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Research Article

Enhanced Immunity of Hybrid Grouper (*Epinephelus sp.*) Against Viral Nervous Necrosis Using a Recombinant Protein Nanovaccine from Chlorella Vulgaris

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Abstract: Hybrid Grouper (*Epinephelus sp.*) is an important export commodity in marine aquaculture, yet Viral Nervous Necrosis (VNN) remains a major threat. Nanovaccines are a type of vaccine developed by combining vaccine ingredients with silver nanoparticles and recombinant protein from *Chlorella vulgaris*. These formulations are designed to effectively boost the body's immune system response to prevent disease infection and destroy pathogenic agents. This study evaluated the efficacy of a recombinant nanovaccine containing *Chlorella vulgaris* protein with chitosan-AgNPs, using a factorial randomized design of eight treatments and three replicates. Treatments included healthy fish (K–), VNN-infected fish (K+), and fish vaccinated with 33, 66, or 112 µL of nanovaccine, with or without subsequent VNN infection. Parameters assessed were hematology, relative CD4/CD8 T-cell levels, antibody titers, and survival. Results indicated that administering the recombinant nanovaccine significantly enhanced the immune response, evident in increased erythrocyte (2.67×10^6 cells/mm^3), leukocyte (1.71×10^5 cells/mm^3), and hemoglobin (7.68 g/dL) levels, as well as higher lymphocytes (85.2%), monocytes (16.8%), neutrophils (17.4%), basophils (0.20%), relative CD4 (0.67%) and CD8 (2.33%) T-cell levels, and survival (88.8%). The ANOVA results indicated that several parameters exhibited statistically significant differences among treatments ($p<0.05$). The highest immune response occurred in fish vaccinated with a 33 µL dose. These findings highlight the potential of recombinant nanovaccines containing *C. vulgaris* protein as a preventive strategy against VNN in Hybrid Grouper.

Keywords: Blood Cells, *Epinephelus sp.*, Immune Response, Recombinant Nanovaccines and Viral Nervous Necrosis

Introduction

The Hybrid Grouper (*Epinephelus sp.*) is one of the leading species due to its excellent growth performance, tolerance to diverse environmental conditions, and high market demand. This species exhibits strong growth and good tolerance to low salinity levels, making it highly suitable for aquaculture (Arrokhman *et al*., 2017; Suyanti *et al*., 2021), and its cultivation has significantly contributed to the increase of national fishery production (Angwarmas *et al*., 2020; Mahasri *et al*., 2023). However, the intensification of grouper farming has also brought about disease-related challenges, particularly viral infections such as Viral Nervous Necrosis (VNN), caused by Betanodavirus. This infection has caused mass mortalities ranging from 80 to 100% in more than 39 fish species, including grouper, affecting both larval and adult stages (Ariff *et al*., 2019). VNN primarily attacks the

nervous system and ocular organs of fish during the juvenile and larval stages (Li *et al*., 2022). Preventive measures against VNN include the implementation of strict biosecurity protocols and vaccination strategies to enhance the immune performance of fish (Zorriehzahra *et al*., 2019).

Vaccination is considered one of the most effective methods in controlling infectious diseases caused by pathogenic agents, by increasing the immune response of fish (Ridzuan *et al*., 2022). VNN vaccines have been widely developed through recombinant vaccines that utilize:

1) Protein coats (Zorriehzahra *et al*., 2019)
2) Vaccine Nervous Necrosis Virus-Like Particle (Barsøe *et al*., 2021)
3) Recombinant vaccines of peridinin protein P-PERCv *C. vulgaris*

The use of recombinant vaccine *C. vulgaris* as an antiviral against VNN in grouper fish has been studied in the laboratory. The peridinin-chlorophyll protein from *C. vulgaris* has demonstrated the ability to boost the immune system performance of grouper fish while also reducing damage to the eye tissue of grouper infected with VNN by enhancing the functional performance of β-actin and MHC proteins. The upregulation of β-actin and MHC proteins in the body contributes to an improved immune response. Vaccines can provide protection up to 65-88% and increase the immune response of grouper fish (Yanuhar *et al*., 2020). The weakness of recombinant protein vaccines lies in their susceptibility to degradation within the body and their ability to induce only a short-term immune response (Maina *et al*., 2020). Efforts to overcome this problem can be made through the development of nanovaccines, with the addition of nanoparticles as an additional ingredient to increase vaccine immunogenicity.

Currently, nanotechnology is being rapidly developed and utilized in the health sector through the development of nanovaccines. These types of vaccines are created by combining vaccine materials with nanoparticles, which serve as a vaccine delivery material due to their ability to penetrate the spaces between cells and enhance the body's immune response (Das and Ali, 2021; Vinay *et al*., 2018). Nanoparticles, such as chitosan, liposomes, polymer-lipid hybrid nanoparticles, and metal nanoparticles, have been utilized in the development of nanovaccines (Kelly *et al*., 2019).

