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Application of Inactivated *Aeromonas salmonicida* Vaccine Towards *Cyprinus Carpio* Koi Fish Imunogenity.

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ABSTRACT

Aeromonas salmonicida is bacterium causing bleeding, muscle lesion, colitis, spleen enlargement and death in gold fish. The bacterium brings disadvantage to fish farmer because it spreads quickly, is lethal and reduces production and as the effect, severely infected fish should be gotten rid of. Therefore, a more natural approach to prevent disease caused by *A. Salmonicida* is needed; one of the method to prevent the bacterial infection is vaccination. The study used independent variable in the form of administration of inactivated *A. Salmonicida* vaccine with the following dosage, namely A: 104cell/milliliter, B: 106 cell/milliliter, C: 108 cell/milliliter, and D: 1010 cell/milliliter with three replications and two controls as comparison, positive and negative control. The parameters were viability test, antibody titer, clinical symptoms, RPS (Relative Percent Survival) and quality of water. Antibody titer, clinical symptoms, RPS (Relative Percent Survival) and quality of water analyses were conducted after 1 to 14 days after the challenge test and administering vaccine 1 and 2 (booster). The findings showed that treatment C (108) was the most significant treatment to prevent infection caused by *A. Salmonicida* bacterial pathogen. The symptoms are the highest increase of antibody titers with 1 vaccination score of 2.107 and the score increases to 2.408 with the highest RPS score of 87.46%. The clinical symptoms reveal that the fish recovers on the 8th-14th day after the infection.

Keywords: *Aeromonas Salmonicida, Cyprinus Carpio,* Antibody Titers, RPS

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INTRODUCTION

A. Salmonicida is obligate bacterium that means type of bacterium that cannot live without its host and is not motile. *A. Salmonicida* obligate bacterium is one of the etiological agents for furunculosis, a disease that causes septicemia, bleeding, muscle lesions, intestinal inflammation, spleen enlargement and death in salmonid fish[1].

The bacterium spreads quickly, is lethal and reduces production and as the effect, severely infected fish should be gotten rid of bringing significant loss for fish farming[2]. Therefore, a more natural approach to prevent disease caused by *A. Salmonicida* is needed; one of the method to prevent the bacterial infection is vaccination. Zafran et al.[3] stated that vaccination was predicted to provide fish specific immunity towards particular disease. Non-specific immunity refers to innate immune system (innate immunity). It involves mechanical and chemical immunity (mucus, skin, scale and gills) and cellular imunity (makrofag cell, leukocytes such as lymphocytes, monocytes, neutrophils, eosinophils and basophils)[4]. The first immunity is physical involving scale, skin and mucus. Mucus is able to prevent microorganism colonization on fish skin, scale and mucosa. Damage on fish skin or scale makes it easier for pathogen to infect host[5].

It is expected that administration of inactivated *A. Salmonicida* vaccine increases fish immunity, protection for certain disease as well as both cellular and humoral response mechanism[6]. Active vaccination is an example of using antigen stimulation for increasing natural and adaptive immune response by producing specific humoral immune response and immunity between cell-mediated immunity towards pathogen and specific antigen[7]. Effective vaccination lowers down fish mortality against certain infection/ disease[8].

MATERIALS AND METHODS

Preparing the Fish

The koi fish (*Cyprinus Carpio*) came from koi fish cultivation in a village called Kemloko, Blitar. 8-10 centimeter fish was placed inside aquarium. Each aquarium consisted of 10 random fish. The fish was acclimatized for seven days before treatment in order to give some time for the fish to adapt to new environment.

Making Inactivated Aeromonas salmonicida Vaccine

The culture of bacteria in TSB medium according to dosage was centrifuged at the speed of 3500 rpm for 10 minutes. The supernatant formed was removed and PBS was again added; the process was repeated 3 times. Once the TSB medium was removed completely from the bacterial sediment, PBS was added to the sediment and they were activated for 24 hours using 2% formalin. The bacteria were incubated at the temperature of 25°C, centrifuged once using PBS at the speed of 3500 rpm for 10 minutes and stored at the temperature of 4 ° C.

