The Status of VNN (Viral Nervous Necrosis)-Infected Grouper Fish Tissue with Chlorella vulgaris Extract as Anti-Virus Candidate

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Abstract. To date, the most common problem in Grouper fish cultivation is VNN (Viral Nervous Necrosis) virus attack. Visual and histological observations on the fish behavior can be done to diagnose the presence of VNN. Chlorella vulgaris is believed to be one of the natural alternative ingredients that have the potential to be a VNN anti-virus. The purpose of this study is to determine the potential use of C. vulgaris extract as a candidate for VNN antivirus by conducting histological observations on the organ tissue of Grouper fish. The method used in this study was a qualitative approach with histopathological techniques examining the fish organs such as brain, kidney and eyes from each treated fish. The clinical symptom observation was carried out for 14 days. Then, the harvested organs were brought for tissue preparation and observed again with a light microscope. In this case, the histopathological changes can be assessed using a semi-quantitative scoring method. The results reveal that the Grouper fish had several symptoms such as darkening body-color, abdominal bloating, abnormal swimming behavior, and decreased appetite. It is also found that there was a change in the histological picture between the infected fish without treatment and the infected fish with the administration of C. vulgaris extract. There were a number of lesions such as vacuolization, hemorrhagic, hypertrophy, and necrosis in the kidney, eye, and brain of the infected fish. Of all the treatments given, treatment (D) shows a little damage to the fish kidney, eye, and brain respectively by 26.7%, 33.3% and 33.3%. Therefore, it can be said that the administration of C. vulgaris as an alternative feed and antivirus candidate can be used as a natural immune-stimulant to enhance the immune system of Grouper fish.

1. Introduction

Grouper fish is one of the leading commodities in marine fish cultivation which has a high economic value in the export commodity [1]. One of which is Humpback Grouper (Cromileptes altivelis) which has a big potential in the fishery export market. Nevertheless, the biggest problem which often occurs
in fish cultivation is a disease such as VNN (Viral Nervous Necrosis) virus [2]. VNN can attack the central nervous system, eye retina, and reproductive organ of the fish. In general, this disease can infect almost all fish growing phase, particularly on the larvae and seed stage. It can also cause death with a mortality rate of up to 100%. The mortality caused by fish disease depends on the type of the disease, the condition of the fish, and the environment. If the environmental conditions decrease, the mortality caused by disease outbreaks is very high and vice versa [3].

Microalgae have an important role in natural renewable source of feed. Microalgae also have big potential as an alternative bioactive ingredient for the health of fish and shrimp. This is very beneficial because, naturally, it will become a source of important nutrients in the early stages of an organism's development [4]. One example of microalgae that is used as a natural feed source and one of the anti-VNN ingredients is Chlorella vulgaris. Chlorella vulgaris contains a lot of contents including protein, vitamins, minerals, carbohydrates, fat, chlorophyll, and beta carotene [5]. Protein is the most dominant content in C. vulgaris (60%). C. vulgaris also has several types of pigments such as violaxanthin and beta-carotene which are known to have high antioxidant content [6,7]. C. vulgaris showed better activity in inhibiting lipid peroxidation and also showed antioxidant properties [8]. Apart from being an antioxidant, the crude extracts of C. vulgaris which contain bioactive such as flavonoids, phenols, and saponins can function as an anti-bacterial. This happens because C. vulgaris inhibits the growth of bacteria in the in-vitro test [9]. In previous studies Yanuhar, et al. [10], it was revealed that the administration of C. vulgaris doses can affect the immune system of VNN-infected fish.

It is assumed that a histological analysis is a very sensitive parameter and a very important instrument in determining the changes of cell structure that occur in internal organs [11]. According to Liebel et al., [12], a histopathological analysis can be used as a biomarker to determine the health condition of fish through structural changes that occur in the organs. It focused on the organs that become the main target of pathogen infections so that they can be used as an early diagnosis of an organism’s health problem. The purpose of this study is to determine the potential use of C. vulgaris extract as a candidate for VNN antiviral by conducting a histological observation on the tissue of Grouper fish organs. By that, it is necessary to examine the status of gill tissue damage by administering the extract of Chlorella vulgaris in the VNN-infected Grouper fish.

