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Influence of a Pigment Protein Fraction from *Chlorella vulgaris* Isolated in Indonesia on β-Actin and MHC-1 Response to Viral Infected Humpback Grouper

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ABSTRACT

Study aims to show the capability of Pigment-Protein Fractions extracted from *C. vulgaris* for treating the infection of Viral Nervous Necrosis (VNN) in Humpback grouper. Study was conducted by isolation and culture *C. vulgaris* after identified using 16 sRNA. PPF was extracted and analyzed using the SDS-page. Fish treatment were divided in four groups: (A) normal fish/control), (B) PPF administration into fish, (C) VNN induction into fish, and (D) both VNN and PPF induction into fish. β-Actin and MHC-1 response were evaluated using ImmunoRatio (IR) analysis. The results show that microalgae that local isolated from Indonesia is confirm as *C. vulgaris* with nucleotide sequences of 424 bp. Extracted PPF of *C. vulgaris* show a 33 kDa of protein molecule weight. Treatment in group B, C, and D able to increases the MHC-1 expression as much as 26.1%, 35%, and 66.9%, respectively. PCR quantification results in all PPF test treatments showed the presence of B-actin expression. Histopathology observation on eye tissue indicates occurred Necrosis (N) in group B, Vacuolation (V) and Necrosis (N) in group C. In group D, there are Vacuolation (V) and Necrosis (N) but less tissue damage compare than group B. It is indicates that *C. vulgaris* have a positive influence on the immune response of groupers (*C. altivelis*) after infected by VNN.

Keywords: β-actin, 16S rRNA, *C. altivelis*, *C. vulgaris*, MHC-1, *Pigment-Protein Fraction*

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INTRODUCTION

Viral Nervous Necrosis (VNN) is virus with single-stranded RNA that causes destructive fish disease with impact on large economic loss in the aquaculture industry. VNN is included in betanodaviruses, a group of virus pathogen, causes mass mortalities up to 100% at fish larval of the aquaculture (Yuwanita *et al*., 2013) and also able to infect some adult marine fish. The use of drugs for the prevention or eradication of diseases is currently not recommended even not allowed because it causes residues and can cause resistance, thus in the prevention and eradication of the disease it is necessary to use natural ingredients (Zorriehzahra, 2020). The use of microalgae has been widely developed, especially in the health sector, which can be used as an antiviral. Protein pigment fragments from N. oculata microalgae can also be used as an anti-inflammatory and immunostimulants against the ssRNA virus attacks groupers (Yanuhar, 2015; Yanuhar and Khumaidi, 2017).

Chlorella vulgaris is marine microalgae having biogenic compounds known as active metabolite chemicals, such as halogenated compounds, alkaloid, terpenoid and tannins in significant amount (Masithaa *et al*., 2019). Active metabolite compounds have function as anti-bacterial, anti-macrofouling, anti-fungal and other possible therapy (Yanuhar, 2016; Yanuhar *et al*., 2011). *C. vulgaris* have several pigments types i.e. *pheophytin-α* and *violaxanthin β-carotene*, *chlorophyll-α*, *chlorophyll β*, *lutein, canthaxanthin, astaxanthin* (Safi *et al*., 2013). The active of pigments of *C. vulgaris* is in the natural form of a pigment–protein complex that potential to be used as a dietary supplement to reduce effect of free radical oxidation (Cai *et al*., 2015). *C. vulgaris* can be used as a source of proteins, vitamins, pigments, fatty acids, sterols in production of aquafeed (Andrade *et al*., 2018; Enyidi, 2017). The use of *C. vulgaris* as a dietary supplement in Caspian salmon (*S. trutta caspius*) cause a resistant to VNN virus in short periods initial infection (Saberi *et al*., 2017). It gives protection to the freshwater fish *O. niloticus* against penoxsulam toxicity (Galal *et al*., 2018). *Chlorella* sp extract significantly increases the heat shock cluster and heat shock protein response of VNN infected fish (Masithaa *et al*., 2019; Yanuhar *et al*., 2019).

The front line of host defense against pathogen is the immune system that comprises humoral and cellular components, and physical barriers (Buchmann, 2014; Hazreen-Nita *et al*., 2019). The actin itself is a protein, and is most commonly found in eukaryotic cells and has an important role in protein interactions. In general, there are three kinds of the actin cytoskeleton in organisms, namely α-actin, γ-actin, and β-actin (Artman *et al*., 2014). Dynamic changes of the actin cytoskeleton have a crucial role in interactions of host-pathogen, such as, phagocytosis and identified as the ligand for a receptor that recognizes damaged and dying cells (Sandiford *et al*., 2015). Viruses can interact with actin, rearranging it for facilitating the interaction of infectious virus (Trejo-Cerro *et al*., 2018). Confirmation of actin has a critical effect on phagocytosis against virus infection (Shu and Zhang, 2017). β-actin proteins have important roles in many cellular functions, i.e. the maintenance of cell shape, motility, and phagocytosis and contributed to much protein-protein interactions compare

than other proteins (Fan *et al*., 2018; Ferreira *et al*., 2017). The expression level of β-action might be an indicator of the severity of fibrosis as the outcome of several diseases, including viral infections (Zhang *et al*., 2019). The major histocompatibility complex class II (MHC-II) is proteins having a role in the immune response to antigenic peptides in vertebrate. As a component of the immune response, it recognize the exogenous epitopes of extracellular pathogens (Ye *et al*., 2020). MHC-I have a role in intracellular antigen present in the cytosol to the cytotoxic T cell (Tc cells), which is usually a bacterial infection or a virus nucleic acid (Yamaguchi and Dijkstra, 2019). The viral peptides recognition in the context of MHC-I molecules by cytotoxic T lymphocytes is a key event in the elimination of virus-infected cells (Wang *et al*., 2019).

Haematology status of the fish blood indicate the fish health condition (Saberi *et al*., 2017). A fish blood examination is a critical factor in helping diagnosis, prognosis, and therapy (Saparuddin *et al*., 2017). Infectious agent that attacked fish cause a severe parameter change in the blood. It shows disturbances are the hematocrit values, erythrocytes content, and changes in leukocytes number and also changes in the tissue (Alamanda *et al*., 2007). Abnormal or normal tissue structures able to be observed by microscope in the histopatology analysis (Yanuhar *et al*., 2020). Study aims to show the capability of Pigment-Protein Fractions extracted from *C. vulgaris* for treating the the Humpback grouper infected by VNN.