Administering the Vaccine

There were four different dosage of the vaccine namely, treatment A: 104cell/milliliter, treatment B: 106 cell/milliliter, treatment C: 108 cell/milliliter, and treatment D: 1010 cell/milliliter, while K: shoulen broth. The vaccine was administrated on the first and eight day (booster) by injecting the 0.1 milliliter of the four vaccines to each fish.

There were several types of vaccine administered for cultivated fish such as whole cell vaccine, cell component, and DNA vaccine. Vaccine administration relied on type of bacterium, fish condition and the environment[9].

Challenge Test of Koi Fish (C.carpio)

Challenge test was conducted one week after booster administration on day 15. The challenge test referred to administering 108 cell/milliliter of *A. Salmonicida* by soaking. Spectrophotometer (λ=625 nm) was used to analyze density. The bacteria used for the challenge test was *A. Salmonicida*.

Post Challenge Test Observation

Antibody titer observation was conducted on day 7 and 14 while clinical reaction and Relative Percent Survival (RPS) analyses was conducted for 14 days after the challenge. At last, water quality observation was conducted during the period of the study.

RESULTS AND DISCUSSION

Viability Test of the Vaccine

In viability test, PBS was added to suspension of bacteria in which 2% formalin was used to inactivate the *A. Salmonicida*. The volume of the PBS was similar to that of the initial volume of the bacterial culture. The *A. Salmonicida* bacterial culture was planted in BHIA (medium) for 72 hours. *A. Salmonicida* was considered inactive when there was not any colony on the medium (BHIA). When the bacterium was no longer grown, the formalin was centrifuged using PBS with the speed of 3500 rpm for 10 minutes. The centrifuge was conducted 3 (three) times. Spectrophotometer (λ=625 nm) was the instrument to analyze density of the inactivated vaccine while McFarland was used as the standard in the analysis.

Antibody Titer

Objective of antibody titer was to describe influence of the vaccine towards the amount of antibody within the koi fish blood serum. Based on an analysis towards the koi fish antibody titer, the researchers obtained antibody titer prior to and after the vaccination and booster. The result of the analysis was described in Table 1.

Table 1. Antibody Titers Score

Treatment	Day	
	Day 7	**Day 14**
K- (0)	1.204	1.204
A	1.505	1.806
B	1.806	2.107
C	2.107	2.408
D	1.505	2.107

Table 1 showed an increase from prior to vaccination (pre-vaccination), after the first vaccination, and after the second vaccination (booster) for all of the treatments. There was sharp increase in antibody titer of the vaccinated fish compared to the control. The antibody titer of the vaccinated fish improved after the challenge test. Released antibody was found in the serum. Major function of the antibody was immunity from viral and bacterial infection (extracellular). Munasir[10] stated that immune response towards extracellular bacteria through phagocytosis mechanism by neutrophils, monocytes as well as tissue macrophages. Bacterial resistance towards phagocytosis and damage in the macrophage showed bacterial virulence. Activation of complement without antibody also played significant role in eliminating extracellular bacteria. Hardi et al.[9] explained that antibody accelerated antigen elimination through opsonization process (antibody as opsonin). It would be easier for macrophage to recognize opsonized antigen and therefore, it was more effective to get rid of the antigen.

