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Antibody Response in The Blood of Cantang Grouper Infected by Viral Nervous Necrosis with Recombinant Protein *Chlorella vulgaris* Vaccination

Choirul Huda[1], Uun Yanuhar[2*], Muhammad Musa[2], Yusuf Arif Wahyudi[3], Nur Sakinah Junirahma[1] and Nico Rahman Caesar[4]

[1]Master Program of Aquaculture, Faculty of Fisheries and Marine Science, Brawijaya University, Jl. Veteran, Malang, East Java 65145, Indonesia
[2]Department of Waters Resources Management, Faculty of Fisheries and Marine Science, Brawijaya University, Jl. Veteran, Malang, East Java 65145, Indonesia
[3]Fish Quarantine Center for Quality Control and Safety of Fishery Products Surabaya I, Ministry of Marine Affairs and Fisheries, Surabaya, East Java 60115, Indonesia
[4] Doctoral Program of Environmental Science, Postgraduate Program, Brawijaya University, Jl. Veteran, Malang, East Java 65145, Indonesia

*Correspondence :
doktoruun@ub.ac.id

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Abstract

Cantang grouper *(Epinephelus* sp.) is one of the Export Oriented commodities which is a mainstay in increasing Indonesian mariculture production. The disease that causes many deaths in cantang grouper is Viral Nervous Necrosis. One of the most effective ways is by giving vaccines to fish. This study aims to develop a recombinant protein Chlorella vulgaris vaccine with the addition of an adjuvant to increase the immunity of cantang grouper infected with VNN. The method used was experimental with a Completely Randomized Design (CRD) consisting of seven treatments and three replications, namely (K+) cantang grouper infected with VNN, (T1) Healthy cantang grouper was given 33 μl of vaccine, (T2) Healthy cantang grouper was given vaccine of 66 μl, (T3) Healthy cantang grouper was given 112 μl of vaccine, (T4) Healthy cantang grouper was given 33 ul of vaccine and then tested against Viral Nervous Necrosis, (T5) Healthy cantang grouper was given 66 ul vaccine and then tested against Viral Nervous Necrosis, (T6) Healthy cantang grouper was given 112 ul vaccine and then tested against Viral Nervous Necrosis. The fish used was 8-10 cm in size and were reared for 56 days. The results showed that the *Chlorella vulgaris* protein recombinant vaccine with the addition of *adjuvant* gave a very significant effect on the immunity of cantang grouper infected with VNN with the best dose of 33 μl (T4) seen from the relative protection level (RPS) (75,6%), survival of cantang grouper (78%) after challenge test and a significant increase in antibody titer.

INTRODUCTION

Aquaculture makes a significant contribution to the animal food-producing sector, which grows and develops the fastest by providing almost half of the total

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fish production in the world, especially in food security and socio-economics in various countries (Dadar *et al.*, 2017). One of them is seawater aquaculture, which is currently growing, both in the use of technology and in the scale of its business. Grouper fish is one of the most important commodities because it is Export Oriented which has a high selling value when the dollar exchange rate strengthens so grouper cultivation is a mainstay in increasing the production of Indonesian mariculture commodities (Widjayanthi and Widayanti, 2020). According to Marine Affairs and Fisheries statistics, the total export value of grouper in Indonesia in 2018 reached 16,8 tons/year which is around 5,32% and increased in 2021 to reach 81,7 tons with a value of 19,9 billion exported to various countries. The type of grouper that has enormous potential to be developed into a sustainable and promising aquaculture commodity is the cantang grouper.

Cantang grouper is the result of crossbreeding between female tiger grouper *(Epinephelus fuscoguttatus)* and male kertang grouper *(Epinephelus lanceolatus)* conducted in 2009 at the Center for Brackish Water Aquaculture (BPBAP) Situbondo (Jiet and Musa, 2018). Cantang grouper has several advantages as a hybrid fish including a high selling price (Suhaili *et al*., 2020), faster growth with a low FCR (Triastuti *et al.*, 2018; Lutfiyah and Budi, 2019), more adaptive and resistance to diseases (Fitrawati *et al.*, 2015; Bunlipatanon and U-taynapun, 2017), tolerance to salinity and low water pH (Firdaus *et al.*, 2016; Arrokhman *et al*., 2017). However, the main problem in the aquaculture industry economically is the emergence of disease outbreaks. One of the diseases that cause death in cantang grouper is Viral Nervous Necrosis (VNN).

