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Research Article

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The making of vaccine from bacteria to prevent *Streptococcosis* disease in tilapia (*oreochromis niloticus*): Study at Sentani lake, Jayapura regency-Indonesia

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ABSTRACT

Diseases in fish could cause a decrease in fish productivity could even lead to the death of fish up to 90% of the total population. Fish disease is anything that can cause disruption in fish, either directly or indirectly, and these disorders are caused other organisms, feed or environmental conditions that do not support fish life. The objective of this study was to determine the doses that effective for inactivating streptococcosis on tilapia. Induction period of the vaccine and challenge test of clinic symptoms and determine the survival rate of tilapia after challenge test. Methods used in this study is completely randomized design (CRD) with three treatments. The use of the vaccine treatment from bacterial isolat O at a density of 10^7 cfu. From density of 10^7cfu vaccine divided into three different doses of the vaccineis: vaccine at a dose of 0.75 ml, vaccine at a doses of 1.25 ml, and vaccine at a dose of 1.75 ml. Parameter swereob served survival rate and clinic symptoms of tilapia. Here, we showed that the vaccine of the bacteria Streptococcus iniae with 10^7 cfu density at a dose of 1.75 ml inactivated by heat kill the density of the most effective and efficient in triggering immunity tilapia against bacteria Streptococcus iniae with the survival rate of 90%. This study provides a new channel of research in making vaccines fish diseases in freshwater fishery more efficient.

Keywords: *Streptococcus iniae*, tilapia, *Oreochromis niloticus*, *Streptococcosis*, vaccines, Sentani lake

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INTRODUCTION

Diseases in fish could cause fish mortality up to 90%, even in extreme environments can reach 100% [1-3]. According Afrianto and Liviawaty, fish disease is anything that can cause disruption in fish, either directly or indirectly. This disorder is caused by another organism (bully), feed or environmental conditions that do not support fish life [4-6].

Parasitic disease that attacks the carp and tilapia are generally caused by viruses, bacteria, fungi, protozoa and worms. *Streptococcosis* is a disease caused by the bacterium Streptococcus genus, one of which is the bacterium *Streptococcus iniae*. *Streptococcus iniae* are gram-positive bacteria which are round with a characteristic form pairs or chains during growth. Previous research has found such a bacterial disease caused by the bacterium Streptococcus iniae is a disease that always occurs in tilapia fish farming, causing huge losses also zoonotic for humans [7-8].

Until present, the solution to overcome the disease continues to be done in the laboratory Streptococcosis. The research is directed to find an alternative disease control Streptococcosis environmentally friendly in line with the regulations prohibition the use of antibiotics in order health, quality and food safety. Streptococcosis disease control

will be safer when done biologically, for example by using microorganisms (bacteria) that is able to inhibit the progression of the disease, but not pathogenic to fish.

This study focused on the manufacture of the vaccine bacteria of Tilapia fish in Lake Sentani, Jayapura regency, Papua province, Indonesia. This research is important to verify whether the bacteria Streptococcus iniea still a major cause of bacterial disease in tilapia are many causes harm to tilapia fish farming as well as causing zoonotic public health problems, in addition to know how to cope streptococcosis by setting the appropriate formula.

The purpose of this study was to determine the disease in tilapia (*Oreochromis niloticus*), which is caused by the bacterium *Streptococcus iniae*, and to determine the concentration of the vaccine with a high response against tilapia (*Oreochromis niloticus*), which is expected to increase the productivity of the fish at the hatchery stage and stage of enlargement, an increase in the provision of protein for humans.

EXPERIMENTAL SECTION

The method in this study through three stages: the method of the making of inactivated vaccine of *Streptococcus iniae*, vaccination methods, and the challenge test method.

Method of making of inactivated vaccine *Streptococcus iniae*
Making the medium Tripticase Soy Broth (TSB) (in test tubes) and Tripticase Soy Agar (TSA) (in Petri dishes), and *Streptococcus iniae* bacterial culture on Tripticase Soy Broth (TSB) medium and 24-hour incubation at room temperature. Move inoculum *Streptococcus iniae* of medium Tripticase Soy Broth (TSB) medium to Tripticase Soy Agar (TSA) and make sure the inoculum evenly throughout the layer agar, incubation for 24 hours at room temperature. *Streptococcus iniae* bacteria harvest, collect the spreader rod and insert into the Erlenmeyer flask using a funnel.