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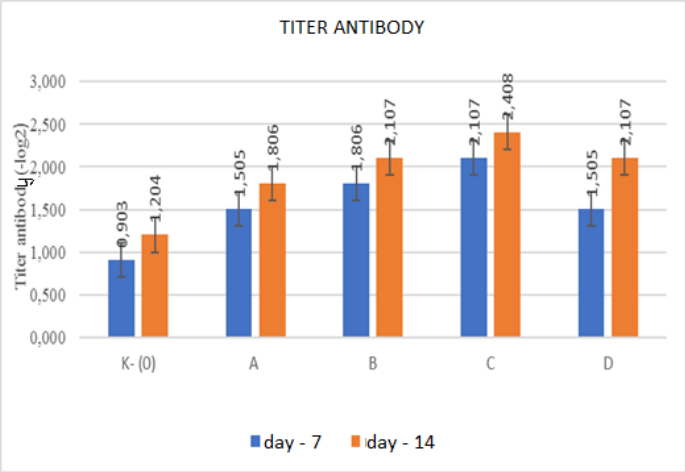
Different increase in the vaccinated fish antibody titer identified the fish specific immunity. Playfair and Chain[12] postulated that adaptive immunity was based on specific characteristics of the lymphocyte (T and B) that was able to selectively respond different antigen resulting in development of specific memory. As the result, specific immune mechanism began with introduction to antigen.

Figure 1 showed increase of antibody titer in treatment A, B and C showing that different dosage of vaccine affected increase of antibody titer. Treatment C resulted the highest antibody titer; the score increased from 2.107 to 2.408. Verma dan Agarwal[13] mentioned the increase explaining that increasing titer happened because immune response would be faster and production of antibody would be higher than the first infection when it was exposed to the same antigen during the challenge test.

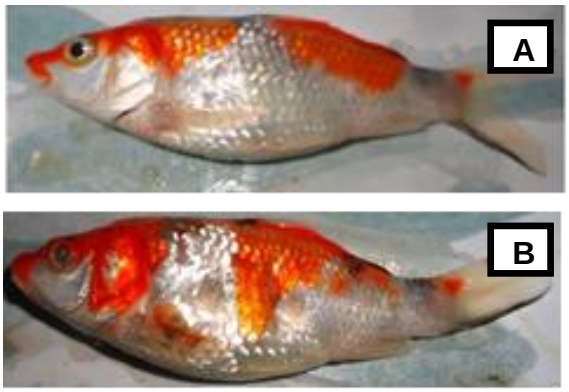
Figure 1. Antibody Titers Score



Immune response towards antigen was divided into two, extracelular and intracelular antigen. Extracellular antigen referred to antigen that enter outer part of the host cells; it did not enter inside part of the cells. Naturally, extracellular antigen took place in general bacterial infection, paracite and fungus. On the other hand, intracellular antigen was one infecting the inner part of the cells such as viral infection and several bacteria that was able to cause infection to the inner cells. Munasir[10] described that immune response was triggered when antigen of microorganism entered the body and met macrophage cell which later became Antigen Presenting Cell (APC).

Clinical Symptoms

Figure 2. (A) Normal Fish; (B) Fish infected by *A. Salmonicida*.



Clinical symptom observation was conducted during the infection period and 14 days after the challenge test because according to Skinner[7], non-specific immune response would be fluctuated right after or

few days after the invasion of antigen while specific immune response was developed within weeks. Both of the immune response played pivotal role in development of immune response mechanism towards pathogen attack in fish. Maftuch[11] mentioned that inflammation took place when there was tissue damage due to infection or antigen-antibody reaction. Figure 2 described both normal fish and fish infected by *A. Salmonicida*.

On the third day after the challenge test, the color of the fish started to fade, the scales started to fall, the fish swell and some of the fish swam in circle. Austin and Austin[1] suggested that histopathologically A.almonicida caused hemorrhage, skin and kidney necrosis, degeneration of skin and liver muscle, as well as fusion in the second lamela of the fish gills. The vaccinated fish had various clinical symptoms but some of them went back to normal on day 8 to 14 after the infection. The fish injected by the bacteria had faster clinical symptom change. Hardi et al.[9] stated that swimming pattern change, response towards fish feed and change in fish eye and clear operculum approximately appeared 6-12 hours post-injection.