According to Khumaidi *et al.* (2019), VNN can cause mass mortality to up to 100% in groupers. VNN infection attacks the central nervous system, retina of the eye, and reproductive organs resulting in damage to tissue structures such as necrosis, hyperplasia, brain vacuolation, and hypertrophy (Yanuhar and Khumaidi, 2017). The clinical symptoms shown vary according to differences in the time of transmission, age of fish, route of infection and the immune system of the fish. There are several clinical symptoms in cantang groupers infected with the VNN virus such as decreased appetite, weakfish movement (passive), pale body color, producing a lot of mucus, fish swimming irregularly and spinning or whirling upside down (Sembiring *et al.*, 2018).

One of the most effective ways to reduce production losses from VNN disease is by administering vaccines to fish. Vaccination provides an effective solution in combating the risk of disease in fish farming by increasing the immune system to fight invasive organisms that enter the body (Yamaguchi *et al.*, 2019). Appropriate vaccine application can prevent disease in fish to provide the best solution to the harmful effect of using antibiotics (Mohd-Aris *et al.*, 2019). The type of vaccine that can be used in the prevention of VNN is the *Chlorella vulgaris* protein recombinant vaccine (P-PERCv).

The P-PERCv recombinant vaccine has advantages including being able to be used immediately, more stable, inexpensive, easy to monitor control, good safety and free from materials that can inhibit or infect species (Yanuhar, 2021). According to Yanuhar (2020), disease prevention in superior fish commodities through the provision of recombinant P-PERCv vaccine can reduce fish mortality rates due to VNN disease ranging from 30-40% compared to without vaccine application which reaches 50-90% and the level of protection against VNN virus infection reaches 70-90%. However, the problem is that it is easily degraded in the body and only induces an immune response in the short term. To overcome this problem, a vaccine was developed that can increase fish immunity faster and maintain antigen integrity by adding an

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adjuvant to the vaccine such as a mineral oil adjuvant.

Completed Freund's Adjuvant (CFA) is an oil-in-mineral emulsion (W/O emulsion) with mycobacteria killed by heat and Incomplete Freund's Adjuvant (IFA) without mycobacteria. Song *et al.* (2018) stated that the addition of CFA adjuvants effectively provided a relative protection level of 75% against bacterial invasion. Furthermore, Zhang *et al.* (2017) the addition of IFA adjuvants gave a relatively high survival percentage of 70,9% and 64,7% respectively in halibut *(Paralichthys olivaceus)*. Therefore, this study aims to develop a recombinant vaccine for *Chlorella vulgaris* protein with the addition of an adjuvant which is expected to able to provide vaccine delivery in fish bodies to be more effective and efficient.

METHODOLOGY

Place and Time

This research was conducted from June to August 2022. Sampling was conducted at UD. Giso Bangkit Situbondo and sample analysis at the Laboratory of Animal Physiology of UIN Malang, Laboratory of Molecular Biology, Faculty of Science and Technology UIN Malang, Laboratory of Anatomical Pathology, Faculty of Medicine, Universitas Brawijaya Malang.

Research Materials

The preparation stage begins with cleaning the plastic tub/bucket using soap, then rinsed with clean water and dried for 1 day. This study uses plastic buckets with a volume of 30 liters as many as 24 pieces filled with 20 liters of water. Each rearing tank contains 20 cantang groupers *(Epinephelus* sp.) and the rearing water is cleaned and replaced every day by as much as 60% depending on water conditions to maintain good water quality.

The test fish used were grouper cantang *(Epinephelus* sp.) measuring ± 8-10 cm with an average weight of 12,4 - 16 g/head obtained from UD. Giso Bangkit Situbondo. Before the fish is used, acclimatization is carried out for weeks. Maintenance was carried out for 56 days and fed *ad libitum,* namely feeding by little until the fish were full. The fish used for the seven test groups were 420 fish.

Research Design

The method used in this study is an experimental method with a factorial Completely Randomized Design (CRD) with seven treatments with three replications to reduce the error rate. The treatments dose used in this study refers to Yanuhar *et al.* (2020) namely (K+) Cantang grouper infected with VNN; (T1) Healthy cantang grouper with a given dose of 33 μl vaccine; (T2) Healthy cantang grouper with a given dose of 66 μl vaccine; (T3) Healthy cantang grouper a given dose 112 μl vaccine; (T4) Healthy cantang grouper is given 33 μl of vaccine then tested against VNN; (T5) Healthy cantang grouper is given 66 μl of vaccine then tested against VNN; (T6) Healthy cantang grouper is given 112 μl of vaccine then tested against VNN.