The next stage, carried inactivation of bacteria: the isolated bacteria are added as much as 2% formalin and then shake with water bath shaker and isolates heated for 30 min at a temperature of 100 °C. Incubation for 24 h at room temperature and to test the viability of bacteria in specific medium NA and incubation for 24 h at room temperature (if the bacteria are still growing, repeat weakening the bacteria by increasing the concentration of formalin solution). If the bacteria are not growing, followed by washing with PBS formalin using centrifuges at a speed of 4000 rpm for 1 h. Sentrifuse done 3 times (each centrifugation, the supernatant discarded).

Vaccination methods
Fish acclimatization test for 1 week, then dilute vaccine 10^7. Perform vaccination orally by inserting the vaccine into the mouth of tilapia with a density of 10^7 vaccine (0.75, 1.25, 1.75). Maintain fish that have been vaccinated for 1 week.

Challenge Test Method
Gives oral vaccine again by the fish that had been vaccinated with pathogenic bacteria and PBS as a control. Observing the fish and record the number of fish deaths during the first week and calculate the value of median survival.

The research design used was a completely randomized design (3 x 3) with three variations of doses treatment (0.75; 1.25; 1.75) and repetitions.

RESULTS AND DISCUSSION

Tilapia were pain with symptoms *Streptococcosis* used in this study was taken from the floating net cages in the lake Sentani, Jayapura regency, after the observation around the Sentani lake obtained samples of tilapia which average almost *Streptococcosis* disease. The fish seem to swim alone with clinical symptoms as in Figure below (**Fig 1**), namely the puffy eyes (exopthalmus), cloudy and there is a prominent and blood spots on the eyes.

Fig 1. Clinical symptoms *Streptococcosis* on tilapia fish coming from the Sentani lake, Jayapura regency, Papua province in Indonesia

Organ samples derived from tilapia which includes the kidneys, brain, eyes and gills isolated in the blood and moved in TSA medium and incubated 29 °C - 37 °C.

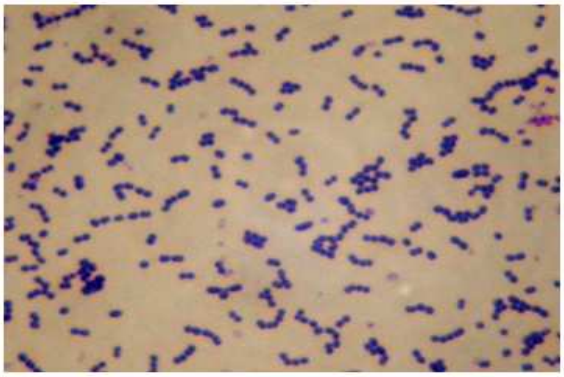


Fig 2. The bacteria *Streptococcus iniae*

In the four isolates were grown, there are some bacteria that have different isolates as in isolates:
1. Kidney, there are 3 (three) isolates, namely:
G.1. Isolates small round, smooth, haemolysis
G.2. Isolates were rounded, slightly cloudy
G.3. Isolates large round, slightly rough

2. The brain, there are four (4) isolates, namely:
O.1. Isolates small round, smooth green, haemolysis
O.2. Isolates small round, haemolysis
O.3. Isolates medium round, haemolysis
O.4. Isolates big round, rough

3. Eyes, there are four (4) isolates, namely:
M.1. Isolates small, haemolysis
M.2. Isolates large, rough, haemolysis
M.3. Isolates small, green, haemolysis
M.4. Isolates medium, haemolysis

4. Gills, there are four (4) isolates, namely:
I.1. Isolates large, rough, haemolysis
I.2. Isolates small, green, haemolysis
I.3. Isolates small, rough
I.4. Isolates medium, haemolysis

In the four samples obtained 15 isolates of bacteria and isolated on TSA media to be identified. From the 15 isolates characterized the bacteria and the results are as shown in Table 1 below.