MATERIALS AND METHODS

Isolation and Cultivation of *Chlorella vulgaris*

Isolation technique is the first step in microalgae culture from marine waters. The purpose of the isolation itself is to get monospecific species by taking seawater samples using plankton net in the Situbondo Sea Waters, East Java, Indonesia. The culture technique used is monospecies or monospecific cultures. Microalgae culture is carried out in stages, starting with the inoculation method using the scratch method, or the pipette method. Microalgae that are planted will usually grow after two weeks (depending on the species planted). Grown cultures transferred to the next culture process, namely on the test-tube, first taken one colony from agar media and given sterile seawater, then checked under a microscope. A sterile culture place into a tuber test. A test tube is given by sterile seawater media that has been fertilized ¾ part using Walne fertilizer and then given a colony of seeds. Microalgae will grow for at least seven days. The results of the next test tube culture can be used as seeds (starter).

First process, *C. vulgaris* was cultivate in five-litre jar and then transferred in ten-litre carboy. The equipment and media for culture should be sterile for avoiding the contaminant and intferensing the growth of *C. vulgaris*. Nutrient for *C. vulgaris* used Walne fertilizer. The water quality should be maintained for optimal growth of *C. vulgaris*. After *C. vulgaris* growth in the exponential growth phase, the harvesting process was carried out.

Cultured *C. vulgaris* were confirmed using molecular identification techniques using 16 sRNA markers. 16 sRNA primers used a Forward Primers (27F: 5'-AGAGTTTGATCMTGGCTCAG-3') and a Reverse Primers (1492R: 5'-TACGGYTACCTTGTTACGACTT-3'). Nucleotide sequence of *C. vulgaris* samples was observed using the DNA sequencer (*BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems*). Sequencing data were initially recorded and edited using MEGA10; the resulting database was compared to the GenBank database through the BLAST (Basic Local Alignment Search Tool) server on NCBI. Phylogenetic analysis was carried out using multiple Clustal W-sequence alignment software packages in MEGA10.

UV – Vis

C. vulgaris extract of 2 ml was placed into the cuvet. UV-Vis measurements were performed at wavelength range from between 0.2 to 0.8 nm. Measurement result was recorded and analyzed using the standard curve line equation to obtained the sample value.

Pigment Protein Fraction (PPF) Extraction

The isolation method refers to (Yanuhar, 2015), i.e. *C. vulgaris* cells were homogenized using mortars for 1 hour with the addition of liquid nitrogen. The addition of 8 mL glycine (concentration of 50 mM) and KCl with concentration 20 mM and pH 7.5 into *C. vulgaris* cells then centrifuge at 12000 rpm for 60 min. at 4°C. Solid solution of ammonium sulfate (SAS) gradually was added into supernatant in a tube until concentration of 30% then continued by centrifugation at 15000 rpm, 4°C, 30 min. The dialysis bag was boiled in Tris-EDTA solution of 0.1 mM (pH 7.3) for 10 min. for sterilization. Samples were dialized in 2000 ml 20 mM Tris-HCl, pH 8.0, for 24h. at 4°C while stirring. After dialysis, Samples were filtered using a Sartorius filter with millipore membrane (0.22 μm). The dialysis and filtering process was repeated once.

Pigment Protein Fraction (PPF) analysis using SDS-PAGE

Analyze of PPF protein induced to Humpback grouper was conducted according to (Yanuhar and Khumaidi, 2017), the process of protein separation using SDS PAGE (Biorad). Electrophoresis was performed using 12.5% and 4% polyacrylamide gel (Merck) with an electric voltage to 100 V, and 400 mA for 100 min. using a coomassie brilliant blue staining (Merck).

The composition of separating gel was acrylamide 30% (Sigma-Aldrich), Sodium Dodecyl Sulfate (SDS) 10%, Tris HCl (Sigma-Aldrich) 1.5 M pH 8.8, dH_2O (Merck KGaA, Germany), (Sigma-Aldrich), Ammonium Persulphate (APS) 10% (Merck KGaA, Germany), tetra ethylene diamine (TEMED) (Thermo Scientific). The composition of stacking gel was acrylamide 30%, SDS 10%, APS 10%, dH_2O, TEMED, and Tris HCl 1.5 M pH 6.8.

Fish models

The fish samples were *C. altivelis*, provided by the Marine Cultivation Unit (UPBL Situbondo) in the size range from 15 to 20 cm. In vivo tests experiments was conducted orally in the laboratory. Fish were divided into four treatments group. Group A is a control; group B is fish administated with PPF of 33 μg/ml; group C is fish administated with VNN; and group D: fish administated with + VNN + PPF of 33 μg/ml. The response of antiviral was indicated by response of MHC and β-actin in the fish. Administration of extracts was three times on the first day, fifth day, and tenth days of the invivo test.

Viral Nervous Necrosis (VNN) Detection

Extraction method refers to (Wahyudi *et al*., 2018). Based on the clinical sign, VNN infected *C.altivelis* was collected. 25 mg of tissue from eye and brain organ of infected *C. altivelis* was used for extracting total RNA using Trizol (Invitrogen, USA). For removing any remaine of genomic DNA, RNase-free DNase I (Qiagen, USA) was used. The reverse transcription was done using One-Step RT-PCR kit (MyTaq™,

Bioline UK) for 45 min. at 45°C, and for 2 min. at 94°C. The system reacts at a final volume of 20 µL comprised of 7 µL H_2O (NFW), 12.5 µL (GoTaq®Green Master Mix), 0.5 µL Tetro™ reverse transcriptase enzyme (Bioline, UK), and 4 µL total RNA (500 ng).