Work Procedure

Production of *C. vulgaris* Protein Recombinant Vaccine

The production of P-PERCv recombinant vaccine refers to the procedure of Yanuhar (2020) through several stages, namely screening and identification of marine microalgae samples. good and potential. Then, microalgae *C. vulgaris* was cultured from laboratory to mass scale. Followed by the isolation of Pigment-Protein Fraction (PPF) from *C. vulgaris* and characterization of the p-Percv target gene protein. The results of *C. vulgaris* RNA isolation were then tested for nano drops to obtain concentrations.

Furthermore, the production and propagation of the recombinant were carried out using a cloning technique, namely transforming into *E. coli* using a pTA2 vector so that there is no content or contamination from other materials. After

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that, the detection of recombinant vaccine genes in plasmid DNA using RT-PCR technique with primers T3 (3'-CTTTAGTG AGGGTTAAT-5') and T7 Promoter (3'-TAATACGACTCACTATAGGG-5'). The results of cloning and recombinant propagation were then stored in the form of glycerol stock and the supernatant was taken for treatment.

Formulation of Recombinant Vaccine with Adjuvant

In the study, the parameter to be tested is the use of adjuvant CFA/IFA in *C. vulgaris* protein recombinant vaccine. P-PERCv recombinant vaccine formulations were added with CFA/IFA adjuvant. The comparison of ingredients P-PERCv recombinant vaccine and adjuvant used in the formulation is 1:0,25. This comparison is obtained from the calculation of the dose to be used given to fish namely (a) 33μl; (b) 66 μl; (c) 112 μl.

In Vivo Test

In vivo testing was carried out to see the effect of administering recombinant protein *C. vulgaris* vaccine which was administered using the oral method, namely through the fish's mouth. The P-PERCv recombinant vaccine was given 2 times, namely the P-PERCv protein recombinant vaccine with adjuvant CFA on day 0 and the P-PERCv recombinant protein booster vaccine with IFA adjuvant on day 4.

Challenge Test

The method of VNN infection is through intramuscular injection and feed in the form of trash fish meat (positive VNN that has been tested previously) then cut into small pieces as much as 5 grams/ head. VNN-infected fish were obtained from Broadstock in Situbondo. VNN testing used a PCR technique with VNN specific primer, target gene 294bp (Wahyudi *et al.*, 2018). This feeding was carried out 4 times, namely on the 0, 2, 4, and 7 days.

Absolute Weight Growth

The absolute weight growth is the difference between the initial length/ weight and the final length /weight of fish using the Febri *et al.* (2020) formula as follows:

$L = L_t - L_0$
$W = W_t - W_0$

Where:
L = total length growth rate (cm)
L_0 = initial length (cm)
L_t = final length (cm)
W = total weight growth rate (g)
W_0 = initial weight (g)
W_t = final weight (g)

Relative Protection Survival

Measurement of the relative protection level of cantang grouper (*Epinephelus* sp.) was carried out to determine the effectiveness of a vaccine. The relative protection level measured by the relative percentage survival value using the Zhang *et al.* (2017) formula is as follows:

$$RPS = 1 - \frac{\%\ \text{mortality of vaccinated fish}}{\%\ \text{control fish mortality}} \times 100\%$$

Survival Rate

The Survival rate of fish is calculated from the percentage of the number of fish that live at the end of the rearing period compared to the number of fish calculated by the formula:

$$SR = \frac{Nt}{No} \times 100\%$$

Where:
SR = survival rate
No = initial number of fish
Nt = final number of fish

Antibody Titer

Measurement of antibody titer using the Jiang *et al.* (2019) method, which is a direct agglutination technique using a 96-well V-bottom microtiter plate. Observations were made on several samples of test fish. The blood serum of the test fish was put into a 1.5 ml microtube. Serum was centrifuged at 3500 rpm for 10 minutes. Then, the agglutination test was carried out in a

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microplate with a serum composition of 50 μl and 50 μl of VNN antigen. The presence of antibody agglutination in the well. A positive reaction was indicated by the absence of serum sedimentation or in the form of dots/clots in a 966-well V-bottom microplate. At the highest dilution level, the agglutination still occurred were calculated as the log 2 value of the antibody titer of the test fish. The antibody titer test data was then converted using the Inayati *et al*. (2020) formula and then analyzed descriptively.

Data Analysis

Data analysis was carried out using the statistical analysis method on the interval of 95% confidence (alpha=0.05). The design used is Complete Random Design (CRD) with one factor using IBM Statistical Software SPSS statistics version 16.0.