Chitosan is one of the materials used as a vaccine adjuvant (Yanuhar *et al*., 2024). The substance is influenced by the ability of nanoparticles as an adjuvant to increase the immunogenicity of vaccines and shield the vaccine components from degradation processes within the body (Vinay *et al*., 2018). Silver nanoparticles (AgNPs) are a type of metal nanoparticle that have been utilized in various fields such as industry, health, and food. In the health sector, AgNPs are used as drug delivery materials, vaccine adjuvants, nanovaccine development, and as diagnostic materials (Zhang *et al*., 2016). According to the previous study (Sanchez-Guzman *et al*., 2019), AgNPs which were used as adjuvants in influenza virus vaccines, are able to increase the adaptive immune response of rats by increasing antibody production against influenza viruses. Therefore, the purpose of this study was to determine the effect of administering recombinant nanovaccines based on *C. vulgaris* protein, which are delivered using chitosan-AgNPs hybrid nanoparticles, on the immune performance of VNN-infected Hybrid Grouper, as assessed by hematological parameters, growth performance, survival rate, relative levels of CD4 and CD8, and antibody titer.

Materials and Methods

Production of Recombinant Nanovaccine Protein Peridinin C. vulgaris

The production of the recombinant *C. vulgaris* protein vaccine involves the utilization of the chlorophyll peridinin protein, which has a molecular weight of 70 kDa and operates within the wavelength range of 310-315 bp, referring to Yanuhar *et al*. (2020). This process involved screening and identifying samples of *C. vulgaris* marine microalgae from Situbondo sea waters, followed by their cultivation on a laboratory and mass scale with a capacity of 500-1,500 liters. The next stage was the isolation of the peridinin protein pigment from *C. vulgaris* and characterization testing. The obtained peridinin protein (70 kDa) was propagated recombinantly using the cloning technique by transforming protein into *E. coli* vector pTA2 bacteria. The bacteria were grown on an appropriate medium, and the detection of the recombinant vaccine gene was carried out using the RT-PCR method with primers T3 (3'-CTTTAGTGAGGGTTAAT-5') and T7 Promoter (3'-TAATACGACTCACTATAGGG-5'). The cloning results were preserved as glycerol stock, while the supernatant was collected for further treatment.

Chitosan nanoparticle synthesis method refers to the previous study (Izaguirre-Hernández *et al*., 2017) and silver nanoparticles were synthesized using $AgNO_3$ (Sigma-Aldrich, USA) as a source of Ag+. The method for nanoparticle biosynthesis was based on the previous research (Badi'ah, 2021; Muthusamy *et al*., 2017). The *C. vulgaris* protein recombinant vaccine was initially combined with chitosan nanoparticles through a combination process method refers to Tattiyapong *et al*. (2022). The recombinant *C. vulgaris* protein-based nanovaccine was formulated with AgNPs nanoparticles at a ratio of 1:0.25, based on the optimization conducted in previous studies (Hartawan *et al*., 2023; Yanuhar *et al*., 2020).

In-vivo Test of Recombinant Nanovaccine of C. vulgaris Protein

This study employed a Completely Randomized Factorial Design (CRFD) with eight treatments and three replicates each, as follows (Table 1).

Table 1: Treatment of recombinant nanovaccines of *C. vulgaris* protein

No.	Treatment	Description
1	K+	Hybrid Grouper infected with VNN (positive control)
2	K-	Healthy Hybrid Grouper fish
3	T1	Healthy fish were given a nanovaccine dose of 33 µL
4	T2	Healthy fish were given a nanovaccine dose of 66 µL
5	T3	Healthy fish were given a nanovaccine dose of 112 µL
6	T4	Healthy fish given a dose of 33 µL of nanovaccine then challenged against VNN
7	T5	Healthy fish given a dose of 66 µL of nanovaccine then challenged against VNN
8	T6	Healthy fish given a dose of 112 µL of nanovaccine then challenged against VNN

The test subjects were Hybrid Grouper (*Epinephelus sp.*), totaling 144 fish with a size range of approximately 10-15 cm. They were sourced from Teluk Kode, Malaka, Pemenang, North Lombok, West Nusa Tenggara, Indonesia. The fish were housed in 40 L plastic tanks, with 18 fish per treatment tank, and were acclimatized prior to experimentation to minimize stress and allow adaptation to the new maintenance environment. Additionally, active and healthy fish were fed ad libitum twice daily to ensure optimal conditioning.

The VNN was collected from fish that had tested positive for VNN infection through the PCR method examination, obtained from the Fish Quarantine Center for Quality Control and Safety of Fishery Products Surabaya I (Juanda). The fish were kept and cut into small pieces to be given to the Hybrid Grouper on the challenge test. The in-vivo nanovaccine test was conducted orally using the sonde method, with nanovaccine dosages based on previous research, namely 33, 66, and 112 µL, as these doses were found to provide optimal results (Hartawan *et al*., 2023). The recombinant *C. vulgaris* protein nanovaccine based on chitosan-AgNPs hybrid nanoparticles was administered on day 0 and boosted on day 7. The Hybrid Grouper was kept for 56 days, and the VNN challenge test was carried out by feeding them with a combination of trash fish meat that was positively confirmed for VNN.

