CD4 (Cluster Differentiation-4) as an immune response to the portal of entry mechanism of VNN (Viral Nervous Necrosis) infection in grouper

U Yanuhar1*, S Anitasari1, Kusriani1, N S Junirahma2 and N R Caesar3

1Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya, Malang Indonesia.
2Master Program of Aquaculture, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya, Malang Indonesia.
3Doctoral Program of Environmental Science, Postgraduate Program, Universitas Brawijaya, Malang Indonesia.

*E-mail: doktoruun@ub.ac.id

Abstract. Viral Nervous Necrosis (VNN) is a Nodaviridae virus that attacks groupers, especially in the larval and seed stages. VNN attacks the brain organs, rapidly attacks the grouper fish receptors and then spreads to the brain via blood circulation. The mechanism of viral infection can occur because of the bond between the VNN adhesive and its receptor molecules in the grouper organ. The aim of this study was to compare the CD4 (Cluster Differentiation-4) values of VNN-infected groupers and healthy fish using immunohistochemical (IHC) analysis. The method used in this study was experimental exploratory by comparing and analyzing the response of CD4 cells in healthy groupers with groupers infected with VNN through IHC observations. The results of IHC test showed that the immunogenic epitope of 32.5 kDa VNN protein had the ability to induce tissue receptors of Humpback grouper (Cromileptes altivelis) by forming immune cells qualitatively. There are CD4 cells that are immune to specific tissue organs, i.e. eyes of the groupers, which are labeled with anti-CD4 secondary antibodies. It seems that immunogenic protein VNN with a molecular weight of 32.5 kDa has the ability to stimulate the expression of Humpback grouper immune cells such as CD4 in tissue organs, especially the eye organs qualitatively.

1. Introduction
Disease is the result of an imbalance between host, pathogen and environment. Caused of diseases in fish are infectious agents like parasites, bacteria, fungi, and viruses, as well as non-infectious agents such as poor feed quality and water environmental conditions that are incompatible with fish life [1]. One of the diseases from the virus class that is often reported to attack groupers is VNN (Viral Nervous Necrosis). The VNN is a Nodaviridae virus that attacks groupers, especially in the larval and seed stages. Nodaviridae is an RNA virus, with a diameter between 20-25 nm. This disease mostly attacks larvae at the age of less than 20 days; even VNN can cause mass mortality up to a prevalence of 100% [2].

In the recent years, VNN has been reported as a cause of the lethal disease in grouper. VNN will attack the brain organs and rapidly attack the grouper fish receptors, and then spread to the brain through blood circulation [3]. It is likely that the virus attacks a weak host via the epithelium of the intestine and peripheral nervous system, immediately reaching the central nervous tissue as a target where the virus can cause death of the host [4]. The portal of entry that occurs in the case of this study is the eye organ, which is one of the main targets in the attack of VNN disease. Clinical symptoms that appear as a sign of VNN virus attack in fish are bulge and enlarged eyes, decreased appetite, very...
weak fish, pale body color, and specific symptoms that cause uncoordinated movement such as undirected swimming, circling, hyperactive, upside down, and often jolting the head on the water surface sporadically [5, 6].

To face virus attack, the fish will activate its body's defense system so that it raises an immunological response. The immune response in the body is in the form of a very complex sequence of events because of an attack by an external antigen and functions to eliminate the antigen. This immune response consists of a set of cellular and humoral components to defend the body against foreign substances such as pathogens, toxins or malignant cells, which respond to factors such as endogenous or exogenous components that stimulate this system. One form of defense by recognizing, weakening and killing pathogens and other foreign substances [7]. The immune system consists of the non-specific (natural / innate) immune system and the specific (adaptive / acquired) immune system. The specific immune system, among others, consists of the humoral specific immune system and the cellular specific immune system. In fish, the cell-mediated immune responses is regulated by various types of leukocytes including T-lymphocytes, which consist of cytotoxic T lymphocytes (CTL) and T helper (Th) cells [8]. Exposure to foreign substances or antigens can generate cellular immune responses through the proliferation and differentiation of dendritic cells into Th1 cells and Th2 cells. The proliferation and differentiation of immune cells such as CD4 (Cluster Differentiation-4) serves to protect themselves from viruses. The T cell receptors are only able to recognize the antigen represented by MHC class II molecules [9]. CD4 is a transmembrane glycoprotein that is expressed on the surface of Th cells and plays an important role in the immune response. T-helper cells expressing CD4 (CD4 + Th cells) coordinate the immune response by acting as effector cells or as memory cells [10]. The function of CD4 + T cells in fish is considered comparable to that of mammals due to the presence of TCR, a CD4-like gene with the same number of domains (D1-D4). Population characterization and function of CD4 + cell has been determined due to the availability of suitable markers for T-lymphocytes in fish. CD4 + Th cells are essential to trigger and maintain natural and vaccine-induced immunity [11].