RESULTS AND DISCUSSION

Absolute Weight Growth

Growth is closely related to feed because feed provides nutrients and energy that are needed for the growth of cantang grouper. The fish feed contains protein, carbohydrates, vitamins and minerals. The results of measuring the absolute weight growth of cantang grouper can be seen in Figure 1.

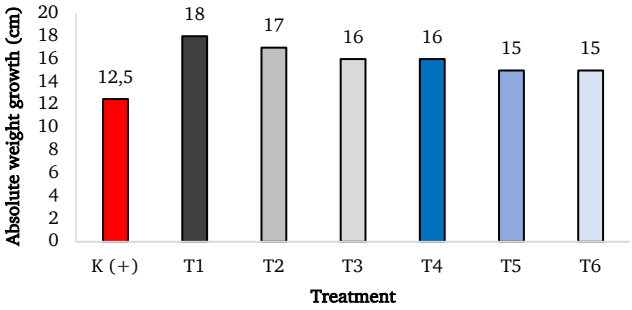

Figure 1. Growth of cantang grouper weight in each treatment.

Weight growth of cantang grouper in each treatment (Figure 10 shows that the absolute weight growth rate of cantang grouper during 56 days of rearing period ranged from 12.5 - 18 cm. The highest value of absolute weight growth was obtained in the T1 treatment (18 cm) and the lowest in the K+ (12.5 cm) treatment. The results of absolute weight growth obtained in T1, T2, T3, T4, T5, and T6 treatments were significantly different ($P<0.05$) with K+ treatment, while T1, T2, T3, T4, T5, and T6 treatments showed different results. significantly different ($P>0.05$).

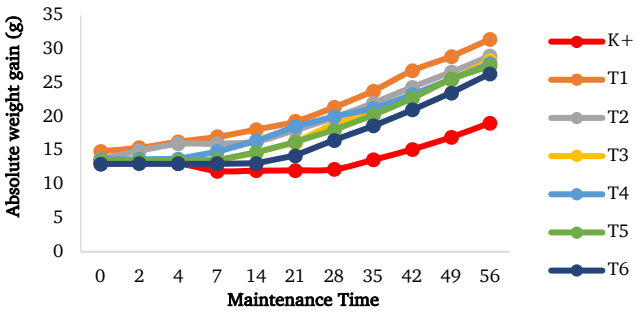

Figure 2. Weight gain of cantang grouper in each treatment.

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Figure 2 shows that the absolute weight gain of cantang grouper during 56 days of rearing period ranged from 19.07-31.47 g. The highest absolute weight growth value was obtained in the T1 treatment (31.47 g) and the lowest was in the K+ treatment (19.07 cm). The results of absolute weight growth obtained in T1, T2, T3, T4, T5, and T6 treatments were significantly different ($P<0.05$) with K+ treatment, while T1, T2, T3, T4, T5, and T6 treatments showed different results. significantly different ($P>0.05$). In the K+ treatment, the lowest value was suspected because VNN infection caused the fish's appetite to decrease so the growth and activity of the fish became weak. This is supported by Sembiring *et al.* (2018) who explain that VNN infection causes decreased appetite, weakfish movements, pale body color, produces a lot of mucus, fish swim irregularly, circling or whirling upside down.

Based on the research data above, shows that the length and weight of the fish's body have a close relationship where the longer the body size, the heavier the fish's body. This is presumably due to the availability of sufficient food and water quality conditions that support the growth of cantang grouper. This is in accordance with Lestari *et al*. (2017) explaining that the length and body weight of fish has a positive correlation. In addition, Rahmandani and Herlina (2021) stated that the increase in length and body weight of fish indicates that fish can digest feed well so that nutrients and energy can be used by the fish body to grow and develop.

Relative Percent Survival

The success rate of vaccination can be seen through the level of relative protection after infection with pathogens, survival and increased immune performance in fish. Challenge tests are conducted to determine the ability of a vaccine to produce an immune system that protects against certain pathogens.The relative protection level of cantang grouper can be seen in Figure 3.