The administrations of VNN infection were carried out three times on the 3rd day, 5th day, and 9th day. During the maintenance process, clinical symptoms and fish behavior were observed to indicate VNN infection.

Survival Rate and Calculation of the Weight-Length of Hybrid Grouper

To evaluate the effect of nanovaccine administration on Hybrid Grouper, we calculated the fish's survival rate using the previously described method (Anita and Dewi, 2020). The survival rate of the Hybrid Grouper was calculated using the formula below:

$$SR = \frac{Nt}{N0} x\ 100\% \quad (1)$$

Notes:
SR : Survival rate
Nt : The Number of fish that are alive at the end of the research
N0 : The number of fish from the beginning of the research

The increase in absolute length and weight of Hybrid Grouper was calculated using the formula presented by Ismi and Budi (2020):

$$L = Lt - Lo \quad (2)$$

$$G = Wt - Wo \quad (3)$$

Note:
Wo : The average weight of the fish at the beginning of the research (g)
Wt : The average weight of fish at the end of the research (g)
Lo : The average length of the fish at the beginning of the research (cm)
Lt : The average length of the fish at the end of the research (cm)

Blood Sampling of Hybrid Grouper

Blood samples were collected for hematological analysis, relative CD4 and CD8 measurements, and antibody assessment, following the method described in a previous study by Junirahma and Yanuhar (2020), which was carried out 10 times on the 2nd, 4th, 7th, 14th, 21st, 28th, 35th, 42nd, 49th, and 56th day. Prior to taking 1 mL of blood using a syringe (Terumo, Japan), the syringe was moistened with 3% Na-Citrate (from Merck, Germany) as an anticoagulant. The blood was drawn from the musculus near the anal fin of the fish. The blood that had been taken was stored in a 1.5 mL microtube (Eppendorf, Germany). For hematological examination, the blood was taken using a syringe without Na-Citrate moistened and stored in an EDTA vaculab tube (OneMed, Indonesia).

Total Erythrocyte Analysis

Total erythrocytes were counted using a hemacytometer (Assistant set Neubauer, Germany). Blood was drawn to the 0.5 mark on a pipette and diluted with Hayem's solution (Merck, Germany) up to the 101 marks, then homogenized by shaking for about 3 minutes. The first drop was removed, and the rest was dripped onto the haemacytometer. The calculation of the erythrocyte cell

count was performed on 5 small squares of the haemacytometer using a microscope at a magnification of 400X (Centofanti *et al*., 2024). The number of erythrocytes was calculated using the following formula:

$$\frac{\sum cell}{\text{Volume of large box}} x\ Dilution\ Factor \quad (4)$$

Total Leukocyte Analysis

Total white-blood-cell counts were determined with a Neubauer haemacytometer (Assistant set, Germany). Blood was drawn into the pipette to the 0.5 mark, diluted with Türk's solution (Merck, Germany) to the 11 mark, and gently mixed for about three minutes. After discarding the first one or two drops, the remaining suspension was transferred to the counting chamber. Once the chamber was completely filled, leukocytes were enumerated in four small squares under a light microscope at 400× magnification (Centofanti *et al*., 2024):

$$\frac{\sum cell}{\text{Volume of large box}} x\ Dilution\ Factor \quad (5)$$

Hemoglobin Level

Hemoglobin concentration in Hybrid Grouper erythrocytes was assessed with the Sahli technique. A 20 mm³ blood sample was drawn using a Sahli pipette and delivered to an Hb-meter tube (OneMed, Indonesia) that already contained 0.1 N HCl (Merck, Germany) up to the 10-mark. The blood–acid mixture was thoroughly stirred, and distilled water was added dropwise until the solution's hue matched the reference standard. Hemoglobin content was then read on the yellow G % scale, representing grams of hemoglobin per 100 mL of blood (Yanuhar *et al*., 2021a).

Differential Leukocytes

Differential leukocyte counts in Hybrid Grouper were determined based on Dalahi *et al*. (2019). Blood samples were placed on glass slides to prepare blood smears. The smears of dried blood samples were fixed with methanol solution (Sigma-Aldrich, USA) for 5-10 minutes, and the fixed blood review preparations were put into the Giemsa solution (Sigma-Aldrich, USA) for 10-20 minutes and dried while glass objects were rinsed using aqueous and dried in air. Blood smears were examined with an Olympus CX23 microscope (Japan) at 400× magnification, and lymphocytes, neutrophils, monocytes, basophils, and eosinophils were identified and enumerated until 100 leukocytes had been counted. The type of leukocytes is calculated using the formula below:

$$\frac{\sum amount\ of\ cells}{100} x\ 100\% \quad (6)$$

Analysis of Flow Cytometry

Analysis of the immune response of CD4+ and CD8+ T cells of Hybrid Grouper was conducted through examination of the flow cytometry method on brain, eyes, and blood. The blood of the Hybrid Grouper fish was taken and stored in a microtube that had been dosed with 3% Na-citrate (Merck, Germany), while the brain and eye organs of the Hybrid Grouper fish were obtained by performing a necropsy procedure. Organs were washed with PBS, homogenized, and spun at 2500 rpm for 5 min at 10 °C in 15 mL tubes. After discarding the supernatant, the pellet was resuspended in 1 mL PBS, divided into 1.5 mL microtubes, and centrifuged again under the same conditions. For extracellular staining, 50 µL of Foxp3, CD4+, and CD8+ PE/Cy5 antibodies were added to the cell pellets and lymphocytes, followed by a 20-min dark incubation at 4 °C. Each sample then received 400 µL PBS and was examined by flow cytometry, with data processed in BD Cell Quest ProTM (Sari *et al*., 2023; Yanuhar *et al*., 2021b).

Titer of Antibody

Antibody titers were measured based on the method described by Jiang *et al*. (2019) using several fish samples. Blood samples from the T1–T6 treatments were collected and stored in 1.5 mL microtubes (Eppendorf, Germany). The obtained blood sample was centrifuged at 5000 rpm for 10 minutes to separate the serum. In the agglutination test on a microplate (ThermoFisher, USA), 12 wells were filled with 25 µL of PBS, and wells numbers 1 and 2 were filled with Hybrid Grouper serum, followed by serial dilution. VNN antigen (25 µL) was added to wells 1 to 12. The presence of antibodies could be detected in 24 hours, indicated by the absence of serum sedimentation or point shape at the bottom of the 96-well V-bottom microplate (ThermoFisher, USA). The antibody titer test data were converted using a formula referring to the previous study McHardy *et al*. (2018) and analyzed descriptively.

Data Analysis

The data were analyzed with a one-way ANOVA, and group means were compared using Tukey's HSD post hoc test; differences were considered significant when $p<0.05$.

Results

Observations of Clinical Symptoms

The results of the observations of clinical symptoms in Hybrid Grouper, which were given a challenge test treatment for 56 days, showed that the fish experienced a decrease in appetite, swam with a tilted body condition and/or in circles, underwent darker body color changes, and some fish even died (Fig. 1). The results of the RT-PCR analysis using specific primers for VNN showed that the sample band was in a parallel position to the positive control at 294 bp, as shown in Fig. 2.

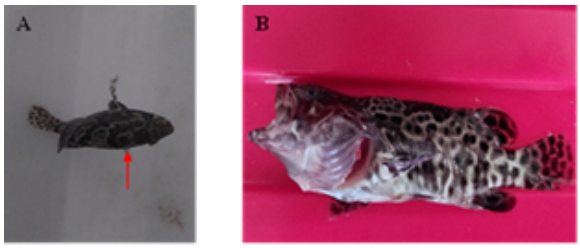

Fig. 1: Clinical symptoms of Hybrid Grouper infected with VNN. Description: (A) Hybrid Grouper experienced changes in body color becoming darker and swam with tilted position, (B) some (other) Hybrid Grouper fish experienced death due to VNN infection

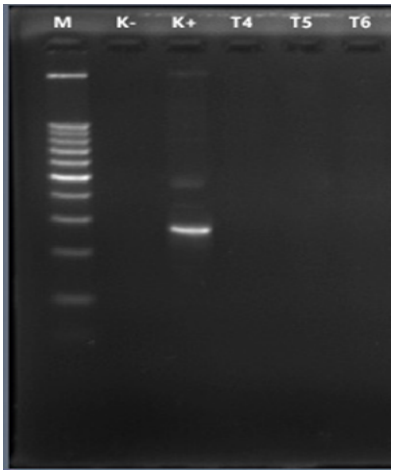

Fig. 2: Results of VNN examination of RT-PCR method. (K-): Healthy Fish; (K+): Hybrid Grouper was confirmed to be infected with VNN; (T4): Healthy fish given a dose of 33 µL of nanovaccine then challenged against VNN; (T5): Healthy fish given a dose of 66 µL of nanovaccine then challenged against VNN; (T6): Healthy fish given a dose of 112 µL of nanovaccine then challenged against VNN

Hybrid grouper treated with the nanovaccine and challenged with VNN showed negative PCR results. The PCR test was conducted after 56 days of the nanovaccine treatment. This indicates that the administration of the nanovaccine enhances the resistance of hybrid grouper to VNN infection.

The Growth of Hybrid Grouper

The observation of growth parameters of Hybrid Grouper revealed an increase in weight and length in each fish, as shown in Fig. 3. The vaccine treatment sample demonstrated the best growth rate compared to the non-vaccine treatment sample, and significant differences compared to treatment K+. Treatment with 33 µL nanovaccine exhibited a growth rate which was significantly higher than the control groups ($p<0.05$) of length and weight in T1 treatment growth rate of length (7.5 cm) and weight (16.6 grams), and T4 treatment growth rate of length (7 cm) and weight (16 grams).