2. Methods

The method used in this study was experimental qualitative research that is done through histopathological observations. The sample of the fish was obtained from floating-net-ponds (Keramba Jaring Apung or KJA) in Pecaron, Situbondo. The fish samples were classified with several treatments including the administration of Chlorella vulgaris which was given by metode penyondean for 3 times. The treatment was given to (A) Healthy fish, (B) VNN-infected fish, (C) VNN-infected fish with 17 μg/mL of C. vulgaris extract, (D) VNN-infected fish with 33 μg/mL of C. vulgaris extract, and (E) VNN-infected fish with 50 μg/mL of C. vulgaris extract. Each treatment consisted of 12 groupers. Then, the fish were observed for 14 days and after 14 days, the fish samples were taken for tissue preparation based on the observation showing the symptoms of VNN infection. The organs observed by the researchers include the brain, eyes, and kidneys of the fish.

Consequently, the brain, eyes, and kidneys preparations were observed under a light microscope at 400x magnification. The assessment of tissue damage for each organ was done using a semi-quantitative scoring method that is based on the research of Pantung et al., [13]. The assessment qualifications were based on the percentage of damage per field of view (%) ranging from 0 (no damage or 0%), 1 (minor damage or <30%), 2 (moderate damage or 30-70%), and 3 (severe damage or <70%).

3. Results And Discussion

3.1. Clinical Symptoms of VNN-Infected Grouper Fish
The main cause of fish susceptibility to disease is stress. Stress can occur due to the bad water environment and the handling process in the rearing. The observation of clinical symptoms in Grouper fish during rearing showed that the VNN-infected fish had a number of characteristics such as bulging eyes, darker body color, abnormal swimming behavior (in circles), enlarged stomach, weakening, loss of appetite, and black gills. This is consistent with the symptoms found by Yanuhar et al. [2], which believed that the common clinical symptoms in VNN-infected fish are abnormal swimming behavior, loss of appetite, darker body color, and floating due to the swelling of the swim bladder.

3.2. Histological Observations

Based on the results of observations using a light microscope, it appears that healthy fish showed less damage compared to infected fish and fish that are treated with *Chlorella vulgaris*. The results of the histopathological analysis on the kidneys, eyes, and brain tissue can be seen in Figure 1 below.

![Figure 1](image)

**Figure 1.** Histological features of healthy Grouper fish (A) and VNN-infected fish (B) in kidneys (1), eyes (2), and brain (3) tissue; Vacuum (V), Hemorrhage (H), Congestion (C).

*Figure 1* portrays the abnormalities in the eyes, kidneys, and brain tissue of the VNN-infected Grouper fish. Whereas, *Figure 2* illustrates several lesions in fish treated with *Chlorella vulgaris*. The changes were mainly found in the brain, eyes, and kidneys on each treatment. In the VNN-infected fish, the types of lesions that appeared in the brain tissue were vacuolization, hemorrhage, and necrosis. While on the other hand, vacuolization, hemorrhagic, hypertrophy, and necrosis were found in the eyes. In the kidneys, there was congestion, vacuolization and cloudy swelling. The assessment of damage to the
brain, eyes, and kidneys was carried out using the scoring method from Pantung et al., [13] based on the observations per field of view (%).

**Figure 2.** The histology pictures of VNN-infected Grouper fish with 17 µg/mL of *C. vulgaris* extract (C), VNN-infected Grouper fish with 33 µg/mL (D) of *C. vulgaris* extract (D), and VNN-infected Grouper fish with 50 µg/mL of *C. vulgaris* extract (D) in kidneys (1), eyes (2) and brain (3); Vacuum (V), Hemorrhage (H).

| Treatment | Total damage (%) | Score* | Damage Rate |
|-----------|------------------|--------|-------------|
| A         | 5.6              | 1      | Light       |
| B         | 70.6             | 3      | Severe      |
| C         | 30.6             | 2      | Moderate    |
| D         | 23.3             | 1      | Light       |
| E         | 28.9             | 1      | Light       |

*Pantung et al., 2008 [13].

Histopathology is the most important indicator in determining the profile of infection caused by bacteria, parasites, and viruses. The organ in the central nervous system like the brain is the main target of the VNN virus. The level of brain damage on each treatment can be seen in Table 1 above. In the research of Yuwanita and Yanuhar [14] emphasized that the brain, spinal cord, and retina are considered to be the target of the virus because the virus can actively replicate in there. This causes extensive tissue...
vacuolization. This study pointed out that almost all treatment showed a vacuolization. According to Amelia and Prayitno [15], Nodaviridae virus caused damage to the eye and brain. This is characterized by abnormalities in the body inclusivity stage of cytoplasm which then caused hypertrophy (cell enlargement) and formed a vacuolization. The tissue in the intracytoplasmic vacuolization stage (enlarged cell cavity) will be accompanied by the degeneration of cells. The vacuole formed will disrupt the work system of the brain. In addition to that, necrosis is found in the brain and eyes. Necrosis is cells that have very low activity and eventually experience a tissue cell death causing loss of function in the damaged area [16].

