In-vivo Test of Chlorella Protein Fragments as Nucleotide Vaccine Candidates in Grouper Viral Nervous Necrosis (VNN) Infection against Haematological Response

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Abstract: Grouper (Cromileptes altivelis) is a species of fish with important economic values both in the national and international markets. The disease that has been reported by researchers is Viral Nervous Necrosis (VNN) which can cause mass death in groupers, especially in larval and juvenile stadia. Based on the problems, a research is needed on haematological analysis of groupers (*Cromileptes altivelis*) infected with Viral Nervous Necrosis by in-vivo testing using protein fragments of *C. vulgaris*. This research employed an experiment method using 5 treatments, namely (A) healthy fish, (B) VNN-infected fish, (C) VNN-infected fish with administration of *C.vulgaris* crude extracts of 17 µg mL^{-1}, (D) VNN-infected fish with administration of *C.vulgaris* crude extract of 33 µg mL^{-1}, and (E) VNN-infected fish with administration of *C.vulgaris* crude extract of 50 µg mL^{-1}. Observations of haematological parameters included erythrocytes, leukocytes, haemoglobin, and haematocrit. The observation results showed an erythrocyte value of 97 x 104 cells/mm^3 in treatment (C), 107 x 104 cells/mm^3 in treatment (D), and 94 x 104 cells/mm^3 in treatment (E). The observation results of leukocyte values were 150,000 cells/mm^3 in treatment (C), 133,300 cells/mm^3 in treatment (D), and 139,000 cells/mm^3 in treatment (E). Furthermore, the observation results of haemoglobin showed a value of 5 gr/100 ml in treatment (C), 6 gr/100 ml in treatment (D), and 5 gr/100 ml in treatment (E). As for the haematocrit parameter, the results obtained from the observation were 18% in treatment (C), 22% in treatment (D), and 15% in treatment (E). Based on this research, the haematological status of VNN-infected groupers was not good. However, the results of the in-vivo testing conducted showed that administration of *C. vulgaris* extract gave a positive result on improving the haematological status of groupers (*C. altivelis*) infected with VNN with the optimal dose of 33 µg mL^{-1}.

1 INTRODUCTION

One of the potential of sea waters that has been developed and is starting to show a growth in international market is grouper. Grouper is widely distributed in waters that are inhabited by coral in tropical and subtropical regions. Some types of grouper that have been targeted in the market are the duck grouper (*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), leopard grouper (*Epinephelus leopardus*) and mud grouper (*Epinephelus coioides*). These types of grouper have a high selling value. In addition, its cultivation process only need and use local components. (Sudaryatma et al., 2012). However, the hybrid of *cantang* grouper is experiencing a decrease in production due to some environmental stresses, for example, poor water quality, which makes the *cantang* grouper is susceptible to viral, bacterial, stressful infections from time to time resulting in poor growth and ultimately death. (Noor et al., 2018).

The obstacle of cultivation in the Epinephelus group (Grouper) in Indonesia is the limited supply of fish seeds due to pathogenic infections which cause more than 80% mortality, even up to 100% (Yanuhar et al., 2012). VNN virus has been reported to infect cultivated marine fish and has been stipulated in Ministerial Decree number 26 Year 2013 as Pests and Diseases of Quarantine Fish (HPIK) Group I. VNN weakens the nervous system of fish so that the fish will lose control nerves, will experience weakness of motion, and eventually death (Yanuhar, 2015).

The development of local natural materials as one of the countermeasures for controlling the spread of the VNN virus is very much needed. Natural

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materials used are derived from natural ingredients that have not been developed much, one of which is the use of microalgae. *Chlorella vulgaris* is a type of one-celled green microalgae that can grow and be found in warm climates. *C. vulgaris* has many ingredients in it which include protein, vitamins, minerals, carbohydrates, fats, chlorophyll and beta carotene (Tang dan Paolo, 2011). The use of microalgae has been developed, especially in the field of pharmacology. Microalgae have benefits as antioxidants for fish because they contain vitamins, polysaccharides, and bioactive compounds (Yanuhar, 2016).

Blood tests are conducted to establish the diagnosis of a disease in fish because physiological disorders in fish will cause changes in blood components which will then be able to determine the condition or health status of the fish (Yanuhar et al., 2019). Based on these problems, research is needed on the hematological analysis of groupers (*Cromileptes altivelis*) infected with Viral Nervous Necrosis (VNN) by in vivo testing using protein fragments from *C. vulgaris*.