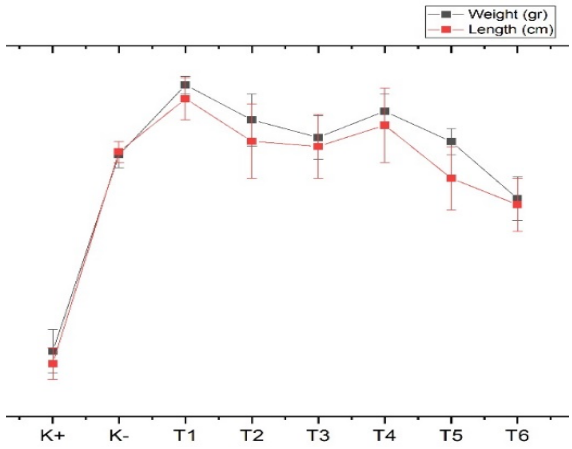

Fig. 3: The graph of the Growth Rate of the Total Length of Hybrid Grouper Weight for 56 Days

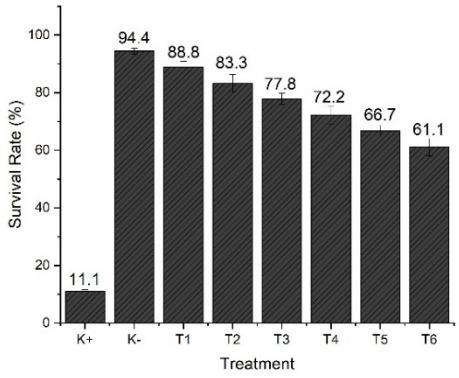

Fig. 4: The survival rate of Hybrid Grouper during 56 days of treatment

Survival Rate

Survival analysis revealed that administering a recombinant C. vulgaris protein nanovaccine formulated with chitosan-AgNP hybrid nanoparticles lowered the mortality of Hybrid Grouper caused by VNN infection. The highest SR values were found in treatments T1 (88.8%) and T4 (72.2%), and both were significantly higher ($p<0.05$) than the positive control group (K+), which had lower survival due to VNN infection. The results are shown in Fig. 4.

Total Erythrocytes

The total erythrocyte counts in Hybrid Grouper after 56 days of treatment, as shown in Table 2, ranged from 2.20 to 2.67x10^6 cells/mm^3. The groups treated with recombinant nanovaccines showed higher values compared to those without nanovaccine administration. Specifically, the recombinant nanovaccine treatment resulted in a significantly higher total erythrocyte count

compared to the K+ control ($p<0.05$, ANOVA followed by post-hoc test), particularly in T1 and T4 groups. Furthermore, the 33 µL treatment (T1 and T4) exhibited higher erythrocyte values compared to the 66 µL (T2 and T5) and 112 µL (T3 and T6) treatments.

Total Leukocytes

The total leukocyte value of the Hybrid Grouper during 56 days of maintenance is shown in Table 2. The results of the calculation of total leukocytes of the Hybrid Grouper for 56 days ranged from 1.12-1.71 x 10^5 cells/mm^3. The results showed that the treatment with nanovaccine and nanovaccine accompanied by a challenge test reached the highest values in treatment T1 (1.68 x 10^5 cells/mm^3) and T4 (1.71 × 10^5 cells/mm^3), which were given a dose of 33 µL of nanovaccine

Hemoglobin Level

The hemoglobin levels of the Hybrid Grouper are presented in Table 2. The results of the analysis of hemoglobin levels of Hybrid Grouper for 56 days ranged from 5.03 – 7.68 g dL^{-1}. The treatment group that received recombinant nanovaccines of *C. vulgaris* protein had higher values than the groups without nanovaccine administration. However, hemoglobin levels in all treatments remained within the normal range. The highest levels were observed in the T1 treatment (7.68 g dL^{-1}) with a 33 µL nanovaccine dose and in the T4 treatment (6.76 g dL^{-1}) with $p<0.05$ after nanovaccine administration followed by VNN infection.

Lymphocytes

The average percentage of lymphocyte cells in Hybrid Grouper is presented in Table 3 and Fig. 5. During the 56-day maintenance period, the average percentage of lymphocyte cells in Hybrid Grouper ranged from 77.3-85.2% with $p<0.05$. The nanovaccine treatment resulted in a higher average percentage of lymphocyte cells compared to the treatment without nanovaccine. The highest average percentage of lymphocyte cells in hybrid grouper was in the T4 treatment (85.2%), which was given a nanovaccine dose of 33 µL and the VNN challenge test.