Table 1. Character isolates bacteria that caused *Streptococcosis* from Sentani Lake

Character	*Streptococcus iniae*	G.1 isolates	O.1 isolates	M.3 isolates	I.2 isolates
Gram	+	+	+	+	+
Shape	cocci	cocci	cocci	cocci	cocci
Motility	-	-	-	-	-
Growing up in the air	+	+	+	+	+
Catalase	-	-	-	-	-
Oxidase	- (*1	+	-	-	+
OF	F (*1	F (w)	F	F	F(w)
Growth at 10 ^{0}C	+	+	+	+	+
Growth at 45 ^{0}C	-	-	-	-	-
6,5% NaCl	-	-	-	-	-
Haemolysis	α/β	β	α	α	α
Esculin	+	-	+	+	-
Lactose	-	-	-	-	+
Mannitol	+	+	+	+	+
Raffinose	-	-	-	-	-
Salicin	+	+	-	-	+
Sorbitol	-	-	-	-	-
Trehalose	+	+	-	-	+
VP test	- (*2	-	+	+	+
Glucose	+ (*2	+	+	+	+
Mac Concay Agar	-	-	-	-	-

Explanation:
*(*1 : Cowan and Steel in Manual for Identification of Medical Bacteria*
*(*2 : Buller in Bacteria from Fish and Other Aqutic Animal.*

From the test results obtained biochemical characteristics for 4 (four) isolates, that is gram positive, character cocci shape with length of chain and a cell diameter varies, catalase and oxidase negative and are fermentative.

Fig 3. The nature of the fermentative isolates O.1 and M.3 after 24 h

All isolates were grown at 10 °C, and could not grow at a temperature of 45 °C. Growth test at a concentration of 6.5% NaCl and all isolates do not grow. All isolates namely G.1, O.1, M.3 and I.2 have properties of haemolysis alpha/beta-hemolytic namely the formation of *halo* around the colony, blurred zone on blood agar media is the result of red blood cell hemolysis is not perfect. O.1 isolates are alpha hemolytic were characterized by the formation of clear zone or zones as a result of bright red blood cell hemolysis perfectly around colonies on media blood agar.

Isolates were purified and isolates obtained in the clear zone and repeat 3 times to make sure the isolate completely pure.

Fig 4. O.1 isolates on media blood agar

Test fermented sugars that conducted include lactose, mannitol, raffinosa, salicin, sorbitol, trehalose and glucose. Bacteria said to be able to ferment the sugar when it changes color from red to yellow medium.

Table 2. Biochemical test four isolates of Sentani lake compared with *Streptococcus iniae*

Character	*Streptococcus iniae*	G.1 isolates	O.1 isolates	M.3 isolates	I.2 isolates
Gram	+	+	+	+	+
Shape	cocci	cocci	cocci	cocci	cocci
Motility	-	-	-	-	-
Growing up in the air	+	+	+	+	+
Catalase	-	-	-	-	-
Oxidase	- (*1	+	-	-	+
OF	F (*1	F (w)	F	F	F(w)
Growth at 10^0C	+	+	+	+	+
Growth at 45^0C	-	-	-	-	-
6,5% NaCl	-	-	-	-	-
A-haemolysis	α/ β	β	α	α	α
Esculin	+	-	+	+	-
Lactose	-	-	-	-	+
Mannitol	+	+	+	+	+
Raffinose	-	-	-	-	-
Salicin	+	+	+	+	+
Sorbitol	-	-	-	-	-
Trehalose	+	+	-	-	+
VP test	- (*2	-	-	-	-
Glucose	+ (*2	+	+	+	+

Explanation:
*(*1 : Cowan and Steel in Manual for Identification of Medical Bacteria*
*(*2 : Buller in Bacteria from Fish and Other Aqutic Animal.*

Based on the above table isolates code O.1 and M.3 has a biochemical test results equal to 100% of bacteria *Streptococcus iniae*. To isolate code G.1 and I.2 are different biochemical test results with *Streptococcus iniae*. Isolates were selected for the vaccine is made isolates O.1 and M.3. From both these isolates that have a very good chance to be made in the vaccine are the isolates O.1, because it has a higher power haemolysis of isolates M.3.