2 METHODS

This study utilized crude extracts from *C. vulgaris* marine microalgae to be tested on *cantang* grouper (Epinephelus sp) infected with Viral Nervous Necrosis (VNN). *C. vulgaris* samples were obtained from the Brackish Aquaculture Fisheries Center (BPBAP) of Situbondo. The research took place at the Laboratory of Environment and Biotechnology Aquatic, Faculty of Fisheries and Marine Sciences, Brawijaya University and Organic Chemistry Laboratory, Faculty of Science and Technology, State Islamic University of Malik Ibrahim Malang.

2.1 Extraction of *C. Vulgaris*

C. vulgaris was extracted by maceration using methanol PA solvent in a ratio of 1:5 for 24 hours. Then it was filtered using filter paper to remove the pulp so that the extract was obtained with a solvent. Furthermore, to obtain the extract, the solvent was removed by using a rotary vacuum evaporator at a temperature of 40 ºC, with a speed of 60 rpm.

2.2 In-vivo Test of C. vulgaris Extract in Groupers

In this study, an in-vivo treatment of extracts from *C. vulgaris* marine microalgae on Groupers (C. altivelis) was carried out. The testing process was carried out orally which refers to Yanuhar (2015), using 5 treatments, namely (A) healthy fish, (B) VNN-infected fish, (C) VNN-infected fish with administration of *C.vulgaris* crude extracts of 17 µg mL^{-1}, (D) VNN-infected fish with administration of *C.vulgaris* crude extract of 33 µg mL^{-1}, and (E) VNN-infected fish with administration of *C.vulgaris* crude extract of 50 µg mL^{-1}. Oral treatment with the help of feeding tube was carried out for 3 times, namely on day 0, 5, and 10. Each rearing tank contained 12 groupers with a size of 10 cm and the test treatment was carried out for 24 days. Hemotological observations were then performed to determine the effect of in vivo test treatments on the Groupers.

2.3 Haematological Response

Observations of measured hematologic responses consisted of erythrocytes, leukocytes, hemoglobin and hematocrit. Blood samples were taken once at the end of the study. The method of blood sampling in fish was carried out according to Svobodova et al. (2006). This blood sampling was carried out using a 0.5 mL syringe that has previously been added with Ethylene Diamine Tetra Acetatic Acid (EDTA) at a dose of 1.50 ± 0.25 mg/mL of blood. The fish was placed with the head on the left side. Blood samples were taken using a syringe that pierced the muscles in the midline of the body behind the anal fin.

2.3.1 Erythrocyte Calculation

The procedure for calculating erythrocytes count was measured according to Blaxhall and Daisley (1973), firstly, blood was sucked with a pipette containing a red stirrer grains to scale 1 (a pipette to measure red blood cells count), then hayem's solution was added to scale 11. The stirring of the blood in a pipette was done by swinging a hand holding a pipette like forming a number, specifically number 8, for 3-5 minutes so that the blood was mixed evenly. The first two drops of the blood solution in a pipette were removed, then the drops were placed on a Neubauer haemocytometer and were covered with a glass cover. Then red blood cells count was calculated with the help of a microscope with 400x magnification. Red blood cells (erythrocytes) count can be calculated by the following formula. According to Blaxhall and Daisley (1973):

Σ erythrocytes found x 10^4 cells/mm^3

2.3.2 Leukocyte Calculation

The procedure for calculating erythocyte count was measured according to Blaxhall and Daisley (1973), blood samples were sucked with a pipette containing white stirrer grains to a scale of 0.5. Then, the truk's solution was added to scale 11. The stirring of the blood in a pipette was done by swinging a hand holding a pipette like forming a number, specifically number 8, for 3-5 minutes so that the blood was mixed evenly (the same as stirring for the calculation of red blood cells count). After that, the first two drops of blood solution from the pipette were removed, then the solution was dropped to the haemocytometer, after which it was closed with a glass cover. The white blood cells (leukocytes) count can be calculated by the following formula. According to Blaxhall and Daisley (1973):

$$\Sigma \text{ leukocytes found} \times 50 \text{ cells/mm}^3$$

2.3.3 Hemoglobin Calculation

Measurement of hemoglobin levels was done by sampling the blood with a sahli pipette up to a scale of 20 mm^3 or on a scale of 0.2 ml. Then the tip of the pipette was cleaned with tissue paper. The blood in the pipette was transferred into a Hb-meter filled with 0.1 N HCl to a scale of 10 (red). The blood was then stirred with a stirring rod for 3-5 minutes. The distilled water was added to the tube until the color of the blood was like the color of the standard solution present in the Hb-meter. The hemoglobin scale can be seen on the gr % (yellow) pathway scale, which meant the amount of hemoglobin in grams per 100 ml of blood.