Monocytes

The average percentage of monocyte cells in Hybrid Grouper for 56 days ranged from 9.1-16.8%, and is shown in Table 3 and Fig. 5. The highest average percentage of monocyte cells was found in the T4 treatment (16.8%) compared to other treatments. The analysis of differential leukocytes, specifically monocyte and basophil cells, in this study revealed was statistically significant increase ($p<0.05$) in the percentage of these cells in the nanovaccine-treated group that was subsequently challenged.

Basophil

Basophil cells were observed in K+, T4, T5, and T6 treatments with a range of 0-0.20%. The highest percentage of basophil cells was obtained in the nanovaccine treatment, which was then challenged with VNN in the T4 treatment (0-0.20%). The percentage of basophil cells in the T4 treatment was higher compared to the T5 (0-0.18%), T6 (0-0.17%), and K+ (0-0.11%) treatments. The results of the average analysis of Hybrid Grouper basophil cells are shown in Table 3 and Fig. 5 (with $p< 0.05$).

Table 2: Total value of erythrocytes, leukocytes, and hemoglobin level of hybrid grouper

Treatment	Total Erythrocytes (x10^6 cell/mm^3)	Total Leukocytes (x 10^5 cells/mm^3)	Hemoglobin level (g dL^{-1})
K+	2.20±0.18	1.54±0.15	5.03±0.47
K-	2.56±0.05	1.12±0.08	6.39±0.08
T1	2.67±0.06	1.68±0.09	7.68±0.08
T2	2.63±0.05	1.67±0.06	7.35±0.07
T3	2.62±0.04	1.64±0.05	7.25±0.05
T4	2.56±0.03	1.71±0.08	6.76±0.09
T5	2.54±0.04	1.66±0.08	6.29±0.08
T6	2.51±0.05	1.60±0.07	6.02±0.07

Table 3: Results of differential leukocyte count in hybrid grouper

Treatment	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	Basophils (%)
K+	5.03	15.1	13.1	0.055
K-	6.39	9.10	6.50	0.000
T1	7.68	15.8	17.3	0.000
T2	7.35	15.5	16.6	0.000
T3	7.25	15.2	14.6	0.000
T4	6.76	16.7	17.4	0.066
T5	6.29	15.9	16.3	0.053
T6	6.02	15.1	14.2	0.043

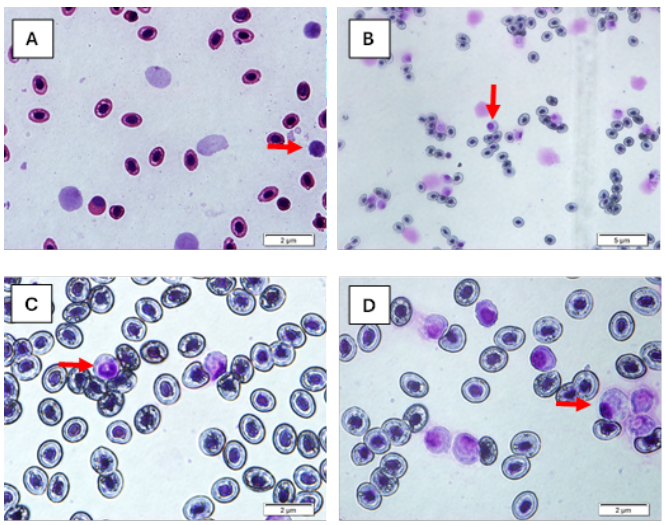

Fig. 5: Blood profile of hybrid grouper with T1 treatment (Healthy fish were given a nanovaccine dose of 33 µL). (A) Lymphocyte; (B) Monocyte; (C) Neutrophil; (D) Basophil

Neutrophils

The results of the analysis of the average percentage of neutrophils in Hybrid Grouper are shown in Table 3 and Fig. 5. The percentage of neutrophils in Hybrid Grouper during 56 rearing days ranged from 6.5% to 17.4%. The highest average percentage of neutrophils was obtained in the treatment of 33 µL nanovaccine and the VNN challenge test, namely the T4 treatment (17.4%).

The ANOVA results showed that several parameters showed statistically significant differences among treatments ($p<0.05$) (Table 4), such as Total Erythrocytes, Total Leukocytes, Hemoglobin Level, Monocytes and Neutrophils.

The Measurement of Cell Immune Response of T CD4 and CD8

The results of CD4 and CD8 T cell analysis through Flow Cytometry are presented in Table 5. Administration of recombinant *C. vulgaris* nanovaccine significantly affected the relative levels of CD4+ and CD8+ T cells ($p<0.05$) of CD4 and CD8 T cells in Hybrid Grouper. The highest average percentages of CD4 (0.67%) and CD8 (2.23%) T cells were observed in the T4 treatment. Additionally, the highest relative levels of these T cells were found in the fish's blood, compared to the eyes and brain.