The next step is to amplify the reverse-transcriptase PCR sequence (AccessQuick™, Promega), followed by nested RT-PCR (GoTaq® Green Master Mix, Promega) using primers (Thiéry *et al.*, 2006), namely:

F2: 5'-CGTGTCAGTCATGTGTCGCT-3'
R3: 5'-CGAGTCAACACGGGTGAAGA-3'
NF2: 5'-GTTCCCTGTACAACGATTCC-3'
NR3: 5'-GGATTTGACGGGGCTGCTA-3'

Specific primers of VNN between F2 - R3 and NF2 - NR3 were detected at 294 bp

β-actin Expression in Humpback Grouper

Extraction of RNA was performed using fish organ. The eye organ of 20-30 mg in Eppendorf was added with 500 µl TRIzol® reagent and destroyed in the mortar. Chloroform solutions (Merck KGaA, Germany) of 100 µl were added and vortexed (Multi-Vortex V 32 - Biosan) at 20 s. 12000 rpm centrifugation (Hettich Zentrifugen Mikro 22R) for 15 min. Supernatant of 200 µl was placed to a microtube, then chloroform 200 µl was added into microtube and vortexed for 20s. Centrifugation process was conducted for 12000 rpm for 10 min. Pellet was obtained after supernatant was removed. The pellet was rinshed using 75% alcohol of 500 µl and centrifuged for 5 min at 9,000 rpm. The pellets were separeted fron alcohol and dried for 10 min., then dissolved using DEPC (Sigma-Aldrich) of 200 µl.

PCR amplification was conducted in RT-PCR equipment (AccessQuick™, Promega) followed by nested PCR using dyes (GoTaq® Green Master Mix, Promega) and same primers. Amplification procedure follows kit protocol until each primer has final concentration of 2.5 µM. β actin primer was Forward F: 5-CGAGCAGGAGATGGGAACC-3 and Reverse R: 5-CGACAACGACGATCATTGC-3.

Major Histocompatibility Complex (MHC) Expression in Humpback Grouper

Detection of both MHC using immunohistochemistry was conducted according to Yanuhar (2015) (Yanuhar, 2015). Tissue was cut using microtome to get 6 µm in thickness then mounted on microscope slides. The grouper eye tissue was expossured by an anti-mouse antibody then fixed in paraformaldehyde 2%, pH 7.3, 10 min. It rinshed and incubated using blocking serum 2% (from goat or horse), for overnight at 4ºC with primary antibody monoclonal anti-mouse IgG anti-MHC. It was countered by secondary antibody anti-mouse IgG conjugated biotin for 30 min. at room temperature. Avidin-biotin Complex peroxides kit (Santa Cruz, Vector Laboratories) was used to detect the biotin using chromogen (diaminobenzidine tetrachloride). The cut tissue was dehydrated by alcohol and cleaned with xylene.

The staining histology of tissue used the MHC fish antibodies as primary antibody (Santa Cruz Biotechnology, Inc.). A positive reaction was indicated by the brown colour on the cell region that show specific reaction with primary antibody. Furthermore, it was carried out under a microscope; then, a quantitative analysis was carried out using the ImmunoRatio (IR) analysis. The ImmunoRatio segments a core area of diaminobenzidine (DAB) and hematoxylin from the microscope image, calculates the labelling index (percentage of the DAB-colored area of the total nuclear area) and produces matching images of segmentation staining (Tuominen *et al.*, 2010).

Blood Samples of Humpback Grouper

Blood samples from each treatment group were collected three times at fifth days, ninth days, and fourteenth days. Before blood suck up, the syringe was wahed with Na-Citrate of 3.8% (Merck KGaA, Germany). *C.altivelis* laid out with position the head on the left. The syringe needle 1cc (Terumo Syringe) stabbed in the musculus at behind the anal fin in the midline of the body. Blood was sucked up and transfferred to Eppendorf tube for observing the blood features include imaging, hematocrit, haemoglobin, leukocytes, and erythrocytes.

Erythrocytes Analysis

Blood samples were sucked up using an 0.5 µL erythrocytes pipette (3B Scientific). Hayem's solution (Sigma-Aldrich) was added into dilute blood until volume of 11 µL. Pippete was shaken until the mixed blood was homogene. As much as 20 µL of the homogeneus mixed blood was covered with a cover glass then placed in the counting chamber (improved Neubauer). Under the light microscope, erythrocytes number was counted (Bijanti, 2005) and total of erythrocytes number was determined by the formula (1) (Blaxhall and Daisley, 1973):

$$\text{Total of erythrocytes number } \left(\frac{cells}{mm^3}\right) = \sum \text{counted erythrocytes} \times 10^4 \left(\frac{cells}{mm^3}\right) \quad \text{.....(1)}$$

Leukocyte Analysis

Pipette containing white grain was used for sucking the blood until scale of 0.5, Turk's solution was added until scale of 11. Pipette was stirred using the hand for 3 to 5 min. to obtain the homogene blood. The first drop pipette of solution was wasted. Next drop was put into a hemocytometer then a slide glass ppasted to cover it. Total number of leukocyte was determined by the formula (2) (Blaxhall and Daisley, 1973):

$$\text{Total number of leucocytes } \left(\frac{cells}{mm^3}\right) = \sum \text{counted leucocytes} \times 50 \left(\frac{cells}{mm^3}\right) \quad \text{..... (2)}$$

Haemoglobin Level

Haemoglobin levels was measured according to Sahli method. Blood was aspirated using a Sahli pipette until as much as 20 mm^3. It was added into a haemoglobin tube that contains HCl 0.1 N until scale of 10 for 3 to 5 min. Tube was stirred while distilled water was dropped until the color gradually change to standard color. The number of haemoglobin was indicated by the Lane scale in gram/100 ml blood.

Hematocrit value

Hematocrit value was measured according to the microhematocrit method. Heparin microhematocrit pipe was filled up by blood about three-quarters by inserted it into the collected blood samples. one end of capillar pipe was blocked by the plug with wax barrier centrifuged by a microhematocrit centrifuge at 1,500 rpm for 5 min. Hematocrit value was determined by a hematocrit reader in a percentage (Vonti, 2008).