In the eyes, vacuolization, necrosis, hemorrhage, and congestion were found. Necrosis of cells causes cells to be damaged, leaving only Empty space in the brain tissue or vacuolization that occurs due to cell damage. this is because VNN infection passes through the bloodstream and enters the brain, causing tissue damage [17]. A similar result was shown by Yuwanita and Yanuhar [14] which revealed that vacuoles occur in the granular layer of the retina in VNN-infected Grouper fish. Meanwhile, congestion, which is the presence of severe swelling of blood vessels can cause blood flow to become obstructed and will cause bleeding. Eventually, tissue oxygenation will be lost causing the rupture of blood vessels to spread to other tissues [18]. The scoring results on the eyes are shown in Table 2 below.

### Table 2. Scoring and Percentage of Eyes Damage

| Treatment | Total damage (%) | Score | Damage Rate |
|-----------|-----------------|-------|-------------|
| A         | 4.2             | 1     | Light       |
| B         | 67.1            | 2     | Moderate    |
| C         | 30.4            | 2     | Moderate    |
| D         | 23.3            | 1     | Light       |
| E         | 27.9            | 1     | Light       |

*Pantung et al., 2008 [13]*

Apart from the central nervous system organs, kidneys are the target organs for the spread of the VNN virus in the osmoregulation system and in the circulatory process. Not only vacuolization, cloudy swelling, and congestion were also found in the kidney. This is in line with the study of Rosmaidar et al., [19] that histological changes in the fish liver are in the form of cloudy swelling with the cloudy cell as well as cloudy and granular cytoplasm. Cloudy swelling is the initial damage that occurs in the epithelium dividing the renal tubules in the form of cell swelling [20]. The results of the kidney scoring can be seen in the following Table 3.

### Table 3. Scoring and Percentage of Kidney Damage

| Perlakuan | Total damage (%) | Score | Tingkat Kerusakan |
|-----------|-----------------|-------|------------------|
| A         | 5.6             | 1     | Light            |
| B         | 63.9            | 2     | Moderate         |
| C         | 32.2            | 2     | Moderate         |
| D         | 26.1            | 1     | Light            |
| E         | 29.4            | 1     | Light            |

*Pantung et al., 2008 [13]*

Based on the results of the scoring that has been carried out on kidneys, eyes, and brain tissue preparations, the highest scores were found in the infected fish with the percentage of total damage to the kidney, eyes, and brain by 63.9%, 67.1%, and 70.6% respectively in the moderate to severe category. This occurs due to the amount of damage in the infected fish tissue. In this case, the infected fish were not being given any treatment. Whereas, the infected fish which were treated with Chlorella vulgaris administration showed a lower level of damage. Qualitatively, this can illustrate the effect of *Chlorella vulgaris* administration on the tissue of VNN-infected fish. In the *Chlorella vulgaris* 33 µg/mL treatment, it is shown that there is relatively lower damage compared to other *C. vulgaris* treatments.
The crude extract of *C. vulgaris* contains several important components such as carotenoids, alkaloids, flavonoids, glycosides, phenols, lignins, saponins, amino acids, and carbohydrates [7,9]. These bioactive contents can be used as a source of antioxidants, antibacterial, antivirus, and anti-inflammatory properties.

4. Conclusion

A histopathological observation on the brain, eyes, and kidneys of VNN-infected Grouper fish treated with *Chlorella vulgaris* proves that there are several types of abnormalities such as vacuolization, congestion, hemorrhage, cloudy swelling, and necrosis. Based on the qualitative scoring analysis, the treatment of *Chlorella vulgaris* 33 µg/mL (D) was relatively better than other treatments. Treatment D showed little damage which was defined as Light according to [13]. This can be said that the administration of *Chlorella vulgaris* extract has the potential to be used as a natural bioactive of antivirus. For this reason, further tests are needed to determine the effectiveness of the dosage used.

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