Table 4: Results of One-way ANOVA in blood cells of hybrid grouper

Parameter	F Value	Sig. (p-value)	Description
Total Erythrocytes	40.557	0.001	SD
Total Leukocytes	20.676	0.001	SD
Hemoglobin Level	12.600	0.001	SD
Lymphocyte	1.296	0.258	ND
Monocytes	2.161	0.042	SD
Neutrophils	11.888	0.001	SD
Basophil	0.703	0.669	ND

Note: SD: Significantly Different; ND: Not significantly Different

Table 5: The results of CD4 and CD8 T cell analysis

Treatment	Antibody	Organs		
		Brain	Eyes	Blood
K+	CD4+	0	0	1.67
	CD8+	0.16	2.54	2.37
K-	CD4+	0	0	0.42
	CD8+	0.14	2.75	0.45
T1	CD4+	0	0.14	1.72
	CD8+	0.27	5.36	0.74
T2	CD4+	0.04	0.14	1.58
	CD8+	0.01	6.27	0.05
T3	CD4+	0.04	0.24	1.24
	CD8+	0.01	4.63	0.47
T4	CD4+	0.04	0.30	1.68
	CD8+	0.36	3.11	3.32
T5	CD4+	0.03	0.33	1.43
	CD8+	0.05	5.68	0.67
T6	CD4+	0.01	1.08	0.05
	CD8+	0.06	5.01	0.03

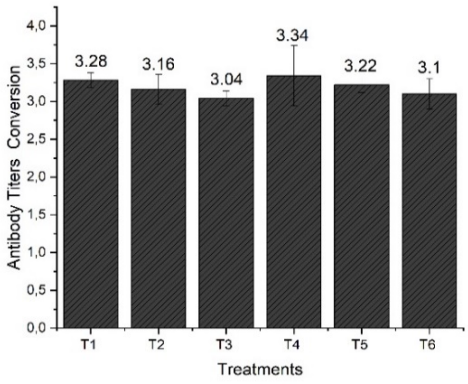

Fig. 6: Hybrid Grouper Antibody Titer Conversion for 56 days of treatment

Antibody Titers

The results of antibody titer (IgM) indicated that the administration of recombinant *C. vulgaris* protein nanovaccine significantly increased antibody levels ($p<0.05$), utilizing Chitosan-AgNPs hybrid nanoparticles increased the antibody titers produced by Hybrid Grouper. Among different doses tested in this current study, the 33 µL-nanovaccine doses demonstrated the highest capacity for generating antibody titers. The highest conversion of antibody titers was found in the T4 (3.34) and T1 (3.28) treatments. The results are shown in Fig. 6.

Discussion

VNN infection in groupers typically manifests as reduced appetite, abnormal swimming behavior (e.g., swimming upside down or in circles), and changes in body coloration (pale or darker), as well as a loss of swimming stability (Yanuhar *et al*., 2021b). The presence of VNN in infected fish can be confirmed through PCR analysis using specific primers, where positive samples are indicated by a band appearing at 294 bp, matching the positive control (Ariff *et al*., 2019; Wahyudi *et al*., 2018).

The administration of the recombinant *C. vulgaris* nanovaccine significantly reduced mortality rates in Hybrid Grouper, as shown in Fig. 4. Moreover, the nanovaccine had a positive impact on growth performance, as evidenced by higher length and weight gains in treated fish compared to both positive and negative control groups (Fig. 3). These findings are supported by Sudheesh *et al*. (2016) reported that vaccination can contribute to improved growth rates in fish by stimulating the immune system, which subsequently enhances metabolic efficiency and supports overall development. Furthermore, optimal growth is also dependent on the energy and nutrient content of the feed consumed, which must meet the nutritional requirements necessary for proper growth and physiological function (Hardy and Kaushik, 2021).

Nanovaccine administration and VNN challenge testing in Hybrid Grouper had a significant impact on hematological parameters (Table 2), covering red blood cell count, hemoglobin concentration, total white blood cell count, and the leukocyte differential. Through the 56-day study, significant fluctuations in total erythrocyte count were observed. Nonetheless, the average erythrocyte count remained within the physiological normal range for this species, indicating that the nanovaccine could enhance resistance to infection while maintaining fish health. Studies have shown that following vaccination administration, there is an immune response characterized by an increase in white blood cells and changes in cellular composition, reflecting the physiological adaptation of fish to the stress posed by viral challenge (Angulo-Pineda *et al*., 2019; Gaafar *et al*., 2018; Hartawan *et al*., 2023; Kumalaningrum *et al*., 2024). These findings highlight the importance of vaccination strategies to support fish health in aquaculture environments while providing insight into the long-term impact on animal health (Natnan *et al*., 2022; Ode *et al*., 2023; Shapawi *et al*., 2018; Yanuhar *et al*., 2021c).

