Impact of the 52 kDa Outer Membrane Protein Comparison of the Conventional Vaccine with *Aeromonas Hydrophila* in Tilapia (*Oreochromis niloticus*)

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Abstract

Oreochromis niloticus, a freshwater fish, proliferates and is simple to culture. The infection of Aeromonas hydrophila, which results in Motil Aeromonas Septicemia (MAS) disease, is one of the challenges in cultivating tilapia fish. Purpose: This study demonstrated the protective effects of vaccination with 52 kDa outer membrane protein. Aeromonas hydrophila to determine the increase in total and differential leukocyte counting in tilapia fish after vaccination with 52 kDa outer membrane protein Infected with Aeromonas hydrophila CFU/ml for four days and Aeromonas hydrophila for one week. Twenty tilapia fish, measuring 10 to 12 cm in length, were Utilized in this research. They were split into four groups and given five repeats in each group. P0(-), P0(+), P1 (vaccinated with whole-cell protein "HydroVac®" and infected), and P2, vaccinated with 52 kDa outer membrane protein. and infected. The study's findings indicate a critical distinction between P1 and P2 post-vaccination and a challenge regarding leukocyte counts. 52 kDa outer membrane protein administration is recommended based on research findings. The Aeromonas hydrophila vaccine can increase total leukocytes and lymphocytes.

Keywords: *Aeromonas hydrophila, Oreochromis niloticus, Motil Aeromonas Septicemia*

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1. Introduction

MAS is a condition that Motile Aeromonas brings on by the opportunistic pathogenic bacteria *Aeromonas hydrophila*. The disease causes mortality in carp, tilapia, and gourami up to 70% - 100% [1-2]. *Aeromonas hydrophila* infection in freshwater fish can cause clinical symptoms, including haemorrhage, dropsy drizzle on the fins and tail, ulcers, exophthalmia, and dropsy. Infected kidneys, liver, spleen, and intestines all experience bleeding and necrosis. The total of bacteria is 10^8CFU/ml, followed by the death of all cells or tissues [3]. In the postmortem findings, necrosis of the musculus, liver, kidney, intestine, and spleen was found [5]. *Aeromonas hydrophila* can infect humans with clinical symptoms of diarrhoea and cause manifestations on the skin and soft tissues [5].

Motile Aeromonas control measures antibiotics are typically given to treat septicemia. In addition to changing the normal flora in the stomach, the use of antibiotics as a treatment strategy can result in the development of antibiotic resistance in fish infections. Then, from germs present in terrestrial animals, antibiotic resistance spreads and emerges more quickly from aquatic microorganisms to humans, bringing potential risks to human health. One of the alternatives that can be used to control this disease and reduce the use of antibiotics is increasing the immune system through vaccination [6].

Vaccination in fish farming has provided satisfactory results, such as an increased Survival Rate (SR) compared to non-vaccinated fish. Fish can develop specific and nonspecific immunity from vaccines, providing long-lasting protection from some infections. The general public frequently utilizes vaccines like the whole-cell protein vaccination. According to research by Mulyani et al., inactivated vaccine preparations still cause a mortality rate of 42.21% [7].

The findings of Sugiani et al. study of the efficacy of the whole-cell protein vaccine freeze-dry preparation resulted in a relative survival rate (RPS/Relative Percent Survival) in tilapia (*Oreochromis niloticus*) of 45.83%, catfish (*Clarias spp.*) 70% and gourami (*Osphronemus gouramy*) 31.67% [8]. The Ministry of Marine Affairs and Fisheries states that the prerequisite for the requirements for producing fish vaccines that can be said to be good and can be circulated to farmers must have an RPS> 50%.

The outermost layer of a cell's surface, the outer membrane proteins, are involved in the induction of immune factors during the defence. Gram-negative bacteria's outer membrane proteins, which are immunogenic, are present in *Aeromonas hydrophila* bacterium. This protein has great potential to be used as a subunit vaccine. Maiti et al. showed that research on outer membrane proteins has proven to be a potential vaccine component to control fish diseases [9].

Based on the description of these problems, this research aims to assess the protectivity of 52 kDa outer membrane protein in tilapia.

2. Materials and Methods

2.1. Material

Materials for research treatment were tilapia (*Oreochromis niloticus*), an Isolate of *Aeromonas hydrophila* from Puspa Agro's, fish feed (Takari, Jakarta), *Aeromonas hydrophila* whole-cell protein vaccine (HydroVac®, Indonesia), Freund's adjuvant complete (Merck® Sigma-Aldrich, United States of America, Cat. No. F5881), Outer membrane Protein with a molecular weight of 52 kDa obtained from previous research.

Dacie's solution, buffer solution, wright dye, and emersion oil were used for blood collection, making blood smears, and counting the number of leukocytes in tilapia.

2.2. Animal trial

The experimental animals used were tilapia (*Oreochromis niloticus*) Jatimbulan strain of 20 fish with a body length of 3.93-4.72 gr. In this study, tilapia fish less than three months old were used so that their sex could not be distinguished. The experimental animals were obtained from Gondang Legi Fish Seed Center, Malang.

2.3. Bacteria characterization

Bacteria characterization confirmed that the bacteria used in the present study was pure *Aeromonas hydrophila*. Bacteria characterization was done using the gram staining method and bacterial validation through Vitex 2. Bacteria were then reisolated on the TSA medium.