The eye tissue histology preparation

Histology preparation of eye tissue was conducted by soaking tissue in a fixation solution for 24h. Eye tissue was

soaked into alcohol 70% for 24h. then consecutively, soaked in alcohol 80.0%, 95.0%, 100%, alcohol and xylol/alcohol mixture (1:3), alcohol and xylol (1:1) solution and xylol for 30 min on each process.

Fixation tissue was conducted by soaking tissue into paraffin-xylol, paraffin I, paraffin II, paraffin III for 30 min at about 50ºC to 60ºC. The tissue was entered into a mould containing liquid paraffin for blocking or embedding process. The tissue was cooled and hardened for at least 24 h at 25ºC. For dehydration process, tissue inside paraffin block was cut by microtome by 5 μm in thickness. The thin tissue was placed in warm water for a while then laid on the object glass and dessicated until tissue was adhered on the surface of object glass. The preparations of tissue were successively dipped into xylol, alcohol 100.0%, 95.0%, 80.0%, and 70.0%, respectively, with duration in 3 to 5 min. then was soaked into destillate water for 5 min.

Staining was conducted by dipping the tissue preparations in dye (hematoxylin) about 5 to 10 min. followed by washing in a flowing water. Next step was dipping in eosin dye about 5 to 10 min. followed by washing in flowing water. The tissue preparations were re-soaked successively at ethanol 70%, 80%, 95% and 100% about 3 to 5 min. subsequented by soaked in absolute alcohol solution about 3 min. The preparation of tissue was then soaked into xylol solution about 5 min.

Tissue preparation was adhered with an adhesive or DPX mounting medium and took a cover glass for covering the preparation. The preparations were wind dried and kept for observation. The indicator of hematoxylin and eosin staining was the red color for cytoplasm while the deep purple cells for the nucleus.

RESULTS AND DISCUSSION

Sequence Results Analysis of *C. vulgaris*

The first step in molecular approach to identify a species is for determining the target genes of specific primer. During PCR amplification, the primers and the targeted gene for identifying *C. vulgaris* must be compatible so, later, it binds to one another. The sequence of *C. vulgaris* show a 424 bp, as shown in Table 1. Gene sequence using 16S rRNA and using NCBI database indicate that local isolate has similarity to several *C. vulgaris* in range 90%. Only a copy of the 16S rRNA gene placed in the algae chloroplast and the primer also has potency for placing 16S rRNA gene fragments within the algae chloroplast (Lakshmikandan and Murugesan, 2016).

Table 1. Sequence results of *C. vulgaris* taken with marker of 16 sRNA

The sequence of *Chlorella Vulgaris*						
1	GTGCGTGCGG	AGCTACCATG	CAGTCGAGCG	CCTCTTCGGT	CTGCGTGGCG	GACGGGTTAT
61	TAACGCGTGA	CAACGTGCCC	ACATCTAGGA	CCTCCTTGTG	AACAGGGAGT	CCACTGTATA
121	CGCCCTTCGG	GGGAAATTTT	ATTTGCTGAG	AATCGAAATG	ATGAATAATC	ACCAGAAGAT
181	GGGCTTGCGG	CTGATTAGCT	TGTTGGTGAG	GTAAAGGCTT	ACCAAGGTAA	TGATCACTAC
241	CTGGTCTGAA	AGGATGATCA	CCCCCACTGG	GACTGAGACA	CGGCCGAGAC	TCCTACCGGA
301	GGCAGGACCA	CCCAATTTTC	CGGAGGCGGT	GCTGCCTCGC	TACCACGCGC	GTGAAGCATC
361	ACGACTTCTT	CAAGTCCGCC	TTGCCCGAAC	GCTACGTCCT	GGAGCGCACC	ATCTTCTTCA
421	AGGT					

Phylogenetic analysis was conducted by using the Maximum Likelihood (ML) method with the Kimura 2-parameter model and 10000x bootstrap value in a software of MEGA 6 (Tamura *et al*., 2013). Results of phylogenetic reconstruction showed that a sample of *C. vulgaris* with a code (CHL B 27F) is a population which originally come from a common ancestor. Although an ecogeographic factor separates all population groups, each population is still genetically similar to populations with a short distance. One of the phylogenetic branches obtains score of 95. This demonstrated that with 10000x boosts (repetitions), the 95% would form the correct branching. Branching of phylogenetic trees with more than 70% is branching with a confidence interval of 95%. So, the phylogenetic reconstruction by using the ML methods with Kimura 2-parameter model and 10000x bootstrap value produces high accurate and consistent branching (Brinkman and Leipe, 2001). The results of the study were supported by (Lakshmikandan and Murugesan, 2016) that show *C. vulgaris* strain MSU-AGM 14 is phenotypically similar to *C. vulgaris* along with the BLAST proof of 97% identic with *Chlorella* sp. ArMOO29B chloroplast complete genome and 99% identic with *C. vulgaris* chloroplast gene. The phylogenetic tree showed a very close relationship with *C. vulgaris*.

