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The Effect of Protein Induction of *Brachionus* sp. Toward In-Vivo Response of Cluster Differentiation-8 (CD8) Cantang Grouper (*Epinephelus* sp.) Infected by VNN (Viral Nervous Necrosis)

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Abstract: The large demand for grouper conduce increase production intensively in various ways, by increasing the production of grouper aquaculture raises causing new problems, one of which is infection with VNN disease. The use of antibiotics to treat VNN infections is prohibited or no longer recommended by the government. An alternative way to deal with VNN infection is by increasing the body's immunity in the fish itself. One effort that can be done to increase the body's immunity in grouper is by administering Brachionus sp. protein extracts. This research was conducted to determine whether the Brachionus sp. has an effect on the immune system in cantang grouper (Epinephelus sp.) as indicated by the emergence of Cluster Differentiation-8 (CD8) expression in fish that have been infected with VNN. The proteins contained in Brachionus sp. that has three type molecular weight (122.73 kDa, 75.49 kDa, and 13.77 kDa) potential as an ingredient for treating VNN-infected fish. It is hoped that this can improve the immune system in Epinephelus sp. infected with VNN. Protein Brachionus sp. given by injection at a dose of 35 µl, 105 µl and 170 µl per 150 gr Epinephelus sp. 105 µl was the best concentration in boosting the immune system in the tested Epinephelus sp., as indicated by a decreased CD8 count.

Keywords: *Brachionus* sp.; Cantang grouper; CD8 (Cluster Differentiation-8)

Introduction

Cantang grouper is a cross breeding between male tiger grouper (*Epinephelus fuscoguttatus*) and female kertang grouper (*Epinephelus lanceoulatus*), and is the result of BPBAP Situbondo research. Cantang grouper has the advantage of being more resistant to disease, faster growth, and tolerance to the environment (Mastuti et al., 2019). Grouper has a fairly high price and is one of Indonesia's export commodities from the fisheries sector and has a promising market segment, both in the domestic and foreign markets (Anita et al., 2020).

The large demand for grouper conduce increase production intensively in various ways. Increasing the production of grouper aquaculture raises new problems, one of the problems that is often experienced by cultivators is the presence of diseases that can attack grouper, especially in the seed phase that we cultivate. Intensive cultivation can cause the spread of the disease to take place more quickly, this is a barrier factor in increasing the production of fishery commodities. Infectious diseases that often attack grouper aquaculture include vibriosis caused by *Vibrio* spp., *streptococcosis* caused by *Streptococcus* spp., sleeping grouper disease

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caused by iridovirus, Viral Nervous Necrosis (VNN) caused by *betanodavirus* (Chieng et al., 2018).

Viral Nervous Necrosis (VNN) is a disease caused by a virus from the *Betanodavirus* genus and constitute main cause of high mortality in fish larvae (Munday et al., 2002). Cases of mass mortality in Epinephelus marginatus fish that have been identified are caused by VNN infection (Boukedjouta et al., 2020; Valencia et al., 2019). VNN attacks the central nervous system, eye retina, and reproductive organs (Yanuhar et al., 2020). The specific symptoms of fish infected with VNN are uncoordinated movements, namely swimming irregularly, spinning, hyperactivity, swimming upside down, and sporadically stomping its head to the surface of the water (Zafran et al., 2000).

Using antibiotics is one attempt that can be made to prevent viral infections on grouper, moreover increasing the body's immunity in the fish itself more highly recommended. One effort that can be done to increase the body's immunity in grouper is by administering *Brachionus* sp. protein extract. *Brachionus* sp. capable of producing stable bioactive compounds (Makridis et al., 1999). *Brachionu*s sp. has a protein content of 40% protein and 13% lipids. The fatty acids contained in *Brachionus* sp. consisting of EPA 23%, DHA 0,6% (Hamre, 2016). The amino acids contained in *Brachionus* sp. itself is more stable than other compounds because it is not affected by enrichment and the environment (Maehre et al., 2013; Srivastava et al., 2006).