The success of vaccination in enhancing the total leukocyte count in Hybrid Grouper was demonstrated by the increased numbers of lymphocytes, monocytes, neutrophils, and basophils (Table 3). The rise in lymphocyte percentages is attributed to the heightened immune response triggered by antigen entry into the body. Lymphocytes are essential components of the adaptive immune system in fish, with B lymphocytes responsible for antibody production and T lymphocytes involved in antigen elimination, cytokine secretion, and overall immune regulation. Vaccination serves as a strategy to stimulate and improve lymphocyte performance in fish, leading to stronger immune defenses (Firdaus-Nawi and Zamri-Saad, 2016). The enhanced lymphocyte activity is reflected in the increased antibody levels produced by B cells, as well as the elevated relative levels of CD4 and CD8 T cells resulting from T cell differentiation. In addition to lymphocytes, the differential leukocyte analysis revealed a significant rise in monocytes and basophils in the nanovaccine-treated group that underwent VNN challenge. According to Agung and Prayitno, (2013), the normal monocyte percentage in grouper ranges from 9 to 15%, while the basophil percentage in fish blood typically ranges from 0.17 to 0.19% (Ibrahim *et al*., 2022). The increase in monocyte levels is driven by the presence of antigens that require rapid elimination. Monocytes play a vital role in antigen phagocytosis and the production of inflammatory cytokines, which are critical in the immune defense response. Furthermore, monocytes can differentiate into macrophages, which act as Antigen-Presenting Cells (APCs) to present antigens to lymphocytes, thereby linking innate and adaptive immunity (Kordon *et al*., 2018; Lu and Chen, 2019).

Increased lymphocyte activation was indicated by elevated relative levels of CD4 and CD8 T cells (Table 5), as well as higher antibody titers in Hybrid Grouper following administration of the recombinant *C. vulgaris* protein nanovaccine formulated with Chitosan-AgNPs hybrid nanoparticles (Fig. 6). Enhanced lymphocyte activation contributes to improved performance of both T and B lymphocytes, which are essential components of the adaptive immune response. According to Yanuhar *et al*. (2020), peridinin protein is capable of enhancing the adaptive immune response by improving the performance of MHC. Enhanced MHC function influences the increased proliferation and differentiation of T lymphocyte cells into CD4 and CD8 T cells.

In this study, chitosan nanoparticles were utilized as adjuvants to enhance the immunogenicity of *C. vulgaris* peridinin-chlorophyll protein and to protect the vaccine components from enzymatic degradation and harsh gastrointestinal conditions. Chitosan, which is biodegradable and biocompatible, binds effectively to vaccine antigens through its NH_2 groups and encapsulates the vaccine material to improve immune response activation and mucosal adhesion via quaternary ammonium interactions (Moine *et al*., 2021). Silver nanoparticles (AgNPs) served as adjuvants to facilitate rapid delivery of the vaccine to target organs such as the anterior kidney and thymus, enabled by their ability to penetrate intercellular spaces and be recognized by Pattern Recognition Receptors (PRRs) and immune cell receptors. The nanovaccine binds to leukocytes through clathrin-mediated endocytosis (Abd AL-Rhman *et al*., 2016; Asgary *et al*., 2016), and once recognized by Antigen-Presenting Cells (APCs), it is transported to lymphoid organs to activate adaptive immune responses involving B and T lymphocytes (Al Ghais *et al*., 2019; Singh, 2021).

Conclusion

In conclusion, the results demonstrated that the administration of the recombinant *C. vulgaris*-based nanovaccine significantly enhanced hematological parameters (including erythrocyte count, leukocyte count, and hemoglobin level), promoted stronger immune responses as indicated by elevated levels of $CD4^+$ and $CD8^+$ T cells, increased antibody titers, and improved survival rates in Hybrid Grouper challenged with VNN. Among the tested doses, the 33 µL treatment produced the most robust immunological response, while all treatments maintained hematological and immune cell values within normal ranges. However, a notable limitation of this study lies in its laboratory-based setting, which does not fully replicate real-world aquaculture conditions and may introduce bias in estimating vaccine efficacy. Furthermore, the immunogenic durability of the nanovaccine over longer

periods and under varying environmental conditions (e.g., temperature fluctuations, salinity, and pathogen co-infections) has not yet been evaluated. There is also a potential risk of immunosuppression associated with excessively high nanoparticle doses. Therefore, future research should focus on optimizing dosage formulations, assessing the long-term stability and immunogenicity of AgNPs in diverse environmental scenarios, conducting large-scale field trials in commercial aquaculture settings, and evaluating the ecological safety and biodegradability of nanoparticle-based vaccines to ensure sustainable application. These findings collectively underscore the promising role of integrating C. vulgaris proteins with nanoparticle adjuvants for advancing fish disease prevention strategies in aquaculture systems.

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Author's Contributions

Uun Yanuhar: coordinated the research, Conception, and study design, data analysis, and manuscript preparation and drafting.

Herly Evanuarini and Heru Suryanto: Conception and study design, data analysis, and manuscript preparation.

Apri Supii: Provide fish sampling.

Nico Rahman Caesar: Sample collection, collecting data, interpretation, drafting the manuscript.

Ethics

The Research Ethics Committee of the University of Brawijaya thoroughly reviewed the study protocol and granted ethical clearance (Approval No. 130-KEP-UB-2024).

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