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Exploration of *Brachionus* sp. Protein on the *In Vivo* Response of CD4 at Cantang Grouper (*Epinephelus* sp.) with VNN Infection (Viral Nervous Necrosis)

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Abstract

This study examines the function of *Brachionus* sp. protein on the Cluster Differentiation-4 (CD4) immune system in cantang grouper infected with VNN. The protein contained in *Brachionus* sp. is expected to be able to induce the immune system in the body of fish to prevent severe damage due to VNN infection. The purpose of this study was to determine the protein content of *Brachionus* sp. which has the potential as an antivirus and to find out the benefits of *Brachionus* sp. on the expression of CD4 as an indicator of increased fish body defense system against VNN. *Brachionus* sp. itself contains three protein bands with molecular weights of 122.73 kDa, 75.49 kDa, and 13.77 kDa, which are expected to increase the immune system against VNN on cantang grouper. Protein injection of *Brachionus* with doses of 35 μl, 105 μl and 170 μl /150 g cantang grouper, showed that the lowest decrease was at a dose of 105 μl/150 gram cantang groupers. The addition of *Brachionus* sp. also affected the immune response of the fish as seen from the decreased expression of CD4 compared to the control fish, this decreased expression indicated that the body had resistance to VNN infection so that cell damage and inflammation were reduced.

INTRODUCTION

Grouper (*Epinephelus* sp.) is the most common commercial marine fish species with strong consumer value and high protein content (Helmi *et al*., 2020). Cantang grouper has the advantages of being more resistant to disease, faster growth, and tolerance to the environment. 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 and Dewi, 2019).

The large demand for grouper in these countries has resulted in an intensive increase in production, this increase in production raises new problems where the emergence of diseases that can attack cultured fish, especially in the seed phase that

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we cultivate. When environmental conditions in aquaculture ponds decline, mortality caused by disease outbreaks is very high, but on the contrary, if environmental conditions are good, mortality from infection with a disease is lower (Cole *et al*., 2009). Viral Nervous Necrosis (VNN) is a disease caused by a virus of the *Betanodavirus* genus. VNN in general can infect almost all stages of fish growth (especially in the larval and fry stages), and the mortality can reach 100%. VNN attacks the central nervous system, the retina of the eye, and reproductive organs. The general symptoms of VNN are decreased fish appetite, pale body color, and specific symptoms in the form of uncoordinated movements, such as undirected swimming, spinning, hyperactivity, upside down, and often pounding their heads on the water surface sporadically (Yuasa *et al*., 2001).

One of the efforts that can be made to increase immunity in grouper is by giving *Brachionus* sp. It has 34-41% dry weight of protein content (Srivastava *et al*., 2006), and according to Eryalçın (2019) the protein content of *Brachionus* sp. of 45.24 g/100 g dry weight. *Brachionus* sp. proteins are expected to induce the immune system in the fish body to prevent severe damage due to VNN infection. In this study, the author used the CD4 response to determine the immune response of fish treated with *Brachionus* sp. which was challenged with VNN.

CD4 (Cluster Differentiation-4) is a transmembrane glycoprotein that is expressed on the surface of Th cells and plays an important role in the immune response (Maisey *et al*., 2016). CD4+ T cells are required as helpers to promote the production of B cell antibodies and are required for cytotoxic CD8+ T cell generation and memory (Ashfaq *et al*., 2019). The use of CD4 to determine the immune response in fish in this study was because the function and characterization of the CD4+ cell population were known and commonly used. This is due to the availability of suitable markers for T lymphocytes in fish, and CD4+ T cells are very important to trigger and maintain natural and vaccine-induced immunity (Yanuhar *et al*., 2021).

The protein contained in *Brachionus* sp. consists of several amino acids. The role of amino acids in the immune system is to activate T and B lymphocytes, macrophage cells, natural killer T cells, production of antibodies, cytokines, and cytotoxic substances (Li *et al*., 2007). The high amino acid content in *Brachionus* sp. is expected to increase the activity of the immune system by increasing the activation of T cells (CD4) in grouper infected with VNN. The purpose of this study was to analyze the expression of CD4 in cantang grouper (*Epinephelus* sp.) that had been infected with VNN, after being treated with *Brachionus* sp. protein extract.

METHODOLOGY

Ethical Approval

There are no animals harmed or improperly treated during this research. The test animals in this study were treated properly according to the optimal environment, starting from temperature, salinity, availability of DO, etc. And it was approved during the due diligence session and proposal seminar at Faculty of Fisheries and Marine Science University of Brawijaya Malang.