CD8 (Cluster Differentiation-8) or cytotoxic T cells are lymphocytes that have the ability to induce damage to infected cells or tumor cells (Musdja et al., 2017). The function of CD8 in the immune system of fish is concurrent with CD4 as a coreceptor and as a marker for detecting cytotoxic T cells (Ashfaq et al., 2019). The function of CD8 is to increasing the affinity of Major Histocompatibility Complex (MHC) in binding to T cell receptors (TCR) (Guo et al., 2013).

The protein contained in *Brachionus* sp. consists of several amino acids that have an important role in activating T and B lymphocytes, macrophages, natural killer T cells, production of antibodies, cytokines, and cytotoxic substances (Kelly et al., 2020). The high content of amino acids in *Brachionus* sp. It is expected to be able to increase the activity of the immune system by increasing the activation of T cells (CD8) in groupers infected with VNN.

Method

Experimental Site

This research was held in laboratory of Reproduction in Faculty of Fisheries and Marine Science University Brawijaya, laboratory of Patology and Biochemistry, Faculty of medicine University Brawijaya, CV. SAA grouper in Banyuwangi and dry laboratory of PSDKU Banyuwangi University Airlangga from November 2020-September 2021. Cantang Grouper obtained from CV. SAA in Banyuwangi. Seed of *Brachionus* sp. obtained from BPBAP Situbondo. The cantang grouper were in healthy condition and not infected by a disease such as *Viral Nervous Necrosis*. Fish positive VNN originated were collected from pond farmers around the Situbondo city.

Research Materials

The materials used in this study were Cantang Grouper with 7-10 cm length, and weight ±15 gram, anti-mouse antibody CD8, Heparin sodium, Freshwater and Marine, protein of *Brachionus* sp., *Brachionus* sp., VNN positive grouper, Aquades water, sea water, 70% Alcohol, separating gel, stacking gel, lower gel buffer, upper gel buffer, PBS solution, T-acryl, ddH_2O, Tetra Methyl Diamine (TEMED) (bio-Rad), ammonium persulfate, Tris (hydroxymethyl), HCl (Merck) pH=8.8 and 6.5, detergent Sodium Dodecyl Sulphate (SDS).

The equipment used in this study were aeration hose, aeration stone, 5 L Erlenmeyer, 2L jar, aerator, heater, centrifuge, section set, volume pipette, mask, hand gloves, microtube, 1 ml syringe, label paper, freezer -90˚C, hot plate, magnetic stirrer, set of electrophoresis (SDS-PAGE), Aquarium 70x70x40 cm, and Filter (Eryalçın, 2019).

Research Design

The present study used completely randomized design (CRD) with five treatment and three times reiteration, which is K+ (fish infected by VNN), K- (healthy fish), P1 (fish infected by VNN + 35 µl protein *Brachionus* sp.), P2 (fish infected by VNN + 105 µl protein *Brachionus* sp.), P3 (fish infected by VNN + 170 µl protein *Brachionus* sp.), P4 (healthy fish + 35 µl protein *Brachionus* sp.), P5 (healthy fish + 105 µl protein *Brachionus* sp.) and P6 (healthy fish + 170 µl protein *Brachionus* sp.). The concentration we use is in accordance with research conducted by Masitha (2019) which states that a concentration of 35 µl can improve the induction response from heat shock proteins as anti-inflammatory markers for VNN-infected grouper fish tissue.

Cantang Grouper Acclimatization

The test fish used were cantang grouper from the BPBAP Situbondo, with 7-10 cm length, and weight ±15 gram. Newly arrived fish acclimatized for 12 hours until the fish show aggressive movements. The feed given to the grouper was Otohime EP3® pellet (48% protein) and trash fish. Feeding was carried out twice per day at 08.00 and 14.00 am WIB.

Culture of Brachionus sp.