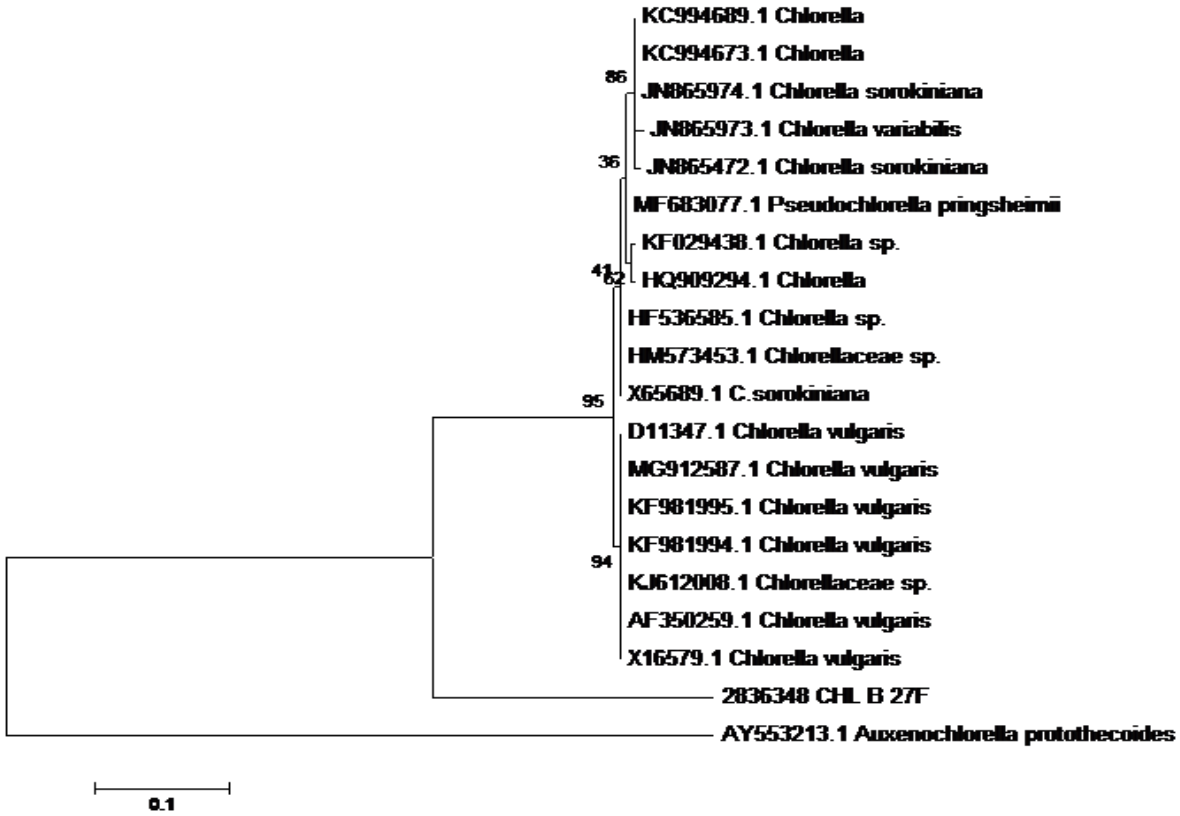

Figure 1. Phylogenetic tree based on the sequence of the 16S rRNA for *C. vulgaris*

Protein Analysis of PPF

The protein analysis of the PPF from *C. vulgaris* isolate Indonesia was shown in Fig. 2. Results show that PPF from *C. vulgaris* was consist by four protein that are 33 kDa, 29 kDa, 21 kDa, and 15 kDa. Several studies related to PPF protein analysis conducted by (Pollard and Cooper, 2009; Yanuhar and Caesar, 2019; Yanuhar and Khumaidi, 2017), state that some microalgae contain Peridinin in two forms a homodimer (short forms) and a monomer (long forms) that have molecular weight range from 14 to 16 kDa and 30 to 35 kDa, respectively. So, it can be suggested that PPF contains peridinin chlorophyll protein (PCP) at 33 kDa and 15 kDa.

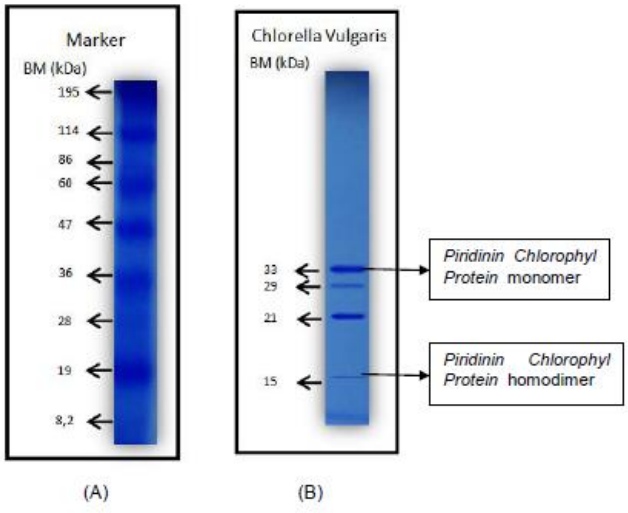

Figure 2. SDS-PAGE pattern of (A) Marker (B) PPF of *C.*

vulgaris

Administration enzymes protein into the fish body have functioned as a biocatalyst to form physiological responses and immune response (Lin and Redies, 2012). Kusuma *et al*., (2020) show that the PCP and VNN treatments showed a greater amount of expression compared to fish-eye tissue that was only exposed to VNN. The combination of VNN and PCP induction increase Protein P56 expression expressed in the tissue of fish organs.

UV-Vis Measurement

The UV-vis measurement of *C. vulgaris extract* results several peaks at wavelength of 663.1 nm, 204 nm, 207 nm, and 232 nm (Table 2. and Fig. 3). It showed that the maximum wavelength was 207.0 nm with a maximum absorbance of 3.421. A wavelength at 663.1 nm is thought to be identified as a chlorophyll a compound. The maximum absorption by chlorophyll a occurs in two wavelength bands, which peak at about 430 and 660 nm (Khotimah *et al*., 2013). At a wavelength of 423.0 nm, the chlorophyll pigment compound is also thought to be the case, as explained by (Barazzouk *et al*., 2012), i.e. chlorophyll pigment has a maximum wavelength in the blue region of 423 nm. The Uv-vis results also contained alkaloids (232.0 nm), tannins (341 nm) and terpenoids (207.0 nm and 204.0 nm).

Table 2. UV-Vis absorption for extract of *C. Vulgaris*

Wavelength (nm)	Abs	Compound	References	
663.1	0.346	Chlorophyll	(Khotimah *et al*., 2013)	
423.0	0.690	Chlorophyll	(Barazzouk *et al*., 2012)	
341.0	0.387	Tannins	(Barazzouk *et al*., 2012)	
232.0	1.021	Alkaloid	(Manasa, 2014)	
207.0	3.421	Terpenoid	(Worsfold *et al*., 2019)	
204.0	3.377	Terpenoid	(Worsfold *et al*., 2019)	

Alkaloids have effects in the health sector as antimicrobials, heart disease medications, sedatives, reducing pain, raising blood pressure, triggers of the nervous system, and others (Aksara *et al*., 2013). Terpenoid compounds are acyclic or cyclic and often have alcohol, aldehyde, or carboxylic acid groups. Terpenoid compounds have effect as anti-virus, anti-bacterial, anti-predator, anti-fungus, and insecticide (Widiyati, 2006). Tannin is a polyphenol compound that has a large molecular weight consisting of hydroxy and carboxyl groups (Sari *et al*., 2011). Tannin has anti-bacterial power, including destruction or inactivation of genetic material functions, enzyme inactivation, and reaction with cell membranes (Martono and Setiyono, 2014).