Place and Time

This research was performed in the Laboratory of Reproduction in the Faculty of Fisheries and Marine Science University Brawijaya, Laboratory of Pathology 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 VNN.

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Fish-positive VNN originated from pond farmers around Situbondo City.

Research Materials

The materials used in this study were Cantang Grouper with 7-10 cm length, and weight ±15 g, anti-mouse antibody CD4, Heparin sodium, Freshwater and Marine, protein of *Brachionus* sp., *Brachionus* sp., VNN positive grouper, Aquades water, seawater, 70% Alcohol, separating gel, stacking gel, lower gel buffer, upper gel buffer, PBS solution, T-acryl, ddH2O, 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 ºC, hot plate, magnetic stirrer, set of electrophoresis (SDS-PAGE), Aquarium 70×70×40, and Filter.

Research Design

The present study used Completely Randomized Design (CRD) with five treatments and three times replication, 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 *et al*. (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.

Work Procedure

Cantang Grouper Acclimatization

Newly arrived fish acclimatize 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.

Culture of *Brachionus* sp.

Culture of *Brachionus* sp. using a jar with a volume of 2 L. The maintenance medium used seawater. Seawater sterilization is done by boiling seawater until it boils, then transferred to a culture container and tightly closed. *Brachionus* sp. is 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 *Brachionus* sp. itself (not from other microalgae).

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

Protein Isolation of *Brachionus* sp.

Protein isolation of *Brachionus* sp. was done by grinding *Brachionus* sp. which was stored in a 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.

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Electrophoresis Protein using SDS-Page

The electrophoresis technique was carried out by preparing a gel in a mold made of glass plates separated by a PVC-spacer and tightly bonded using “Glisseal" oil (Borer Chemic AG, Zuchwil/Switzerland). After filling the gel solution is then coated with distilled water. Electrophoresis using SDS-PAGE (Biorad) was carried out on a 190 x 130 mm gel in a vertical well used for electrophoresis (Laemmli, 1970). The separating gel was polymerized from a 12.5% acrylamide solution; 0.344% bis-acrylamide; 3.5 mM SDS (Sodium Dodecyl Sulfate); 375 mM Tris-HC1 buffer; pH 8.8. Polymerization was started by adding 0.05 ml of 40% APDS and 0.025 ml of TEMED (tetra ethylene diamine) per 50 ml of gel solution.

The gel containing the substrate was prepared by adding 0.1 mg of fibrinogen/ml of the separating gel solution and stirring for 45 minutes before polymerization. Separating gel is made of 4.38% acrylamide; 0.12% bis-acrylamide; 3.5 mM SDS in 125 mM Tris-HC1 pH 6.8. Polymerization was started by adding 0.025 m140% APDS and 0.01 ml TEMED per 10 ml gel solution. The sample was dissolved in buffer S containing 80 mM SDS and 0.1 M DTT. The electrode buffer consisted of 192 mM glycine, 25 mM Tris, and 3.5 mM SDS. With a 2 mm gel electrophoresis (at 6 °C) starting with 30 mA, and increasing to 50 mA after 60 min, the current intensity was halved by 1 mm gel. Electrophoresis was stopped when Bromophenol Blue was about 1 cm from the bottom edge of the gel.

The results of the SDS-PAGE electrophoresis in the form of bands (protein bands) were determined to determine the molecular weight by calculating the Rf (Retardation Factor) value of each band with the following formula.

$$Rf = \frac{\text{the distance the protein moves from its starting point}}{\text{the distance the color moves from the starting place}}$$

Then the Rf value is entered in the linear regression equation with the formula below.

$$Y = a + bX$$

Where:
Y = molecular weight
X = sample Rf value

Electroelution and Protein Dialysis

Brachionus sp. proteins obtained from SDS-PAGE were identified and selected with the highest molecular weight. The protein bands were separated by the electroelution method using horizontal electrophoresis (Biorad). Protein pieces were inserted into the cellophane membrane and electrophoresed at a voltage of 120 V, 400 mA for 120 minutes. Then the protein was analyzed using PBS at pH 7.4 at 40 °C for 48 hours.

The liquid contained in the cellophane bag was taken and put in a microtube, then precipitated by incubation in acetone solution (1:1 v/v) overnight at 40 °C. The protein solution and acetone were centrifuged at 12,000 rpm for 20 minutes at 40 °C. The protein formed is in the form of pellets. The pellet was then stretched and dissolved in 100 L of 0.5 M tri HCl and pH 8.6. The protein concentration of *Brachionus* sp. was measured using a NanoDrop 1000 Spectrophotometer using wavelength 280 nm.