2.3.4 Hematocrit Calculation

The examination of hematocrit values was performed using the microhematocrit method. Microhydematocrit with heparin was inserted into the collected blood sample, until the blood filled approximately three quarters (3/4) of the capillary tube. In addition, one end of the capillary tube was blocked by sticking it in the wax stopper. Then it was centrifuged for 5 minutes using a microhematocrit centrifuge with a speed of 1,500 rpm. In addition, the results were read using a hematocrit reader and were expressed in % (Vonti, 2008).

3 RESULTS AND DISCUSSION

3.1 Erythrocyte Calculation

Based on the calculation, red blood cells count (Figure 1.) in treatment (A) was 203 x 104 cells/mm^3, in treatment (B) was 48 x 104 cells/mm^3, in treatment was 97 x 104 cells/mm^3, in treatment (D) was 107 x 104 cells/mm^3 and in treatment (E) was 94 x 104 cells/mm^3. The highest erythrocytes count was found in treatment (A), i.e. healthy grouper without treatment and the lowest was found in treatment (B) VNN-infected fish.

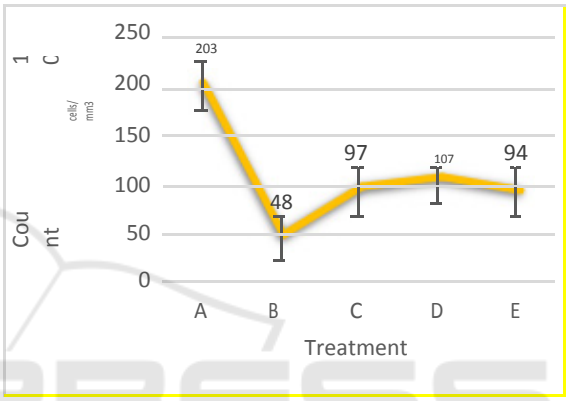

Figure 1. Erythrocyte calculation results. (A) healthy fish, VNN-infected fish, (C) VNN-infected fish with administration of C.vulgaris crude extracts of 17 µg mL-1, VNN-infected fish with administration of C.vulgaris crude extract of 33 µg mL-1, and (E) VNN-infected fish with administration of C.vulgaris crude extract of 50 µg mL-1.

Among the three treatments of *C. vulgaris* extract tested, treatment (D) at a dose of 33 µg mL^{-1} gave the highest increase in the erythrocytes count at 107 x 104 cells/mm^3. It showed that administration of *C. vulgaris* extract at a dose of 33 µg mL^{-1} increased the erythrocytes count of groupers infected with VNN. The fish blood cells count in teleostei fish ranged from 1.05 × 106 cells/mm^3 - 3.0 x 106 cells/mm^3. Low erythrocytes count are an indicator of anemia, while high erythrocytes count indicates that fish are under stress (Sababalat, 2015).

3.2 Leukocyte Calculation

White blood cells are immune cells that will respond to the presence of pathogens or foreign objects that enter the body, the higher the pathogenicity, the body will produce more white blood cells. In addition, according to Muiswinkel and Vervoorn (2006), leukocytes have a variety of functions,

closely related to the removal of foreign matter (including pathogenic microorganisms). Based on the calculation of the leukocytes count (**Figure 2.**) it shown the results of leukocyte calculation, as follows: treatment (A) was 91,350 cells/mm^3, treatment (B) was 182,050 cells/mm^3, treatment (C) was 150,000 cells/mm^3, treatment (D) was 133,300 cells/mm^3 and treatment (E) of 139,000 cells/mm^3.

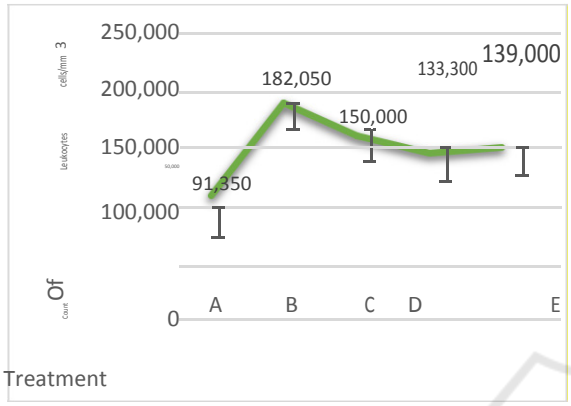

Figure 2. Leukocyte calculation results. (A) healthy fish, VNN-infected fish, (C) VNN-infected fish with administration of C.vulgaris crude extracts of 17 µg mL-1, VNN-infected fish with administration of C.vulgaris crude extract of 33 µg mL-1, and (E) VNN-infected fish with administration of C.vulgaris crude extract of 50 µg mL-1.