Fig 5. Results of the vaccine isolates O.1

Tabel 3. Clinical symptoms of fish that are infected with a bacterium *Streptococcus iniae* of Sentani lake for 3 days

	Clinical symptoms							
Day - *n*	**O.1 isolates**	**% M**	**M.3 isolates**	**% M**	**I.2 isolates**	**% M**	**fish control**	**% M**
1	+	10,30	+	6,66	+	6,66	N	0
2	++	66,6	++	46,66	++	46,66	N	0
3	++	100	++	100	++	76,66	N	0

Notes:
Symptoms disorders included: anorexia, weakness, and swim at the surface.
+ < 3 fish have symptoms of disorders
++ : ≥ 3 fish test have symptoms of disorders
% M: % Morbidity (prevalence) number of sick fish/number of test fish x 100%.

Koch's postulates performed to see if the bacteria is causing the symptoms. Table 4 shows that the 3 (three) isolates were injected into the fish causing the fish weak condition, anorexia and tend to swim at the surface. Conditions characterized by weak fish fish swim just passively in the water.

Table 4. Molarity and the average survival rate of test fish for 1 (one) week challenge test period

Doses of treatment	Mortality of fish test Repetition							Amount	Median survival (%)
	1	2	3	4	5	6	7		
0.75 ml		3		2		3		8	26.66
1.25 ml		2	3	1			4	10	33.33
1.75 ml					2			28	93.33
Control			1					29	96.66

Information:
A : Tilapia vaccinated with the density of the bacteria *Streptococcus iniae* 10^7cfu with dose of 0.75
B : Tilapia vaccinated with the density of the bacteria *Streptococcus iniae* 10^7 cfu with dose of 1.25
C : Tilapia vaccinated with the density of the bacteria *Streptococcus iniae* 10^7 cfu with dose of 1.75
D : Tilapia vaccinated with 0.1 ml Phosphate Buffer Saline (PBS)

Observations on mortality and survival rate of tilapia every day for one week test period challenged with *Streptococcus iniae* bacteria were inactivated with head kill 100 °C for 30 min showed that each density of bacteria with different doses give different survival rates.

Table 5. The average survival rate of fish and significance of giving treatment

Treatment	The average of survival rate	Sig (50%)
A. (0.75 ml)	26.66	a
B. (1.25 ml)	33.33	b
C. (1.75 ml)	93.33	cd
D. (control)	96.66	cd

Notes : Each live notations indicate that there are differences in treatment that has the same notation.

In Table 5, shown in the treatment c of bacterial density 10^7 cfu with with a dose of 1.75 ml greater than the other treatments, namely 93.33%. It is claimed that the vaccine of *Streptococcus iniae* bacteria with a dose of 1.75 ml with a density of 10^7 cfu is the density of bacteria that could trigger fish immunity against attacks *Streptococcosis* can be seen from the clinical symptoms milder than the other treatments.

Table 6. The average survival rate of fish and fish relative survival challenge test

Treatment	The average of survival rate	Sig (50%)
A. (0.75 ml)	26.66	26.58
B. (1.25 ml)	33.33	33.48
C. (1.75 ml)	93.33	95.55
D. (control)	96.66	-

Relative survival > 50%, it given an effective vaccine to be used, the average survival is calculated by the formula Ellis [9]

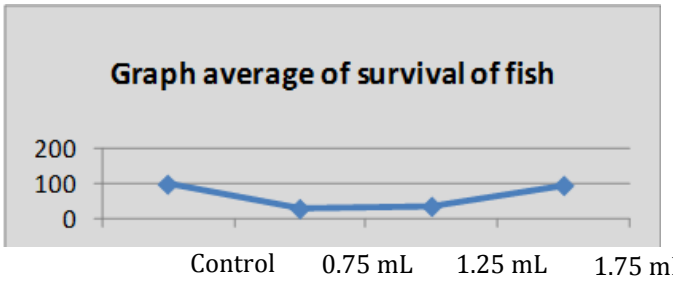
Table 7. The range of water quality maintenance media and the results of water quality measurements for research