Culture of *Brachionus* sp. using a jar with a volume of 2 L. The maintenance medium used sea water. Seawater sterilization is done by boiling seawater until it boils, then transferred to a culture container and tightly closed. *Brachionus* sp. given feed (nutrition) in the form of baker's yeast mixed with fish oil in a 1:1 ratio. The purpose of giving yeast feed is that at the time of testing the extract given is pure from the protein of *Brachiounus* sp. itself (not from other microalgae).

After being cultured for approximately 7 days, *Brachionus* sp. harvested using a 40 m plankton net and carried out in a container filled with ice. *Brachionus* sp. filtered is transferred into an Ependorf tube using a pipette which has been stored in Ependorf wrapped in aluminum foil and stored in the freezer at -20^{o}C.

Protein Isolation of Brachionus sp.

Protein isolation of *Brachionus* sp. done by grinding *Brachionus* sp. which was stored in 15 mL microtube at -20 °C in a mortar, then homogenized with 80% methanol (1:2 ratio = plankton sample: methanol). The homogenate was soaked for 24 hours, then centrifuged at 300 rpm for 15 minutes. In this process, precipitate 1 and supernatant 1 were obtained. In Precipitate 1, 1:2 methanol was added, then incubated for 8 hours and centrifuged for 15 minutes at 300 rpm. precipitates 2 and supernatant 2. The next step is supernatant 1 and 2 with precipitates 1 and 2 obtained by evaporation using a rotary vacuum evaporator to obtain extracts of *Brachionus* sp.

In-Vivo Test on Epinephelus sp.

Brachionus sp. protein isolate given to test fish by intraperitoneal injection at a dose of 31.6 µg/ml per 150 g of fish body weight with a volume of 0.1 ml/fish on days 0, 7, 14, 21 and 28 (Yanuhar, 2011). VNN infection in the test fish was carried out orally, namely by chopping and giving it to the test fish twice (Yanuhar et al., 2017). VNN infection was carried out on day 5 and day 10 adlibitum (until full) (Masitha, 2019). Observations on behavioral responses, weight gain and length were made during the rearing period.

Immunohistochemistry Analysis

The preparation of immunohistochemistry preparations in this study was carried out according to the method of (Yanuhar et al., 2017). The first step is the preparation of organ tissue that has been exposed to immunogenic substances and fixation using 10% formalin, then embedding using paraffin wax. The embedded tissue was cut using a microtome at a thickness of 4-5 m and placed on glass slides for special immunohistochemical preparations. The slide preparations were paraffinized by heating the slides at a temperature of 60-80^{0}C, after which they were immersed in the xylol solution for approximately 5 minutes. Then the slides were dehydrated using absolute alcohol by rinsing, this step was repeated 2 times at a concentration of 90%, 80% and 70% each for approximately 5 minutes. The slide preparations were rinsed again with 20 m dionized water 3 times for 5 minutes each, then rinsed with distilled water and prepared the preparation in the refrigerator (overnight).

The next step is using the SCytek kit. The slides were rinsed using PBS (pH 7.4) as much as 20 L 3 times for 5 minutes each. Then incubated with Peroxide Blocking for Image Analysis for 4 minutes at room temperature and rinsed with PBS 3 times. The next step is to incubate the Super Block for 24 hours at 4^{o}C. After 24 hours, the preparations were rinsed using PBS 3 times. Prepare primary antibodies (CD8) which have been dissolved in blotto solution in a ratio of 1:1000, then incubated with primary antibodies that have been diluted in a blocking super block overnight at 4^{o}C. Slides were rinsed with PBS (pH 7.4) for 3 of us for 5 minutes each. The rinsed slides were incubated with the CRF Anti-Polyvalent HRP secondary antibody for 1 hour at room temperature, then the slides were rinsed with PBS 3 times and incubated with the ultratek HRP enzyme for 40 minutes at room temperature.