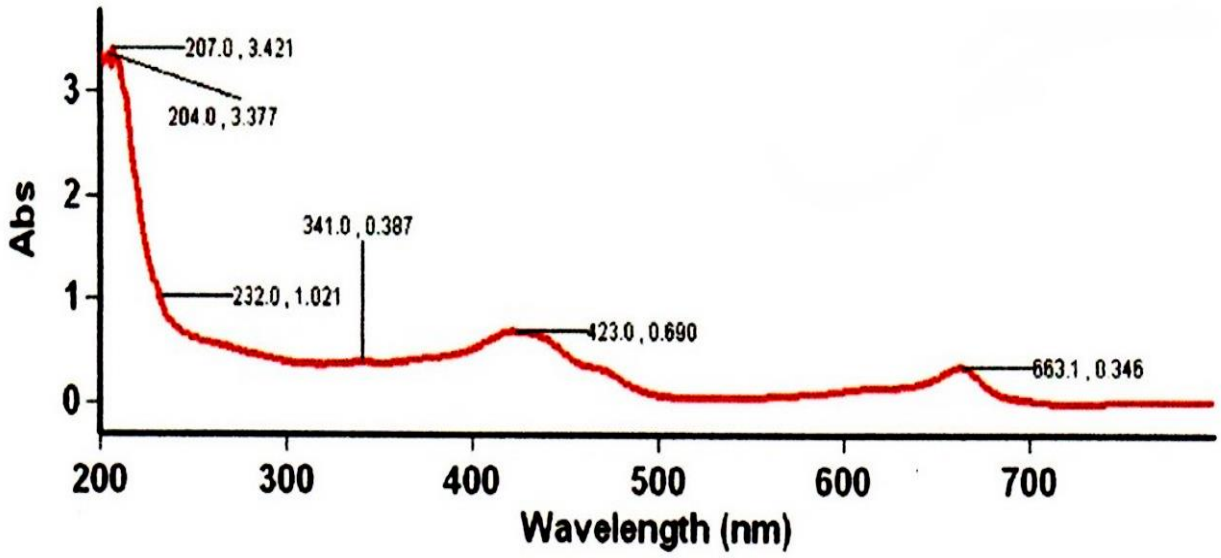

Figure 3. UV-Vis absorption spectrogram of *C. vulgaris* extract

Polymerase Chain Reaction (PCR) analysis of VNN

VNN infections mostly occurs in larvae and juveniles and shows strong vacuolation (damage) in the retina and central nervous system (Thiéry *et al*., 2006; Wahyudi *et al*., 2018). Infected adult fish exhibit swimming feature with various erratic behaviors such as swimming bubbles, swollen spinning, spirals, while in the larval fish infected VNN, these symptoms are not appeared. PCR analysis was conducted to assure that *C. altivelis* truly infected by VNN. The eye organs was used in the PCR analysis. The amplification using nucleic acids from infected fish yielded a amount of 294 bp. DNA product corresponding to that obtained from VNN RNA. In this case, itcan be conclude that the sample is positive infected by VNN (Fig. 4).

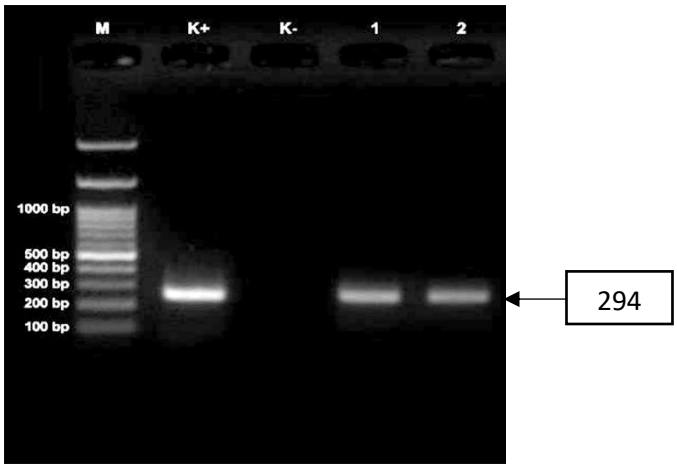

Fig. 4. PCR identification of VNN. (M) Marker (1000bp); (K+) Positive Control (294bp); (K-) Negative Control; (1) Brain tissue of *C. altivelis* infected with VNN; (2) Eye tissue of *C. altivelis* infected with VNN.

Analysis of MHC-1 and β actin response

β-actin in cells acts as a facilitator by providing mechanical strength in cell polarization and assembling or transporting molecules so that the expression of β-actin in cells continues. Under conditions of the stressor (VNN infection) in the body, the immune system in fish will express β-actin related from the actin remodelling system. The results of PCR amplification using β-actin specific primers gave rise to a band at 150 bp (Fig. 5). This is supported by research conducted by (Hausser *et al*., 2015), the target, squeeze of β-actin is at an amplicon length of 150 bp. These results show a form of fish defence that is strong enough to fight infection with viral attacks due to PPF administration. The performance of PPF as a biocatalyst in the formation of the immune system runs well. PPF act a good inducer in increasing β-actin expression.

β-actin is a proteins that having significant role in the cells immune system (Jönsson *et al*., 2012), and cell regulatory gene (Pollard and Cooper, 2009). So that, this gene or protein is necessary for maintain the cell (Wang *et al*., 2019) and believed as a housekeeping gene. When VNN infects the fish, β-actin is activated immune response to form a self-defence against VNN infection.