Leukocytes or white blood cells are an important part of the body's defense system which has the nature to prey on pathogens that enter the body. Therefore, leukocytes are very closely related to the immune system. Leukocytes have a role in cellular defense and humoral organisms against foreign substances. Fish have white blood cells called leukocytes which range from 20,000 - 150,000 cells/mm^3 (Irianto, 2005). Nearly all treatments showed leukocyte counts in the normal category except for VNN-infected fish.

3.3 Hemoglobin Calculation

According to Almanda et al. 2007, low Hb levels caused the metabolic rate to decrease and the energy produced to be low. This makes the fish weak and has no appetite and looks still at the bottom or hangs under the surface of the water. Normal hemoglobin levels in fish range from 5.05 to 8.33 grams/100 ml of blood or grams/%.

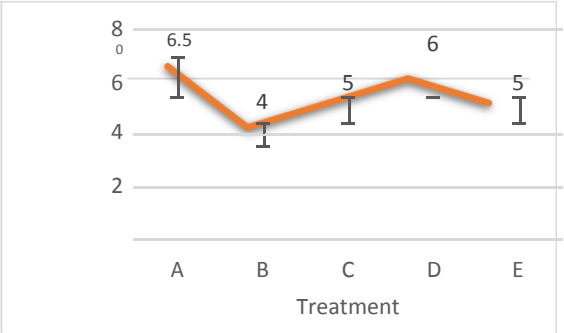

Figure 3. Hemoglobin calculation results. (A) healthy fish, VNN-infected fish, (C) VNN-infected fish with administration of C.vulgaris crude extracts of 17 µg mL-1, VNN-infected fish with administration of C.vulgaris crude extract of 33 µg mL-1, and (E) VNN-infected fish with administration of C.vulgaris crude extract of 50 µg mL-1.

The observation results of the highest hemogloblin levels (**Figure 3.**) other than healthy fish treated (A) were found in treated groupers (D) VNN-infected fish with *C.vulgaris* crude extract of 33 µg mL-1with hemoglobin levels of 6.5 gr/100 ml. The lowest hemoglobin concentration was observed in treatment of VNN-infected fish with a hemoglobin level of 4 gr/100 ml.

3.4 Hematocrit Calculation

Hematocrit is a comparison between red blood cells and blood plasma, and it affects the regulation of red blood cells. Hematocrit is a means for aquaculture to find out whether the fish being cultivated has anemia or not. Hematocrit is the percentage of the volume of erythrocytes in the blood and its value is related to red blood cells count. The increase in hematocrit levels is influenced by two factors, namely changes in environmental parameters, especially the temperature and physiological conditions of fish related to the energy needed (Jawad et al., 2004).

Based on hematocrit observations (**Figure 4.**) it obtained results as follow: namely at treatment (A) by 29%, treatment (B) by 13%, treatment (C) by 18%, treatment (D) by 22% and treatment (E) by 15% . The highest hematocrit value was in the treated groupers (D), namely the VNN-infected fish with *C.vulgaris* crude extract of 33 µg mL-1,which was around 30%. Whereas the lowest hematocrit value was in treatment (B), namely VNN-infected fish, which was around 13%.

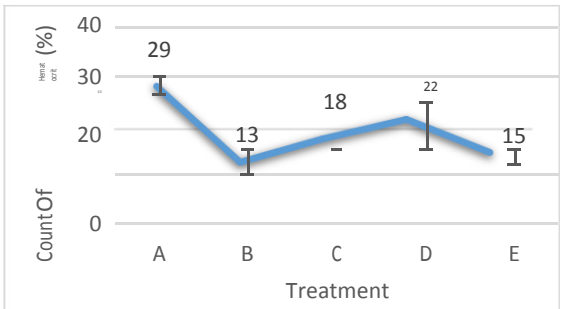

Figure 4. Hematocrit calculation results. (A) healthy fish, VNN-infected fish, (C) VNN-infected fish with administration of C.vulgaris crude extracts of 17 µg mL-1, VNN-infected fish with administration of C.vulgaris crude extract of 33 µg mL-1, and (E) VNN-infected fish with administration of C.vulgaris crude extract of 50 µg mL-1.

4 CONCLUSIONS

The health condition of fish can be seen from the hematological status which changes the amount value at the normal range. Based on this study, the hematological status of groupers infected with VNN was not good. However, based on the results of in vivo tests conducted, it was showed that the administration of *C. vulgaris* extract gave positive results on improving the hematological status of grouper (C. altivelis) infected with VNN with the most optimal dose of 33 µg mL^{-1}.

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