After being incubated with the ultratek HRP enzyme for 40 minutes at room temperature, the slides were rinsed with distilled water until the PBS disappeared, then dried from the remaining PBS that was still attached. The slides were then incubated using DAB Chromogen kit for 20 minutes and washed with PBS (pH 7.4) 3 times for 5 minutes each. The slide preparations were counterstained with hematoxylin for 10 minutes, then rinsed with DH2O 3 times for 5 minutes each and air-dried. When the slide is completely dry, the slide is covered with entelan. The slides can be observed using a microscope at a magnification of 400x, taking pictures of the CPI results using an Olympus digital camera. The observed images were analyzed with the application of Image J software.

Data Analysis

CD8 expression was analyzed statistically using ANOVA (Analysis of Variance) to determine the effect of the treatment given, then followed by Duncan's test to determine differences between treatments. The results of the ANOVA analysis are presented in a narrative manner with tables, graphs and pictures. Data on water quality, growth and behavior were analyzed descriptively.

Result and Discussion

Differentiation-8 or CD8 is found in cytotoxic T cells and binds to class I MHC molecules. CD8 itself is a transmembrane glycoprotein which is a co-receptor for T cells (Nakanishi et al., 2015). Cytotoxic T cells that have a CD8 surface protein are called CD8+ T cells. The use of CD8 as a parameter in this study is because CD8 cells are able to describe their immunomodulatory potential, which in this study used the protein *Brachionus* sp. An analysis that can be used to see the presence of CD8 expression is by immunohistochemistry staining (IHC), which is a histological staining technique that allows the detection of tissue antigens (markers) in various specimens using the principle of specific antigen-antibody interactions.

The results of observations on control fish can be seen in Figure 1. Immunoratio analysis is shown by DAB values ranging from 90-92% (Figure 1a), the orange color represents the bond between antigen and antibody. The histogram (semicumulative analysis) can be seen in Figure 1c, measurements with histograms were carried out to show the intensity of the target gene. The semicumulative value using the histogram is divided into 4 zones, namely strong positive (0-60), positive (61-120), weak positive (121-180) and negative (181-255). In control fish, the target gene has a value of 46-51 which is included in the strong positive category (Figure 1c).

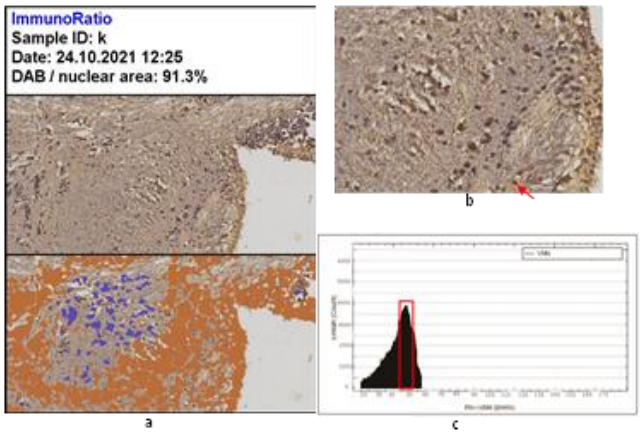

Figure 1. Organ profile of fish brain induced by *Brachionus* sp. protein and infected with VNN, a) Immunoratio analysis, b) Immunohistochemical results, c) Histogram imageJ. Description: ( ) T cell expression of CD8

Cantang grouper infected by VNN with treatment of *Brachionus* sp. protein was given from the first day during the in-vivo test and was carried out every 7 days until the study ended, this was done so that the test fish formed an immune system before being infected with VNN and given on day 5 to day 10.

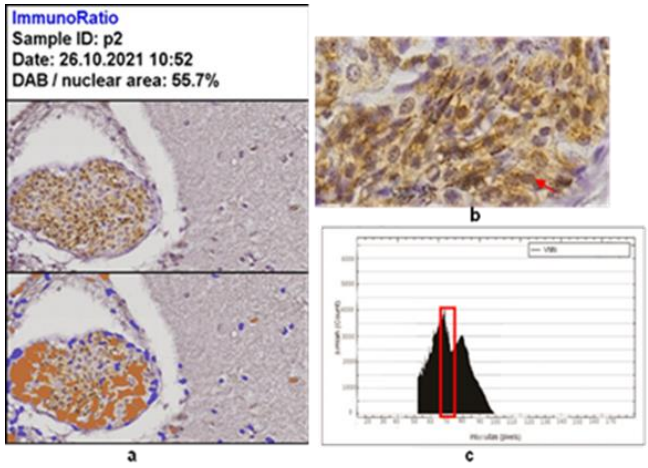

Figure 2. Brain organ profile of fish induced by *Brachionus* sp. protein and VNN infection, a) Immunoratio analysis, b) immunohistochemistry results, c) Histogram imageJ. Description: ( ) T cell expression of CD8