No	Parameters	Units	Quality Standards	Test Results
1	Temperature	^{0}C	Deviation 3	27.1
2	pH	-	6.0 – 9.0	6.88
3	Dissolved Oxygen (DO)	mg/L	≥ 6	6.65
4	Ammonia as (NH_3-N)	mg/L	0.5	1.24
5	Nitrate as (NO_3-N)	mg/L	10	14.0
6	Nitrite	mg/L	0.06	0.065
7	Sulfide (S-H_2S)	mg/L	0.002	0.008

Value parameter of water quality maintenance media for research in the range appropriate to the maintenance of tilapia (*Oreochromis niloticus*)

Tilapia fish samples taken from floating cages of Jayapura regency almost all *Streptococcosis* disease that causes the bacterium *Streptococcus iniae*. In the previous table, explaining that the rate of spread of the disease *Streptococcosis* against other tilapia occurred on the third day around 76.66% - 100%. This shows that Streptococcosis disease that causes bacterial pathogens. If tilapia disease *Streptococcosis* not handled properly will result in huge losses for cultivators of tilapia.

Survival of the fish is a fish life level after vaccinated. The survival rate in this study 90%, it is influenced by the physical state of the aquarium maintenance, water quality, the number of fish and how to of maintenance.

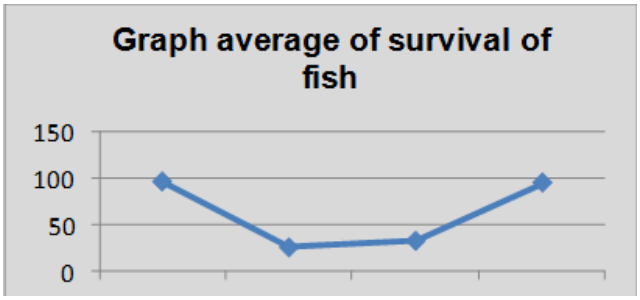


An average survival or the relative protection level is used to indicate the level of effectiveness of the vaccine or a vaccine for protecting fish from bacteria. According Kamiso, that the results of laboratory tests in which average of survival vaccination around 58-100%. MTD (*Mean Time To Death*) or average day of death, vaccination does not always affect the fish's death. On vaccination to prevent bacteria *Streptococcus iniae* say that although vaccination increases the level of protection of fish, it turns out average of time of death was not different between vaccinated and control fish [10-15].

Vaccination of fish increases the durability of not only humoral but also cell in the body's defenses. Between cellular and humoral work not only individually but also cooperate with each other. When the defense, both humoral and cellular increases, the antibodies will also be increased.

Vaccine antigen has a very close relationship because the vaccine is a material or antigen is intentionally inserted into the body to stimulate specific immune in fish. Antigen also has a close relationship with antibody titers, because the type of antigen will determine the high titer antibody. Furthermore, antigenic variation of bacteria used not only on the type of antigen but also the amount of antibody titers formed. Thus, the high titer antibodies depending on the type of antigen used and antigenic variation of the bacterium *Streptococcus iniae*. If the antibody titer is high then the rate of survival of fish will also be high. Because the better the efficacy of vaccines used to stimulate lymphocyte cells into forming antibodies the better the humoral and cellular defense. This will suppress the high mortality rate due to bacterial infection and also will increase the growth rate.

The test results of the bacteria *Streptococcus iniae* vaccine with bacterial density 10^7cfu given doses of 0.75 ml, 1.25 ml and 1.75 ml give different results. In the previous showed that mortality and survival rates of fish every day for a week during the challenge test with a dose of of 0.75 ml showed an average survival of fish 26.66%; 1.25 ml dose showed average survival of fish 33.33% and 1.75 ml dosage showed average survival of fish 93.33%. In this case the dose that can inhibit *Streptococcosis* disease in tilapia vaccine with a dose of 1.75 ml.



Based on the results of this study concluded that the vaccine of *Streptococcus iniae* bacteria isolates O.1 with a dose of 1.75 ml and a density of 10^7 cfu were in the inactivation with heat kill the density of the most effective in triggering the immune tilapia against *Streptococcus iniae* bacteria to prevent disease *Streptococcosis* on tilapia (*Oreochromis niloticus*) with a 90% survival value.

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