Another important role of the grouper defense system against VNN is the receptor protein, which is expressed on the body parts of the grouper. The mechanism of viral infection can occur because of the bond between the VNN adhesin and its receptor molecules in the grouper organ. Viral adhesin can be a basic component of the virus, namely coat protein and nucleic acid. The VNN coat protein is a major factor in the mechanism by which the virus infects the host where the protein plays a role in the attachment of the virus to the host receptor. It is known that one of the adhesins of VNN is haemagglutinin [9]. More complete information on molecules related to the immune system are needed that can be used also for further applications in aquaculture, among others for the development of biomarkers, for vaccine or auxiliary development, and for the development of transgenic fish [12]. Therefore, this study aims to compare the CD4 value of VNN-infected groupers and healthy fish by using immunohistochemical (IHC) analysis.

2. Materials and Method

2.1. Research and sampling method

The experimental exploratory method was used in this study by comparing and analyzing the response of CD4 cells in healthy and VNN infected groupers through histology and immunohistochemistry (IHC) observations. Humpback groupers (Cromileptes altivelis) from floating net cages (KJA) Cultivation Center in Situbondo Waters, East Java were used in this study. As many as 10 samples of grouper at larva stadia, size 10-15 cm for both healthy and VNN infected fishes were maintained homogenously at controlled salinity sea water of 30-33 ppm. The immune response is shown by the CD4 cell response in healthy and VNN infected fishes.
2.2. Isolation of grouper eye organs

Isolation of grouper eye organs refers to method of [9], and positive samples of grouper fish infected with VNN were stored in liquid nitrogen. Grouper eye organs from VNN positive samples were isolated in laminar airflow using a set of sterile scalpels. The eye organs were homogenized with a sterile mortar and nerve cells were isolated by adding an extract buffer with an organ ratio of 2 ml: 1 mg. Furthermore, the homogenate was centrifuged by using ultra high centrifugation at 50000 rpm for 1 hour to separate the impurities from the nerve cells. The morphology of the degraded nerve cells was examined and then the nerve cell receptor protein was isolated. To obtain protein of VNN, the collected samples were again centrifuged at 150000 rpm for 3-5 hours. Supernatant, which is crude VNN protein was separated from pellets, packaged in sterile eppendorf and then stored in freezer at -80°C until further testing.

2.3. Measurement of CD4 cell response

Measurement of CD4 cell response based on the method proposed by [9]. This measurement was carried out by directly inducing the VNN adhesion protein material which cross-reacted with the 32.5 kDa receptor through 300 gram of grouper. Before measurement, fish was induced with a complete adjuvant (CFA) and incomplete (IFA) as many as three times booster. After the third booster, the fish serum containing antibodies was purified and the measurement of CD4 cell response in healthy fish and fish infected with VNN to the receptor was carried out using immunohistochemistry method.

2.4. Immunohistochemistry (IHC) techniques

Immunohistochemical staining is mostly done through immunohistochemical by using primary and secondary antibodies and streptavidin–avidin to show cross-reactivity between antigen and antibody performed. In the cross-reaction test method of CD4 cell proliferation and expression using secondary antibodies of anti CD4 cells from IgG of mice with the initial tissue incubation technique for 30 minutes under normal conditions [9]. Drop the serum and wash off the rest, no need to rinse. Incubate for 30 minutes with each of the following 3 reagents; rinse with and place 3-5 minutes in the wash buffer after each step. Furthermore, primary antibodies are given to anti CD4 cells, then labeled with a biotinylated secondary antibody, and prepared for at least 30 minutes for the use of the streptavidin-avidin complex. Incubate the wash substrate until the desired staining intensity develops. Rinse with distilled water, counterstained and covered, then observed under light microscope.

3. Results and Discussion

3.1. Clinical symptoms of VNN-infected grouper

Observation of clinical symptoms in groupers selected as samples of VNN-infected fish showed several external morphological signs such as bulging eyes, abnormal swimming behavior (swirling), darker body color, enlarged stomach, lose appetite, and blackish gill. This result is in accordance with the symptoms presented by Yuwanita et al. (2013) [4] and Sembiring, et al., (2018) [5] that the clinical symptoms that can occur in VNN-infected fish are abnormal swimming behavior, floating due to swelling of the swim bladder, darker body color and loss of appetite. These signs, especially the swirling swimming behavior is mostly associated with the presence of vacuoles in the central nervous system and in the retina.