Immunoratio analysis on CD8 showed a decrease compared to control fish (CPI data can be seen in Figure 2 and Table 1). The decrease CD8 levels in fish indicated that the *Brachionus* sp. protein treatment. In fish is able to inhibit the development of VNN so that cell damage can be minimized. The histogram (Figure 3c) showing the intensity of the target gene is 65–70 indicating a positive value.

Fish tested positive for VNN with treatment *Brachionus* sp. This is a fish that is resistant to the end of the study compared to control fish. Test fish that were able to survive until the end of the study indicated that these fish were able to form a stronger immune system by administering *Brachionus* sp. protein. Before being infected with VNN. The protein content contained in *Brachionus* sp. able to work as a biocatalyst during the formation of the immune system (Jeeja. et al., 2011)(.

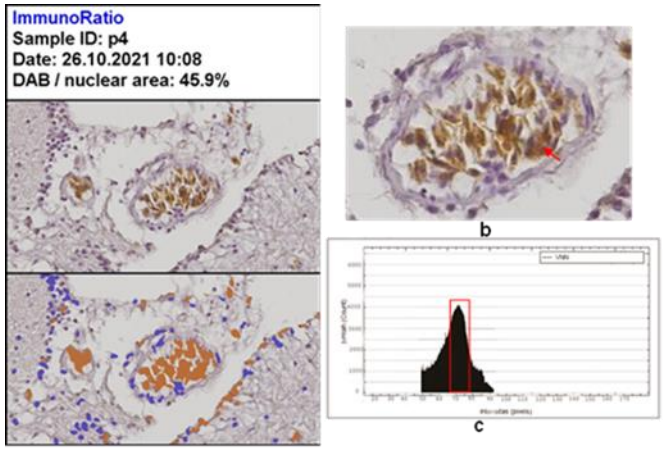

Figure 3. Organ profile of fish brain induced by *Brachionus* sp. Protein but without VNN, a) Immunoratio analysis, b) Immunohistochemical results, c) Histogram imageJ. Description: ( ) T cell expression of CD8

Normal cantang grouper treated with *Brachionus* sp. showed different results compared to control fish (positive VNN) and VNN fish treated with *Brachionus* sp protein. Figure 3a is the result of immunohistochemistry which was analyzed by immunoratio having a DAB of around 44-56%, while the analysis of the histogram results in Figure 14c shows an intensity value of 69-78 (positive category). This indicates that the protein *Brachionus* sp. given to healthy groupers can help synthesize proteins that play a role in antibody cells.

Table 1. Analysis Data IHC with Immunoratio and Semicumulative (Histogram)

Treatment	IHC Analysis	Histogram Analysis
Control	91,80±0,44[a]	48
P1	68,10±0,26[b]	64
P2	59,60±0,56[d]	66
P3	65,80±0,46[c]	66
P4	57,13±0,93[e]	71
P5	53,83±0,35[f]	75
P6	56,50±0,72[e]	77

Notes: Control is fish infected with VNN, P1) normal fish+VNN+ 35µl of *Brachionus* sp. protein, P2) normal fish+VNN+ 105µl of *Brachionus* sp. protein, P3) normal fish+VNN+ 170µl *of Brachionus* sp. protein, P4) normal fish+VNN+ 35µl of *Brachionus* sp. protein, P5) normal fish+VNN+ 105µl *Brachionus* sp. Protein, P6) normal fish+VNN+ 170µl *Brachionus* sp protein.