Immunohistochemistry Analysis

The preparation of immunohistochemistry preparations in this study was carried out according to the method of Khan *et al*. (2014), and Yanuhar and Khumaidi (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 °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

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concentration of 90%, 80%, and 70% each for approximately 5 minutes. The slide preparations were rinsed again with 20 m deionized water 3 times for 5 minutes each, then rinsed with distilled water and prepared 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 °C. After 24 hours, the preparations were rinsed using PBS 3 times. Prepare primary antibodies (CD4) 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 °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 Ultratech 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 the 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 400×, 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

CD4 measurement data were analyzed statistically using ANOVA (Analysis of Variance) with SPSS software to determine the effect of the treatment given. If from the analysis it is known that the treatment shows a significantly different effect or is very significantly different, it will be continued with Duncan's test to determine the difference between treatments. The results of the ANOVA analysis are presented narratively with tables, graphs, and pictures. Water quality data were analyzed descriptively.

RESULTS AND DISCUSSION

Fragment Profile Extract Protein of *Brachionus* sp.

Brachionus sp. was analyzed in the extraction to obtain crude protein. The protein quantity of *Brachionus* sp. was analyzed using the Nanodrop method with a Spectrophotometer ND-1000, and read at a wavelength of 280 nm with 1 ml sample size. The quantity of the supernatant *Brachionus* sp. after being tested using nanodrop is 92.9 mg/ml and has an absorbance value of 1.67. While the pellet quantity of *Brachionus* sp. is 61.5 mg/ml with an absorbance value of 1.12. Maftuchah *et al*. (2014) stated that the absorbance value of 1.7-2.0 with a ratio of A260/A280 is a protein that has good quality, thus the protein possessed by *Brachionus* sp. is good.

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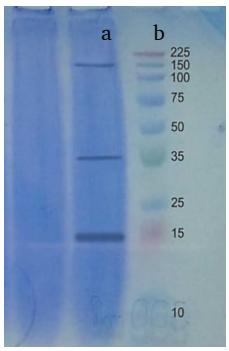

Figure 1. SDS-page electrophoresis results, (a) supernatant protein of *Brachionus* sp., (b) protein marker.

SDS-page analysis is carried out to detect protein molecules based on their molecular weight so that certain types of proteins are known (Roy and Kumar, 2014). According to Mahasri *et al.* (2010), the determination of protein molecular weight can be done by calculating the RF (Retardation Factor), namely, the distance of the protein movement from the initial place divided by the distance of the color movement from the starting place of each protein band. The results of the calculation of the protein band *Brachionus* sp. can be seen in Table 1. The molecular weight of the sample protein is determined by calculating using RF and plotted on a logarithmic graph of the RF marker protein whose molecular weight is known (Darmawati *et al.*, 2010). The calculation of the linear equation can be seen in Figure 2.

Table 1. Data on calculation of molecular weight of protein bands of *Brachionus* sp.

BM	Log BM (y)	A (mm)	B (mm)	Rf (x)
225	2.35	20	92	0.22
250	2.18	25	92	0.27
100	2.00	30	92	0.33
75	1.88	35	92	0.38
50	1.77	44	92	0.48
35	1.54	52	92	0.57
25	1.40	61	92	0.66
15	1.18	74	92	0.80
10	1.00	80	92	0.87

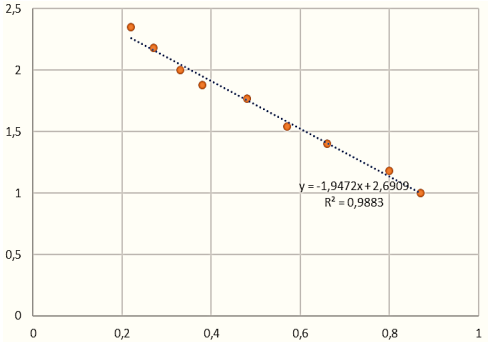

Figure 2. Linear graph of protein marker bands on *Brachionus* sp.

The results of the graph in Figure 2 above show that the linear equation for the marker protein band is y= -1.9472x + 2.6909. Furthermore, the molecular weight of *Brachionus* sp. the protein band

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was calculated and the results of these calculations are listed in Table 2.

Table 2. Calculation of molecular weight of *Brachionus* sp. protein bands.