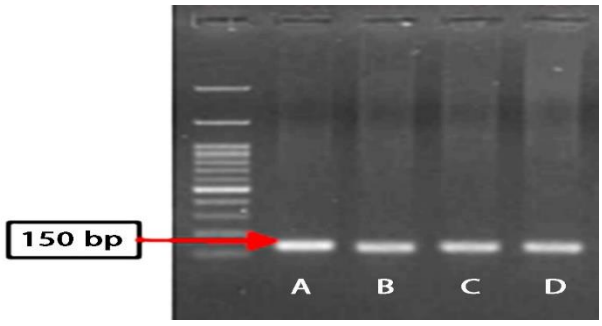

Figure 5. PCR amplification results taken primer of β-actin. (A) control; (B) Treatment with PPF of 33 µg/ml; (C) Treatment with VNN; and group D: fish administated with + VNN + PPF of 33 µg/ml

Analysis of MHC grouper expression after administration of PPF and VNN by ImmunoRatio tecknic showed that DAB percentage values of group A, group B, group C, and group D were 1.0%, 26.1%, 35%, and 66.9%, respectively (Fig. 6). PPF of microalgae reduce levels of free radical in infected fish, and PCP from microalgae has capability as antiviral genes inducer like TNFα, IL-6, and MHC (Yanuhar, 2015). During mitotic cell process, β-actin have a role on the cellular shaping (Joseph *et al*., 2012). The β-actin expressions have an impact on the increased cell migration and bulge. The installation of actin makes protrusions on the front that push the membrane of the cell. β-actin arrange to transport MHC molecules to membranes of the cell (Yamaguchi and Dijkstra, 2019).

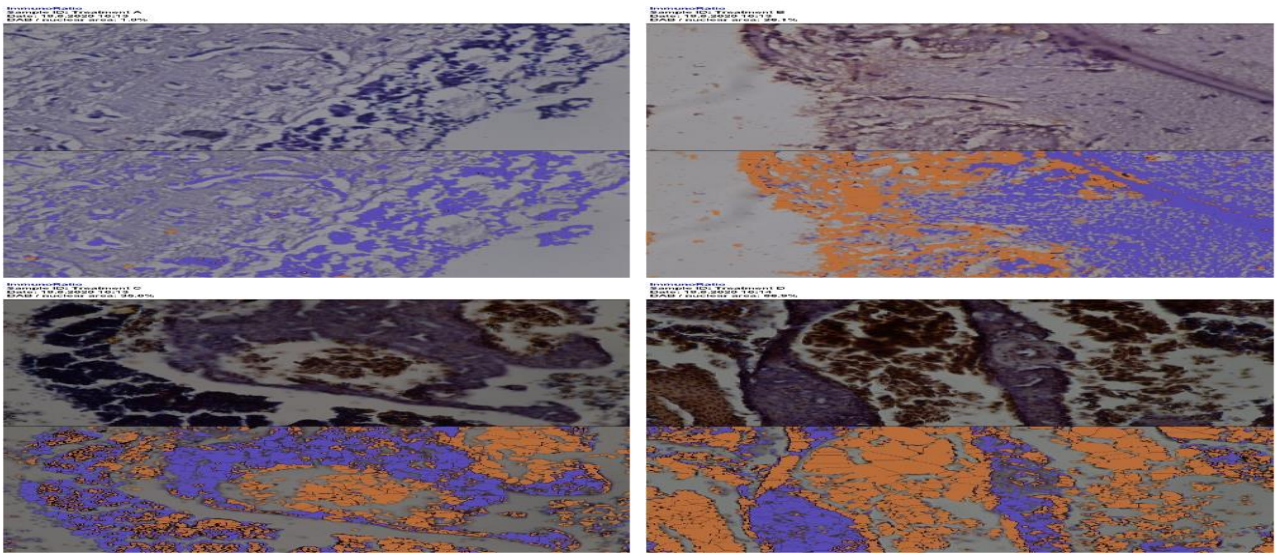

Figure 6. MHC expression of eye tissue using immunohistochemistry at (A) Group A with a DAB 1.0%; (B) Group B with DAB 26.1%; (C) Group C with DAB 35%; (D) Group D with DAB 66.9%.

The analysis showed that the DAB value increase after treatment group D compared to group A (control). It indicates the infected fish have a good immune response. Increasing MHC value was possible because of the PPF contained in *C. vulgaris* is administrated to groupers sample. It is correlated to the role of MHC as a gene functioning to neutralize viruses. The presence of MHC-I and MHC-II molecules is associated with the immune response. Phagocytic cells will be activated by MHC-II for producing antibodies and activating immunological characters

involved for neutralizing viruses, eliminating bacteria and parasites (Sucipto *et al*., 2009).

Fish Hematology

The results of the hematologic quality observation of *C. altivelis*, including erythrocyte, haemoglobin, hematocrit, and leucocyte, are shown in Fig. 7. Blood components was changed by disease attack to fish. In general, haematology status of *C. altivelis* at Group A to Group D showed a decreasing in the blood components number.

