7
C	76,8	56	29	45	26	7
D	82,6	55	22	46	32	7

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E	82	55	24	48	28	8

Leukocytes are blood cells that play a role in the immune system. Leukocytes help rid the body of foreign bodies, including pathogen invasion through the immune response system and other responses. Blood leukocyte levels of fish after vaccination have increased, both the treatment of vaccinated fish and unvaccinated fish (control). This shows that specific and non-specific immunity work together after vaccination, Rastogi (1977) states that normal fish blood counts range from 20,000-150,000 cells/mm3. Vaccine treatment is higher than control, this shows that vaccine treatment can increase the ability of immune system cells (leukocytes) to proliferate and differentiate due to bacterial infection. Sakai et al. (1995) stated that the leukocyte population increases due to an infection.

Phagocytosis is a defense mechanism in the body of an organism by swallowing foreign objects and then destroying them (Kamiso and Triyanto 1990). Phagocytosis is a natural defense mechanism against disease. While phagocytosis activity is the eating or ingestion of particulate objects by certain cells (Michael and Chan 2008). Phagocytic cells consist of monocytes, macrophages, and granulocytes. Phagocytic cells will recognize and engulf antigenic particles, including bacteria and damaged host cells through three stages of the process namely attachment, phagocytosis, and digestion (Irianto, 2005). Phagocytosis activity of vaccinated fish treatment is higher than unvaccinated fish. This also proves that vaccination can boost the immune system in fish through the mechanism of phagocytosis.

Leukocytes are the main cells of the body's defense system, so it is very important to know changes in the number or appearance of two groups of leukocytes in the blood circulation, namely agranulocytes and polymorphonuclear granulocytes. Tilapia has a fairly complete type of leukocyte, consisting of; agranulocytes are lymphocytes and monocytes and polymorphonuclear granulocytes are neutrophils, eosinophils and basophils. This is in accordance with what was revealed by Clem et al. (1985) and China but et al. (1991) that fish leukocytes consist of three types including lymphocytes, monocytes and neutrophils. Post-vaccination leukocytes observed were lymphocytes. Lymphocytes act as memory cells that form antibodies. The increase in lymphocytes was also in line with the formation of antibodies in fish after 5-10 days after vaccination. Monocytes and neutrophils play a role in the process of phagocytosis. Monocytes are more likely to phagocytize large particles while neutrophils are more likely to phagocytize small particles. The leukocyte differential values of vaccinated fish and control fish were relatively the same. This is a sign that the fish is fighting the infection. The overall leukocyte differential data showed that the vaccine components given were able to increase fish-specific and non-specific defense cells.

Antibodies are protein molecules produced by plasma cells as a result of interactions between antigen-sensitive B lymphocytes and antigens, where antibodies have a special ability to bind to antigens and accelerate their destruction and removal. New antibodies are discovered about a week after the first injection and their serum levels rise to a peak after 10-14 days (Tizard 1982). The antibody titer value indicates the formation of antibodies in fish after the vaccine administration.

The results of the antibody titer agglutination test analysis showed that the treatment of vaccine administration to fish through the vector Artemia sp. can form protective antibodies in the test fish. Based on Table 1, it can be seen that the antibody titer value of the fish during the study showed a value of 6 in treatment A, in treatment B, C, D the antibody titre value was 7. The highest titre value was shown in treatment E, with a value of 8. This indicates that the vaccine given was able to increase the immune system in the test fish to form specific antibodies. Differences in antibody titers between treated and control fish. The antibody titers in the treated fish were relatively higher than the control fish. This indicates that the vaccine is able to stimulate

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immunity in the body of the test fish. Tizard (1988) said that several factors that affect antibody responses are vaccine dose, time of vaccine administration, antigenicity of the bacteria, and the immunogenic response of the vaccinated fish. New antibodies are discovered about a week after the first injection and their serum levels rise to a peak after 10-14 days. The lowest titer value was shown in the control treatment (without vaccine administration), this indicated that the fish immune system was weak in protecting fish, characterized by high fish mortality, which was an average of 50% (Table 2). In the control treatment, antibody titers were still found, although the amount was small. This indicates that naturally tilapia already has an immune system. Vaccination will stimulate the natural immune system resulting in an increase in antibody titers.

Conclusion

Conclusion of efficacy testing of whole cell vaccines administered through Artemia sp. is a vaccine treatment able to provide protection against test fish from virulent bacterial infection after the challenge test with an RPS value of 80-93%, with a low mortality rate of vaccinated fish of 3.33-10% compared to the mortality of unvaccinated fish (control) which is an average of 50%. While the advice that needs further research is testing the efficacy of vaccines given through Artemia sp. with different time intervals.

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