Immune Response of VNN (Viral Nervous Necrosis) Infected Grouper Utilizing Chlorella vulgaris Extract as an Anti-Virus Candidate

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Abstract. The quality and health of fish guarantee the success of aquaculture. However, grouper aquaculture are susceptible to diseases such as VNN (Viral Nervous Necrosis). It is necessary to determine the fish immune response system by observing the changes in the number of blood cells. Utilizing alternative substances, Chlorella vulgaris for instance, may minimize infection. This study aims to determine the effect of C. vulgaris extract treatment, as a candidate of VNN anti-virus, through hematological observations. The research method used qualitative experimental design by describing the fish immune response based on the number of blood cells. Fish blood was collected from each treated fish in the following treatments: (A) healthy fish, (B) VNN-infected fish, (C) VNN-infected fish treated with 17 µg / mL C. vulgaris extract, (D) VNN-infected fish treated with 33 µg / mL C. vulgaris extract, and (E) VNN-infected fish treated with 50 µg / mL C. vulgaris extract. The blood cells are observed using a light microscope. The water quality parameters analyzed were Temperature, pH, Salinity, and Dissolved Oxygen (DO) as supporting data. The research results showed that healthy fish has a high number of erythrocytes (28 cells/field of view). Treatment (D) shows a value close to healthy conditions, namely 22 cells/field of view. C. vulgaris extract treatment on VNN-infected groupers improves the immune system as indicated by an increased number of erythrocytes and decreased number of basophils cells (11 cells/field of view) and neutrophils (16 cells/field of view). Examination of water quality parameters shows tolerable conditions for the growth of groupers based on quality standards.

1. Introduction
Grouper aquaculture is highly favored as it has an economic value in the international market. Humpback grouper (Cromileptes altivelis) has high export potential. However, the grouper cultivators face a problem as humpback groupers have a low survival rate due to disease that attacks grouper seeds. The disease was caused by environmental pressure in the form of decreasing water quality. The low water quality induces stress and increases bacterial, parasitic, and viral infections [1]. One of the diseases infecting groupers is Viral Nervous Necrosis. The disease causes a high mortality rate of grouper fish, especially in the larval and juvenile stage. VNN weakens the grouper nervous system, causing it to lose nerve control, weaken motions, and lead to death [2].
Microalgae have an important role as a renewable natural food source. In addition, microalgae is a potential alternative to bioactive substances in improving fish and shrimp health. Natural food is an important source of nutrition for the early stages of organism growth [3]. VNN generally attacks the eyes and brain nervous system which causes behavioral deviation. The fish will swim in circles and float on the surface of the water belly side up. In addition, VNN causes darker color pigmentation on fish [4]. VNN diagnosis is conducted through visual observation on fish behavior and clinical symptoms. Further observations are conducted microscopically through hematological observation on Grouper Fish blood.

VNN countermeasures through natural substances use the microalgae bioactive compound of *Chlorella vulgaris*. *Chlorella vulgaris* is a type of microalgae containing protein, vitamin, mineral, carbohydrate, fat, chlorophyll, and beta carotene. *C. vulgaris* has several types of pigments such as β-carotene, astaxanthin, canthaxanthin, lutein, chlorophyll-α, chlorophyll β, pheophytin-α, and violaxanthin [5]. Chlorella is one of the microalgae containing carotenoids such as canthaxanthin, astaxanthin dan lutein [6]. Astaxanthin is used to prevent the inflammation process. For instance, colitis and several types of cancers. The effect of *Chlorella vulgaris* have been researched on the blood and immunology parameter of Caspian salmon (*Salmo trutta caspius*) [7].

Natural immunostimulants such as microalgae increase resistance to infectious diseases. Microalgae increase specific immune response and non-specific immune defense [7]. VNN infection triggers inflammation in fish tissue. Responses to stimulants, in the form of viral infection and inflammation, starts after a few minutes in the respective host using a functional innate immune system. The innate immune system is a major contributor to inflammation. Immune cells such as macrophage, dendritic cells, mast cells, neutrophils, and lymphocytes play an important role in the inflammatory response [8]. White blood cells or leukocytes and its derivatives; neutrophils, basophils, eosinophils, lymphocytes, macrophage mastocytes, and plasma cells is crucial for inflammatory response [9]. This research aims to determine the potential of *C. vulgaris* extract as the candidate of VNN anti-virus. This research observed the immune response of VNN-infected groupers by administering *Chlorella vulgaris* extract and conducting the hematological observation.