3.2. Histology and CD4 response observation

Information on change of tissue microscopically from fish eye tissue can be used to develop cellular biology and provide a pathological evaluation of disease, and show the presence of lesions. Histological observations on the eye tissue of infected grouper found that there are lesions in the granular layer of the retina, including Edema, Hypertrophy, Vacuolation, and Haemorage as shown in Figure 1.
Vacuolation in the retina of the eye can be indicated by impaired vision of groupers as seen in clinical symptoms observation. This is consistent with the result of Yuwanita et al. (2013) [4] who found that in the eye, receptor interaction is done by laminin that can be found on the epithelial surface and on the retina. VNN attacks can cause exophthalmia, which is a decrease in the ability of the cornea.

Viruses can get access to the central nervous system via the peripheral nervous system, which consists of nerves emerging from the brain and spinal cord, then the eyes, gills, liver, kidneys, intestines and other organs of fish. Viral proliferation causes blockade of blood flow and increased capillary pressure. Due to blockage, fluid is increasingly forced across the capillary membrane into the interstitial space. Accumulation of extracellular fluid causes edema and ultimately results in rupture of the inner eye lining, altering the normal physiological state of the eye [13]. In addition, rupture of blood vessels in the lining of the eye results in the appearance of Hemorrhage (bleeding) in the eye tissue as a sign of disease attack in infected fish. Hemorrhage and Hypertrophy found in this study were also found in the study of Yuwanita et al. (2013) [4].

The CD markers are antibodies to recognize cell surface antigens and detect certain CD antigens used in immunohistochemistry to differentiate various types of immune cells. The CD4 gene is responsible for encoding the surface glycoproteins of CD4 T cells that are expressed in a subset of lymphocytes, T regulatory cells, and T helper cells. Results of IHC examination performed on groupers that have been clinically tested with VNN immunogenic protein 32.5 kDa. This test was performed on sample of grouper eye organ tissue as a portal of entry site for VNN infection. Some of the CD4 cell expression results proved positive by the brownish reaction in the organ of the grouper eye tissue. This is used to see the expression of immune cells in the grouper tissue that has been tested with the immunogenic protein VNN 32.5 kDa as presented in Figure 2.

The test results are carried out to detect the expression of CD4, which is an immune cell that is formed to respond the presence of anti CD4 antibodies. Response of CD4 can be seen by looking at the bond between the antigen (VNN 32.5 kDa immunogenic protein) and the anti-CD4 monoclonal secondary antibody to the biotin conjugate antibody. The results showed that the expression of a specific 32.5 kDa VNN immunogenic protein antigen determinant to induce immune cell receptors through expression on grouper CD4 cells. The reaction, which forms a golden brown color (shown in a circle), indicates a strong bond between the epitope of the 32.5 kDa immunogenic VNN protein and anti-CD4 antibody that identifies a particular part of the immunogenic protein. In infected fish, the expression of CD4 response is relatively higher; this is because immune cells are used for the protection system against viruses.
Figure 2. Immunohistochemical observations used secondary antibodies to mouse conjugate anti-CD4 IgG antibodies in eye tissue of fish; a) CD4 expression in normal eye tissue; b) CD4 expression in the VNN-infected eye tissue; c) CD4 expression in normal eye tissue with the addition of 32.5 kDa protein adhesion; d) CD4 expression in the eye tissue of VNN infected with the addition of 32.5 kDa protein adhesion

Besides VNN infection in grouper, previous studies to strengthen this research also examined *Mycobacterium tuberculosis* infection in carp (*Osphronemus gourami* Lac.) as well as in hybridoma cells, which are specified for the manufacture of *M. tuberculosis* monoclonal anti-adhesion antibodies [14]. Previous studies have developed an in vivo immune response related to vibriosis infection [15, 16], then an in vivo immune response related to the development of a vaccine for anti-VNN and a diagnostic tool for VNN in fish [17]. In addition to the expression of CD4 cells, the study of Yanuhar et al., (2012) [12] also reported that the highest expression of IFN-γ cells in groupers was found in the eye organ while the highest expression of NF-kB was shown in the brain organ. This was due to proliferation and the expression of immune cells in groupers against the viral response to the VNN protein immunogenic that is regulated by central nerve cells. The number of expressions which are induced by immune cells varies depend on the target cell of each organ. Furthermore, in that study, the attachment of crude VNN protein with a molecular weight of 45.9 kDa in expressing grouper cells proved CD4 cells and CD8 + cells as well as IFN-γ and NF-kB.

4. Conclusion

Based on the results of this study, it was concluded that the immunogenic protein VNN with a molecular weight of 32.5 kDa had the ability to induce the expression of immune cells in grouper. Expression of immune cells such as CD4 as an active immune response works qualitatively on tissue organs, especially eye organs of Humpback grouper through immunohistochemistry (IHC) staining. The lesions found on the retina of the eye are Edema, Hypertrophy, Vacuolation, and Haemorage.
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