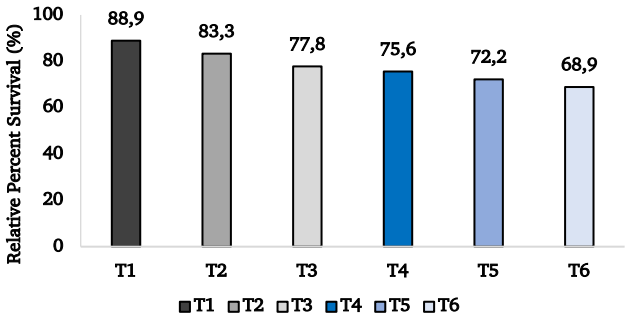

Figure 3. Relative percent survival in each treatment.

Based on the results of the study in Figure 3, the relative protection level of cantang grouper given the *C. vulgaris* protein recombinant vaccine ranged from 68.9 - 88.9%. The highest relative protection level was found in the T1 treatment (88.9%). The administration of vaccines with different doses did not have an effect between treatments ($P>0.05$). This indicates that the P-PERCv recombinant vaccine is able to increase the immune system of cantang grouper (*Epinephelus* sp.) and statistically there is no difference in the effect of vaccine doses. This is supported by the opinion of Sudirman *et al*. (2021), which states that the quality of a vaccine is considered good if it is able to provide a relative protection level (RPS) > 50%. In addition, Wang *et al.* (2016) also explained that the addition of adjuvants to the vaccine will increase the immunogenicity of the vaccine by increasing the level of relative protection

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after the challenge test on the 14th and 28th days.

Survival Rate

The survival rate of cantang grouper after being given the *C. vulgaris* protein recombinant vaccine showed that the T1, T2, and T3 treatments were significantly higher than the T4, T5, and T6 treatments that were given the vaccine and then infected with viral nervous necrosis virus, but between treatments did not give a different effect. real ($p>0.05$). The survival rate of cantang grouper can be seen in Figure 4.

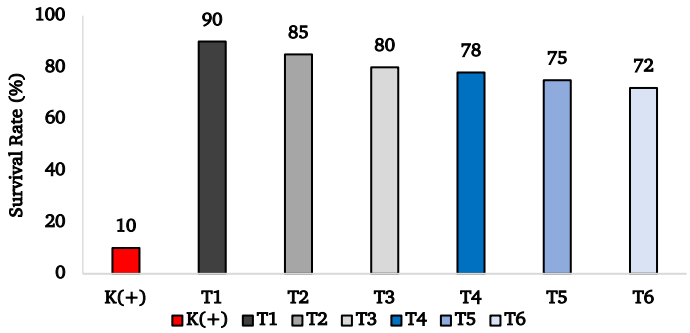

Figure 4. Survival rate in each treatment.

Based on the results of the study in Figure 4, the survival rate of cantang grouper (*Epinephelus* sp.) during 56 days of rearing ranged from 10 - 90%. The highest survival rate was found in treatment T1 (90%) while the lowest was in treatment K+ (10%). This shows that the administration of recombinant protein *C. vulgaris* vaccine triggers the body's defense response and the survival rate of fish increases. These results are following the research of Yanuhar *et al.* (2020), which explains that the administration of the *C. vulgaris* protein recombinant vaccine provides better survival for superior fish commodities. This is in line with Suprapto *et al*. (2017) who showed that vaccine administration can have an effect on the survival rate of cantang grouper (*Epinephelus* sp.) with a survival rate of 70%. In addition, it is supported by Hardi *et al.* (2019) that the use of adjuvants in vaccines is proven to increase the immunity ability of vaccines given to fish.

Antibody Titer

Antibody titers have an important role as the fish's body's defense system in paralyzing incoming pathogens. Antibodies produced in the body are indicated by the presence of an IgM antibody response in fish. The immunoglobin M (IgM) antibody titer response test was carried out on the 2nd, 4th, 7th, 14th, 21st, 28th, 35th, 42nd, 49th, and 56th days. The results of the observation of the hemagglutination test during the 56-day study can be seen in the scatter plot. More details can be seen in Figure 5.