2. Methods

The research method used a qualitative experimental design that is presented descriptively through hematological observations. Fish samples were obtained from floating net ponds (KJA) in Pecaron waters, Situbondo, Indonesia. Fish samples were grouped into several *Chlorella vulgaris* treatments. The treatments were conducted 3 times using a feeding tube during the study period. The grouper fish treatment is as follows: (A) Healthy fish, (B) VNN-infected fish, (C) VNN-infected fish treated with 17 µg/mL of *C. vulgaris* extract, (D) VNN-infected fish treated with 33 µg/mL of *C. vulgaris* extract and (E) VNN-infected fish treated with 50 µg/mL of *C. vulgaris* extract. Each treatment group consisted of 12 groupers. The fish are reared for 14 days. Behavioral observation was conducted on the groupers. Blood sample was collected from groupers exhibiting the symptoms of VNN infection. The blood sample of healthy fish was collected as a control group.

Fish blood collection referred to [10] method. The preparation of blood smear was observed under a light microscope. Measurement of red blood cells (Erythrocytes) referred to [11] Formula:

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\Sigma \text{Erythrocytes} = \frac{\text{Cell}}{\text{mm}^3} = \frac{\text{Average of Counted Erythrocytes} \times (\text{Diluent / Volume})}{1}\n\]

The percentage of leukocyte cells was calculated by observing 10 fields of view. Each differential type of leukocytes was counted and grouped according to its type (neutrophils and basophils). The calculation of the number of neutrophils and basophils cells referred to [12] formula:
The water quality parameters were measured as supporting data. The measured parameters are temperature, pH, DO, and CO$_2$. Water temperature measurement used a thermometer, Salinity measurement used an Atago refractometer, the DO values measurement used a DO mete, and the pH value measurement used a pH meter.

3. Findings And Discussion

3.1. Clinical Symptoms of VNN-Infected Grouper

Stress is the primary cause of fish susceptibility to disease. Stress can occur due to bad water environmental factors and the handling process of maintenance. Observation of groupers' clinical symptoms was conducted during the rearing process. VNN-infected groupers exhibit the following characteristics: bulging eyes, darker body color, abnormal swimming in circles, enlarged stomach, limp, and loss of appetite. In addition, dissected VNN-infected groupers exhibit black gills. The symptoms are similar to [13] findings. Common clinical symptoms of VNN-infected fish are abnormal swimming behavior, loss of appetite, and darker body color. In addition, the fish may float due to swollen swim bladder.

3.2. Immunological Response of Grouper Blood Cells

The immunological response is a body response, in the form of a very complex sequence of events, as a result of external antigen attack and functions to eliminate these antigens. The immune response consists of a set of cellular and humoral components to defend the body against foreign substances such as microorganisms, toxins, or malignant cells, which respond to factors such as endogenous or exogenous components that stimulate this system [14]. Whereas, this study focused on VNN infection. One of the body immunological responses is seen from the blood cells. As a result of VNN infection, the body will experience inflammation as a non-specific response to tissue damage. Furthermore, the body uses the innate immune system and the adaptive immune system to fight pathogens. The released inflammatory mediators increase blood flow. Blood cells that play a role in the inflammatory event are white blood cells or leukocytes with its derivatives; neutrophils, basophils, eosinophils, lymphocytes, macrophage mastocytes, and plasma cells [9]. Figure 1 shows the result of treatments and observation of the changes in groupers' blood cells.

Erythrocytes are biconcave, non-nucleated discs with a diameter of ± 8 μm, edge thickness up to 2 μm, and core thickness up to 1 μm. The main component of erythrocytes is the hemoglobin protein which transports mostly oxygen (O$_2$) and a small fraction of carbon dioxide (CO$_2$) [15]. The average number of erythrocyte cells in treatment A was 22.4x10$^6$ cells / mm$^3$. In normal conditions without viral infection, erythrocytes are the largest blood component [16]. The average number of erythrocyte cells in treatment C was 11.4x10$^6$ cells/mm$^3$, treatment D was 17.6x10$^6$ cells/mm$^3$, and treatment E was 13.3x10$^6$ cells/mm$^3$. The number of erythrocyte cells decreases after infection [17,18]. Also, the previous study of [19] showed that fish infected by VNN experienced a decrease in erythrocytes followed by a decrease in hemoglobin which caused the fish to become weak. VNN-infected grouper treated with C. vulgaris extract in C, D, and E treatments shows increased erythrocyte value compared to treatment B.
This indicates that \textit{C. vulgaris} treatments can increase the immunological response of VNN-infected groupers.