A (mm)	B (mm)	Rf (x)	Y= -1,9472x+2,6909	BM (sample)
28	92	0,30	2,08896	122,73
38	92	0,41	1,87787	75,49
73	92	0,79	1,13907	13,77

The protein used in this study used proteins derived from the results of electroelution and protein dialysis only. We do not test crude extract protein. After the calculations as shown in Table 2, the protein fragments of *Brachionus* sp. It was found that there were 3 protein bands with a molecular weight of 122.73 kDa; 75.49 kDa; and 13.77 kDa. Weiss *et al*. (2002) stated that based on the results of SDS-Page analysis, a protein with a molecular weight of 122.73 kDa is a threonine protein, while 75.49 kDa is apyrase and 13.77 kDa is aspartate acid. The proteins are aspartate acid (30-35 kDa), apyrase (65-86 kDa), and threonine (119-125 kDa) (Makridis and Olsen, 1999).

Apyrase is a calcium or magnesium-activated plasma membrane-bound enzyme, its job is to catalyze the hydrolysis of ATP (Adenosine Triphosphate) to produce AMP (Cyclic Adenosine Monophosphate). Apyrase in the immune system plays a role in protecting the host from cell death. Apyrase is very efficient at preventing the production of TNF and IL-1 cytokines that increase inflammation and are destructive, correlated with reduced leukocyte infiltration, mitochondrial and tissue damage (Cauwels *et al*., 2014).

The role of MHC is as a gene that functions to neutralize viruses, the presence of MHC-I and MHC-II molecules is associated with the immune response. Phagocyte cells will be activated by MHC-II to produce antibodies and activate immunological responses involved in neutralizing viruses, killing bacteria and parasites (Yamaguchi and Dijkstra, 2019).

Threonine (Thr), better known as α-amino-β-hydroxybutyric acid. Threonine is an important bioactive molecule in mediating protein synthesis, energy metabolism, and nutrient absorption. Threonine can increase animal growth, improve immune function and maintain digestive tract health (Ross-Inta *et al*., 2009). When the intestinal tract is in an inflammatory state, threonine is used to regulate immune cell differentiation, cytokine expression, and immune-associated signaling (Tang *et al.*, 2021).

CD4 (Cluster Differentiation-4)

CD4 (Cluster Differentiation 4) is a molecule involved in T-cell development and activation, and is a significant cell surface marker used to identify T-helper cell subsets. T cells that express the CD4 surface protein play an important role in the humoral immune response to viruses (Somamoto *et al*., 2014). Analysis that can be used to see the presence of CD4 expression is immunohistochemistry (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 the immunohistochemical staining can be seen in Figure 3, then statistical analysis was carried out using SPSS which can be seen.

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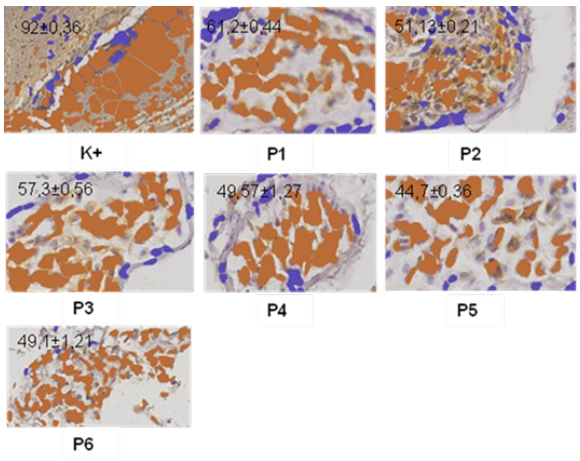

Figure 3. Immunohistochemistry results from CD4 expression in the brain of Cantang Grouper.

Description: (K+) Fish infected with VNN without *Brachionus* sp. protein, (P1) VNN+35μL *Brachionus* sp. protein, (P2) VNN+105μL *Brachionus* sp. protein, (P3) VNN+170μL *Brachionus* sp. protein, (P4) 35μL *Brachionus* sp., (P5) 105μL *Brachionus* sp. protein, (P6) 170μL *Brachionus* sp. protein. Remarks: brown color indicates the expression of CD4.

The results obtained from the analysis using SPSS, namely treatment with a dose of protein *Brachionus* sp. different groups showed a significantly different effect (P<0.05) on the CD4 immunological response. The results of SPSS showed that the K+ treatment produced the highest CD4 expression (92.00% ± 0.36), while the lowest was at p5 (49.10% ± 1.21). CD4 expression on K+ was significantly different with treatments P1 (61.20% ± 0.44), P2 (51.13% ± 0.21), P3 (57.30% ± 0.56), and P5 (44.70 % ± 0.36). P4 (49.57% ± 1.27) and P6 (49.10% ± 1.21) were not significantly different.