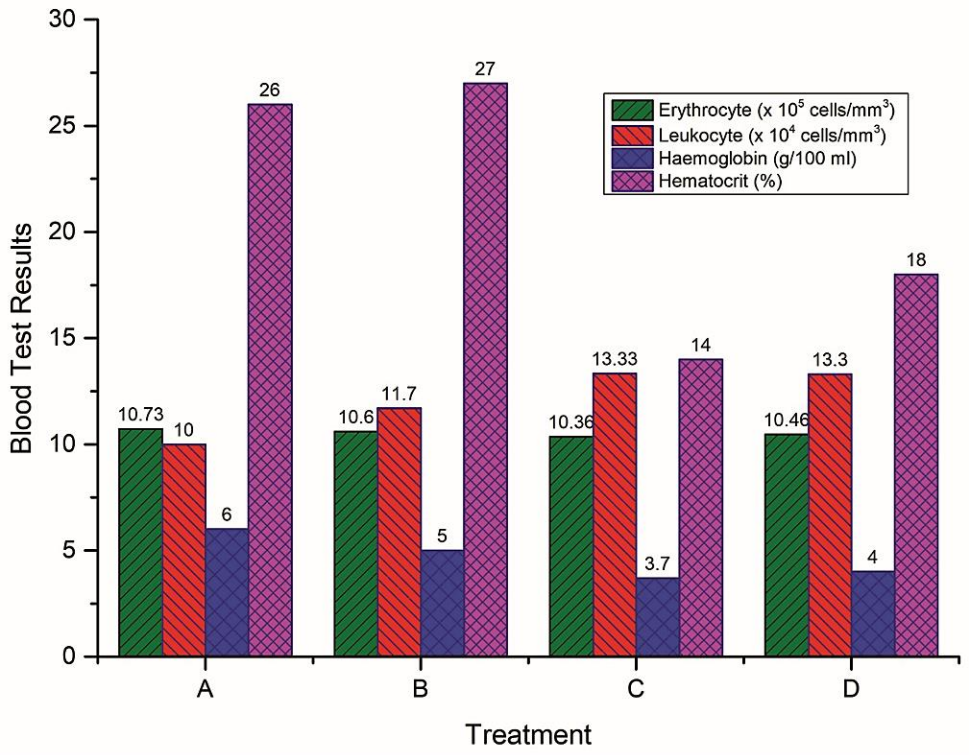

Figure 7. Hematology observation on *C. altivelis*

The total number of erythrocytes in the group A, group B, group C, and group D was 10.73 x10^5 cells/mm^3, 10.6 x10^5 cells/mm^3, 10.36 x10^5 cells/mm^3, and 10.46 x10^5 cells/mm^3, respectively. The number of erythrocyte was below the normal level in fish with range from 1.05x10^6 to 3.0x10^6 cells/mm^3 (Yanto and Hasan, 2015). The oxygen in blood was affect erythrocytes levels. Reducing the amount of oxygen lead to a low viscosity red blood cells so that oxygen diffusion from blood into tissue becomes unfavourable.

The level of leukocyte in the group A, group B, group C, and group D was 10x10^4 cells/mm^3, 11.7x10^4 cells/mm^3, 13.33x10^4 cells/mm^3, and 13.3 x10^4 cells/mm^3, respectively. The level of haemoglobin in group A, group B, group C, and group D was 6 gr/100 ml, 5 gr/100 ml., 3.7 gr/100ml., and 4 gr/100 ml, respectively. It shows that the haemoglobin levels in non-VNN treatment was in the normal range of haemoglobin for *C. altivelis*. While VNN-treated treatment yields low levels of haemoglobin that decreasing the rate of metabolism and resulting low energy. It cause the fish weak, has no appetite, hanging below the surface of the water or seen at the bottom.

The level of hematocrit in group A, group B, group C, and group D was 26%, 27%, 14%, and 18%, respectively. This results indicate that the level of hematocrit on non-VNN treatment (group B) was still in the normal condition for groupers. After treated by VNN, the level of hematocrit was reduces. PPF administration increase the level of hematocrit of VNN-infected grouper.

The histopathology analysis

The histopathology observation on eye tissue indicate that group B shows occurred Necrosis (N), group C and group D shows necrosis (N) and vacuolation (V), and group D show less tissue damage (Fig. 8). It is indicated that VNN infection showed the existence of very severe necrosis and vacuolization of the central nervous system (brain, spinal cord) and on the retina. The cells are infected tissue which exhibits pathological degradation characteristics that include core damage, vacuolation and decreased cytoplasmic surface power (Pokorova *et al.*, 2005). On observation, vacuoles appear as colorless regions and appear as holes in the cytoplasm, as shown by (Praveenraj *et al.*, 2018). Vacuolation occurs due to cell damage due to pathogen infection; then the cell undergoes destruction so that the remaining space on the tissue (Shubin *et al*., 2016). The same thing was discovered in the study of (Yuwanita *et al*., 2013), that vacuoles occur in infected VNN groupers in the retinal granule layer of the eye. Normal cells can repair and treat the damage. When the damage occurs beyond ability of these cells, then the cell to destroy itself though apoptosis or necrosis mechanism.

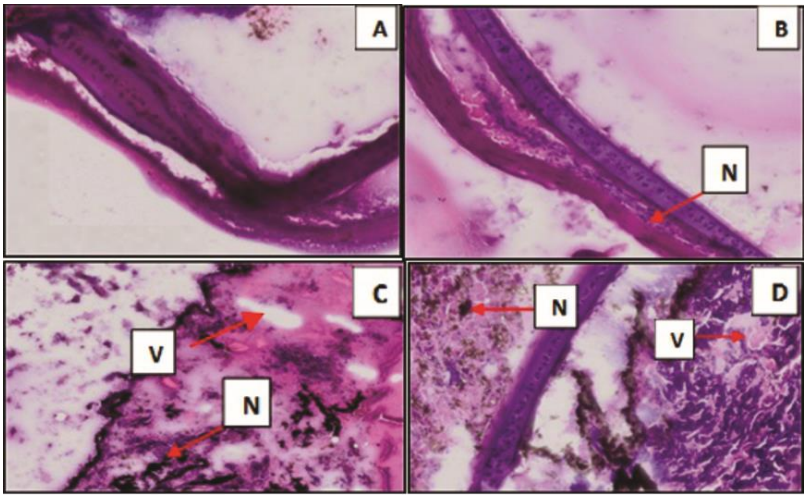

Figure 8. Histology observation on eye tissue of grouper in (A) Group A; (B) Group B; (C) Group C; (D) Group D. N: Necrosis; V: Vacuolation

Visible necrosis is a type of chronic tissue damage that cannot be recovered. Necrosis indicated in treatment (C) can be characterized by swelling in cell organelles and loss of integrity of the plasma membrane where this damage can reduce the ability of cells to produce *adenosine triphosphate* (Zong and Thompson, 2006). Cell necrosis is caused by phagocytes lymphocyte activity which causes reduction or shrinkage in the overall nucleus size. So that the condition of necrosis in organ, the tissue will result in loss of function in the area of necrosis (Yuwanita *et al*., 2013).

Conclusion

PPF from microalgae *C. vulgaris* has administrated into VNN infected grouper. PPF increase the expression of MHC-1 and β-actin that indicating the growth of immune response in *C.altivelis* that impact on decreasing on eye tissue damage of VNN attacked fish. In the future, this finding can be developed as an immunostimulant agent for increasing the fish immune system.

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