Administration of *Brachionus* sp. in healthy fish has an effect on increasing growth in fish (Table 1), this is due to the content of aspartic acid and threonine which can support growth in cantang grouper (Tang et al., 2021). Administration of *Brachionus* sp. in VNN fish it had an effect on the survival of fish that were still healthy until the end of the study (Table 1). This can be seen in Figure 2 that the levels of expressed CD8 decreased compared to the control fish. In control fish, CD8 expression was the highest, this was because fish infected with VNN showed that the immune cells in fish that are useful for protecting the body are fighting viruses or incoming antigens (Yanuhar, 2011). The appearance of a high CD8 indicates that there is damage to cells that have been infected by the virus. CD8 or cytotoxic cells themselves are lymphocytes that have the ability to induce damage to virus-infected cells (Musdja et al., 2017).

Decreased CD8 levels in VNN fish treated with *Brachionus* sp. indicated that the protein *Brachionus* sp. which is injected into fish is able to inhibit the development of the virus so as to minimize damage to cells. The decrease in CD8 expression indicated that the fish treated with *Brachionus* sp. able to minimize damage caused by cell infection. This also happened in a study conducted at rainbow trout that had been treated with DNA vaccines showed a decrease in CD8 expression (Castro et al., 2013).

CD8 has the ability to kill cells infected by viruses, the role of CD8 takes place by means of CTL recognition of intracellular antigens which causes cytotoxic release, namely the release of lymphokines (perforin and granzymes) and the apoptotic pathway is activated through FAL/FasL interactions to destroy virus-infected cells so that expression CD8 is lower than in control fish (Musdja et al., 2017; Tizard, 2004).

Normal fish treated with *Brachionus* sp. Protein also express CD8. Fish stimulated with polysaccharide (LPS), polycytidylic acid (polyI:C), concanavalin A (ConA) induce CD8 expression because fish respond to foreign substances (antigens) that enter their bodies (Kono et al., 2013). *Brachionus* sp. contains the amino acids threonine, aspartic acid and apyrase. According to Pinto et al. (2021) threonine is an amino acid that can increase growth in *Sparus aurata* fish. Aspartate acid and threonine can act as mediators in protein synthesis, energy metabolism and increase nutrient absorption, thereby increasing growth in fish (Gonzalez-Silvera et al., 2018; Tang et al., 2021).

The content of amino acids in *Brachionus* sp. can increase the immune system in fish, amino acids play a role in inducing an immune response and regulating the activation of T lymphocytes, B lymphocytes, NK cells and macrophages, the production of antibodies, cytokines and other cytotoxic substances (Kelly et al., 2020). When the virus infects fish, the threonine contained in *Brachionus* sp. may assist APC in promoting antibody production in lymphocytes via protein synthesis and cellular signaling (Duval et al., 1991).

Apyrase (Cauwels et al., 2014) and threonine (Chen et al., 2015) can play a role in suppressing inflammation, so that cell damage when infected with a virus can be reduced. This can be seen by the decreased expression of CD8 in fish infected with VNN after being treated with *Brachionus* sp. Aspartic acid can also improve fish health by maintaining antiprotease enzymes so that it can suppress the neuroendocrine stress response (Gonzalez-Silvera et al., 2018) as indicated by normal appetite, normal swimming habits, and responsiveness to movement in fish infected with VNN after treatment. with *Brachionus* sp.

Conclusion

Administration of *Brachionus* sp. in cantang grouper able to support fish growth seen from length and body weight which tends to increase compared to control fish (VNN fish), high appetite, and responsive behavior (normal), this indicates that the fish are in good health (no symptoms appear VNN). Administration of

Brachionus sp. also affected the immune response of fish as seen from the expression of CD4 and CD8 which decreased compared to control fish, this decrease in expression indicated that there was body resistance to VNN infection so that cell damage and inflammation were reduced.

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Conflicts of Interest
There is no conflict of interest in this manuscript between among all authors upon writing and publishing this manuscript.

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