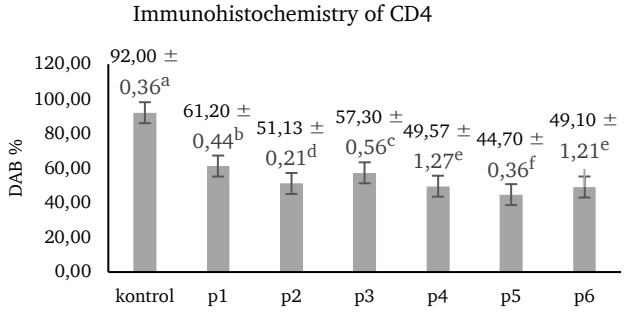

Figure 4. Immunohistochemistry Data of CD4 expression.

Description: (K+) Fish infected with VNN without *Brachionus* sp. protein, (P1) VNN+35μL *Brachionus* sp. protein, (P2) VNN+105μL *Brachionus* sp. protein, (P3) VNN+170μL *Brachionus* sp. protein, (P4) 35μL Brachionus sp., (P5) 105μL *Brachionus* sp. protein, (P6) 170μL *Brachionus* sp. protein. Different letters above the bar indicate a significant difference (P<0.05) based on Duncan's Multiple Range Test (DMRT).

The results obtained from the analysis using SPSS, namely treatment with a dose of protein *Brachionus* sp. different groups showed a significantly different effect (P<0.05) on the CD4 immunological response. The results of SPSS showed that the K+ treatment produced the highest CD4 expression (92.00% ± 0.36), while the lowest was at p5 (49.10% ± 1.21). CD4 expression on K+ was significantly different with treatments P1 (61.20% ± 0.44), P2 (51.13% ± 0.21), P3 (57.30% ±

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0.56), and P5 (44.70 % ± 0.36). P4 (49.57% ± 1.27) and P6 (49.10% ± 1.21) were not significantly different.

In the treatment of fish infected with VNN by giving *Brachionus* sp. (P1, P2, and P3), the best treatment was on P2 with CD4 expression which is 51.13%. The %DAB value in treatment P2 (105μL protein *Brachionus* sp.) was lower than P1 and P3. The best treatment for *Brachionus* sp. which used normal fish was found in P5 (49.57% ± 1.27) because the value was smaller than P4 and P6.

CD4 is a transmembrane glycoprotein that is expressed on the surface of Th (T-helper) cells and plays an important role in the immune response. CD4-expressing T-helper cells ($CD4^+$ Th cells) coordinate the immune response by acting as either effector cells or memory cells. $CD4^+$ Th cells are essential for triggering and maintaining innate immunity in fish induced by antigens (Ashfaq *et al*., 2019). The control fish (K+) had the highest CD4 expression, this was indicated because the control fish had the highest damage to the target organ (brain). The high expression of CD4 in VNN-infected fish indicates that immune cells in fish that are useful for protecting the body are fighting viruses or incoming antigens (Yanuhar *et al*., 2021).

Another indication is that in control fish that have the highest CD4 expression, there is a lack of nutrients, especially amino acids. This can be seen in immunohistochemistry and flow cytometry tests that control fish (VNN positive, without *Brachionus* sp. protein treatment) had the highest CD4 expression compared to fish that had been treated with *Brachionus* sp. protein. Amino acids have a major role in the defense mechanism of the fish body because they are directly involved in the preparation of antibody protein synthesis and the main control of immune regulation (Kiron, 2012).

Fish that were treated with *Brachionus* sp. showed a decrease in CD4 count, especially in the P2 treatment (administration of *Brachionus* sp. 105μL protein dose). Decreased CD4 levels in fish indicated that *Brachionus* sp. which is injected into fish is able to inhibit the development of the virus so as to minimize damage to cells. Sumsanto *et al*. (2019) stated that decreased CD4 expression indicates reduced *Myxobolus* sp. givens deltamethrin to koi fish.

Normal fish treated with *Brachionus* sp. also express CD4. According to Kono and Korenaga (2013), fish stimulated with polysaccharide (LPS), polycytidylic acid (polyI:C), and concanavalin A (ConA) induce CD4 expression because fish respond to foreign substances (antigens) that enter their bodies. This incoming antigen will be recognized by APC and forwarded by MHC class II to induce the emergence of CD4.

CONCLUSION

After conducting research on the immune response of cantang grouper infected with VNN (Viral Nervous Necrosis), it can be concluded that the administration of *Brachionus* sp. can reduce VNN infection in cantang grouper. In addition, the administration of *Brachionus* sp. also affects the immune response of fish as seen from the decreased expression of CD4. Fish treated with 105μL protein *Brachionus* sp. is the best concentration in reducing VNN infection seen from the lowest CD4 expression.

CONFLICT OF INTEREST

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

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