![Figure 1](image_url)

**Figure 1.** Graph of grouper immunological response: (a) total erythrocyte (cells / mm$^3$); (b) the percentage of neutrophil and basophil cells (%). Description: (A) Healthy fish; (B) VNN-Infected Fish; (C) VNN-infected fish treated with 17 $\mu$g / mL extract of \textit{C. vulgaris}; (D) VNN-infected fish treated with 33 $\mu$g/mL extract of \textit{C. vulgaris}; and (E) VNN-infected fish treated with 50 $\mu$g/mL extract of \textit{C. vulgaris}.

The basophils in blood circulation have been observed in only a small number of the existing fish species. Basophils are less common and less likely to show in Giemsa staining [20]. Neutrophils are polymorphonuclear cells found in the blood, lymphoid tissue, and peritoneal cavity which can phagocytose foreign particles [14]. The primary function of neutrophils is the destruction of foreign material through the phagocytosis process. Based on the calculation result, the average percentage of basophil cells in treatment A was 18%. Basophil cells are part of leukocyte cells which is influenced by infection. The basophil cells in the blood will increase during viral infection. This is supported by the results of treatment B observation. The average percentage of basophils cells in treatment B was 62%. C, D, and E treatments show slightly decreased basophil cell percentage value compared to treatment B, namely 35%, 32%, and 46% respectively. Similar to basophil cells, neutrophil cells are massively produced during infection. The number of neutrophils cell production is relatively low during normal conditions. The percentage of neutrophils cells in Treatment A was 26%. On the contrary, Treatment B shows increased neutrophils cells up to 88%. Treatment C, D, and E show the percentage of neutrophils cells of 54%, 49%, and 58% respectively. The increased neutrophil cells indicate an increased collection of macrophages at the site of infection. As a result, it is easier for macrophages to destroy foreign particles [21]. The samples treated with \textit{C. vulgaris} showed a slight decrease compared to treatment B. It indicates that the administration of \textit{C. vulgaris} affects the number of erythrocytes, basophils, and neutrophils cells of VNN-infected groupers.

### 3.3. Water Quality Measurement

Environmental factor highly influences the growth of grouper. Water quality factors that affect the growths of grouper are dissolved Oxygen (DO), pH, temperatures, and salinity [1]. Table 1 shows the observation result on the water quality parameters of grouper culture media.
Table 1. Observation results of water quality parameters

| Treatment | A     | B     | C     | D     | E     |
|-----------|-------|-------|-------|-------|-------|
| Temperature (°C) | 29-30 | 29-31 | 29-31 | 29-31 | 29-31 |
| Salinity (%)o   | 33-35 | 33-34 | 33-34 | 33-36 | 33-35 |
| pH             | 8.1-8.4 | 8.0-8.4 | 8.2-8.3 | 8.0-8.3 | 8.0-8.4 |
| DO (ppm)       | 8.9-9.2 | 8.6-9.0 | 8.7-9.1 | 8.8-9.0 | 8.8-9.0 |

Description: (A) Healthy fish tank; (B) VNN-infected fish tank; (C) VNN-infected fish tank treated with 17 µg/mL C. vulgaris extract; (D) VNN-infected fish tank treated with 33 µg/mL C. vulgaris extract; and (E) VNN-infected fish tank with 50 µg/mL C. vulgaris extract.

Based on the observation data on the water quality of the fish culture media, all water quality parameters were in normal conditions for the growth of groupers. The temperature value ranges from 29 - 31 °C. Temperature is one of the environmental factors that may induce stress on fish. Large and sudden changes in temperature induce stress. Stress is the inability of an organism to maintain homeostatic conditions due to the disruption of the individual by external stimuli called stressors. Therefore, stress causes vulnerability to VNN attacks [22]. The salinity values ranged from 33%-36%. The pH values ranged between 8-8.4. The dissolved oxygen in the water ranged between 8.6-9 ppm. This is in accordance with [23] research which stated the range of good quality parameters for grouper growth. The parameters are temperatures ranging from 29 - 30 °C, salinity between 30-33 ppt, dissolved oxygen> 3.5 ppm, and pH range between 7.8 - 8.0.

4. Conclusion

Based on blood cell observations, the administration of C. vulgaris extract to VNN-infected groupers increased the immune system indicated by an increased number of erythrocytes and decreased number of basophil and neutrophil cells. Treatment D (33 µg/mL C. vulgaris extract treatment) shows relatively better blood cell conditions. Observation of Temperature, pH, DO and salinity on water quality parameters are classified as good and tolerated for grouper habitat. Blood cell analysis can be used as an early indicator in determining grouper health. However, further research is needed to optimize the effective dosage for treating VNN-infected groupers.

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