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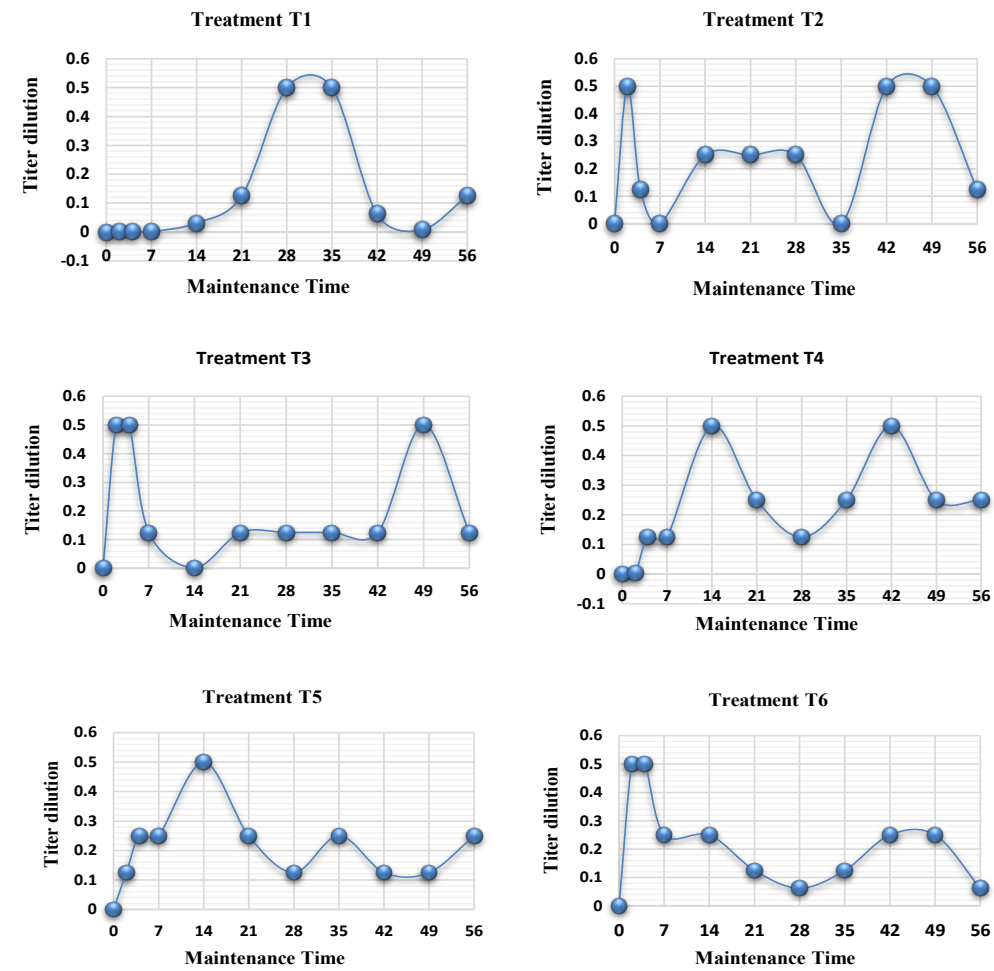

Figure 5. Antibody titer test result.

Based on the results of the study, it was shown that the administration of recombinant *C. vulgaris* vaccine could significantly increase antibodies in cantang grouper. In the T1 treatment, there was a significant increase in antibody titer after being given the booster vaccine until the 35th day. Meanwhile, in T2 and T3 treatments, after being given the booster vaccine, the antibody titer decreased in cantang grouper. It is suspected that the decrease in antibody titers in fish is influenced by several factors, one of which is the dose of vaccine given. This is supported by research by Bahar *et al*. (2017) which states that increasing the vaccine dose is not always proportional to the increase in antibody titer so it will affect the immunogenicity of the vaccine and there are certain doses that provide maximum immune response. In addition, it is also supported by Yanuhar *et al.* (2020) who explain that vaccines given to fish must be antigenic, immunogenic, and protective which can trigger the formation of an immune system in fish.

Figure 5 illustrates that administering recombinant *C. vulgaris* vaccine into fish bodies succeeded in increasing the cellular defense response that occurred in T4, T5 and T6 treatments. This is supported by Hardi *et al.* (2019) who explain that the addition of adjuvants in vaccines is proven to increase the ability of immune performance in fish. In the T6 treatment, there was a decrease in antibody titer from the 4th day to the 28th day. It is suspected that the dose and concentration of the vaccine given exceeds the limits of the fish's body so that the vaccine is immunosuppressive (suppresses

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the emergence of the immune response) which causes the fish's body to be unable to respond to antigenic stimuli which will inhibit the specific immune response in forming antibodies. This is in accordance with Bahar *et al*. (2017) who explain that if the vaccine dose given is too high, it does not necessarily provide a high immune system in the fish body.

CONCLUSION

Based on the result of the study, it was found that administration of *C. vulgaris* protein recombinant vaccine with the addition of adjuvant gave body resistance to cantang grouper and the best dose of 33 μl gave a relative protection level (75,6%), cantang grouper survival (78%) after challenge and a significant increase in antibody titers.

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