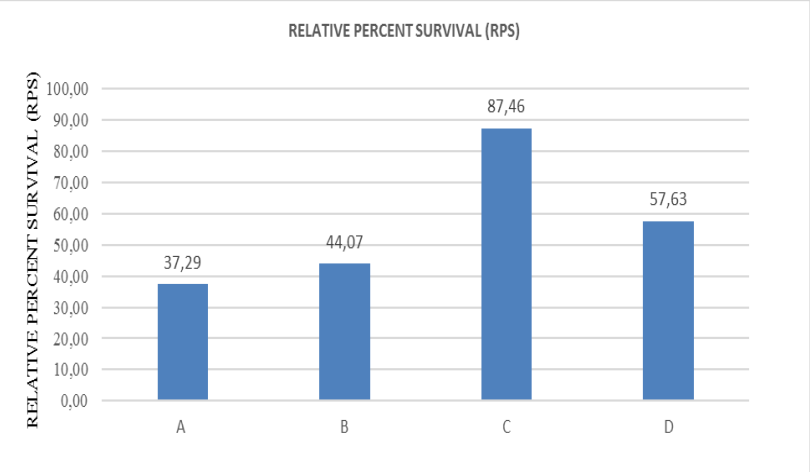
Relative Percent Survival/ RPS)

Table 2 showed the RPS scores of the infected koi fish. High and low RPS scores in the study referred to dosage of the vaccine. The density of the inactivated bacterium in the vaccine dosage had major influence towards the fish health. The fish was challenged with *A. Salmonicida* on day 15 and then analyzed whether it was still alive or not for 14 days after the challenge test.

Table 2. Relative Percent Survival/RPS Score

Treatment	RPS
A (10^4)	37.29%
B (10^6)	44.07%
C (10^8)	87.46%
D (10^{10})	57.63%

Figure 3. Relative Percent Survival/RPS Chart



Based on the Relative Percent Survival (RPS) analysis, the RPS of treatment A where bacterial density was 104 was 37.29%, the RPS of treatment B where bacterial density was 106 was 44.07%, the RPS of treatment C where bacterial density was 108 87.46%, and the RPS of treatment D where bacterial density was 1010 was 57.63% Relative Percent Survival (RPS) referred to comparison of relative percentage between mortality of vaccinated fish and that of the control fish. Figure 3 indicated that different dosage of vaccine affected level of mortality.

Based on the RPS chart, some fish died even though they had been vaccinated. However, Grisez and Tan[14] argued that the minimum fish RPS was 60% when vaccination was successful. On the other hand,

Purwaningsih et al.[4] mentioned that the level of mortality of vaccinated fish was lower than 24%. Referring to the treatment of the study, the RPS of treatment C was higher than 60%, and therefore, it was suitable dosage/ combination for vaccine.

Water Quality

Quality of water was a vital component to keep the fish alive. The methods to maintain the quality of water were circulation system, changing water constantly or recirculation with help of filter. Table 3 described the parameters indicated the quality of water in the study.

Table 3. Water Quality Parameter Scores

Parameter	Findings	Literature
Temperature	26-27 °C	25-30 °C
pH	7.69-8	6.75-8.2
DO	5.7-6 mg/l	>5 (Partosuwiryo and Warseno[15]).

Based on Table 3, it was concluded that the quality of water was within the normal range. Ghufran et al.[16] postulated that analyzing quality of water was one of the pivotal steps in aquaculture since it determines productivity of fish and other marine organisms. Components frequently analyzed to analyze water quality were, for example, temperature, clarity, pH, DO, CO2, alkalinity, hardness, of water, phosphate, and nitrogen dan lainnya. Firdaus[17] stated that in high yet tolerable temperature, fish would accelerate production of antibody and increase reaction of antibody being produced

CONCLUSION

The conclusion is inactivated Aeromonas salmonicida vaccine in treatment C (108) has the most significant influence to prevent infection caused by A.salmonicida bacterial pathogen. The symptoms are the highest increase of antibody titers with 1 vaccination score of 2.107 and the score increases to 2.408 with the highest RPS score of 87.46%. The clinical symptoms reveal that the fish recovers on the 8th-14th day after the infection.

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