2.4. Experimental design

This study used four treatments, with each group consisting of 5 replicates. Tilapia were acclimatized for one week, and the experimental animals were vaccinated except P0 (-) and P0 (+) or negative and positive controls. Research by Mulia et al. states that antibodies can be formed within one week [10]. The increase in antibody titer one week after vaccination demonstrates this. Then, one week after vaccination, the experimental animals were infected with *Aeromonas hydrophila* bacteria except P0 (-) or negative control. Clinical symptoms occurred after being infected with *Aeromonas hydrophila* for 96 hours.

P0(-) : Fish not vaccinated or not infected with *Aeromonas hydrophila*.

P0(+) : The fish was not vaccinated but was infected with *Aeromonas hydrophila* (dose 10^7CFU/ml).

P1 : Fish were vaccinated using Whole Protein Cell (Hydrovac®) as much as 0.1 ml and then infected with *Aeromonas hydrophila* (dose 10^7CFU/ml).

P2 : Fish vaccinated using 52 kDa Outer Membrane Protein as much as 0.1 ml (0.05 ml Freund's Adjuvant Complete + 0.05 ml 52 kDa Outer Membrane Protein) and then infected with *Aeromonas hydrophila* at a dose of 10^7CFU/ml.

2.5. Post-vaccination challenge test

One week following vaccination, a bacterial challenge test was performed. The challenge test was performed with an intramuscular injection of 0.1 ml containing 10^7CFU/ml *Aeromonas hydrophila* LD_{50} dosage. The following formula was used to compute relative percent survival:

$$\left(\frac{1 - mortality\ in\ vaccinated\ fish\ (\%)}{mortality\ in\ control\ fish\ (\%)} \times 100\right)$$

For four days after infection, clinical symptoms and death were recorded daily.

2.6. Blood collection for leukocyte cell count examination

The blood was drawn using the Caudal Vein Puncture method. The researchers used the improved Neubauer counting chamber to check leukocyte counts in some of the tilapia blood and performed a leukocyte type count by making blood smear preparations.

2.7. Data Analysis

The data acquired as the number of tilapia leukocytes is organized in a table and examined. ANOVA (Analysis of Variant) will explore the different increases in leukocyte count brought on by administering *Aeromonas hydrophila* bacterium infected with different doses. If there is a difference between treatments, the Duncan Multiple Range test with a significance level of 5% is used to decide which treatment is superior. SPSS 20 was used to analyze the data.

3. Results

3.1. Observation of clinical symptoms

Figure 1. Group treatment P0(-). Description fish no clinical symptoms.

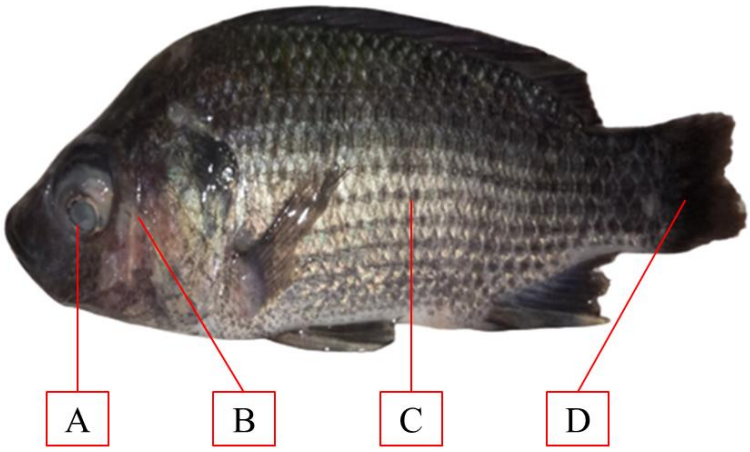

Figure 2. Group treatment P0(+) fish showing clinical symptoms including A. Exopthalmia, B. Hemorrhage, C. Dropsy in the abdominal cavity, and D. Drizzle in the fins and tail.

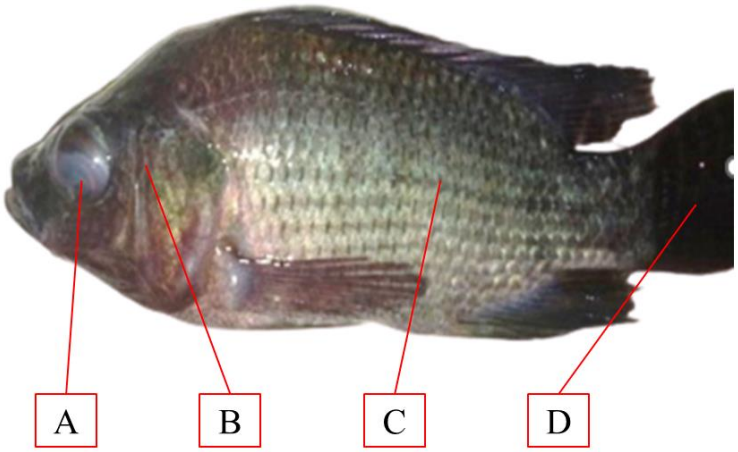

Figure 3. Group treatment P1 fish showing clinical symptoms including A. Exopthalmia, B. Hemorrhage, C. Dropsy in the abdominal cavity, and D. Drizzle in the fins and tail.

Figure 4. Group treatment P2 showed no clinical symptoms.

3.2. Relative Percent Survival (RPS)

Table 1. Relative Percent Survival (RPS)

Treatment	Number of Mortality	Number of Survival	Survival Rate (%)
P0 (-)	0	5	100%
P0 (+)	5	0	0%
P1	3	2	40%
P2	1	4	80%

3.3. Profile of leukocyte

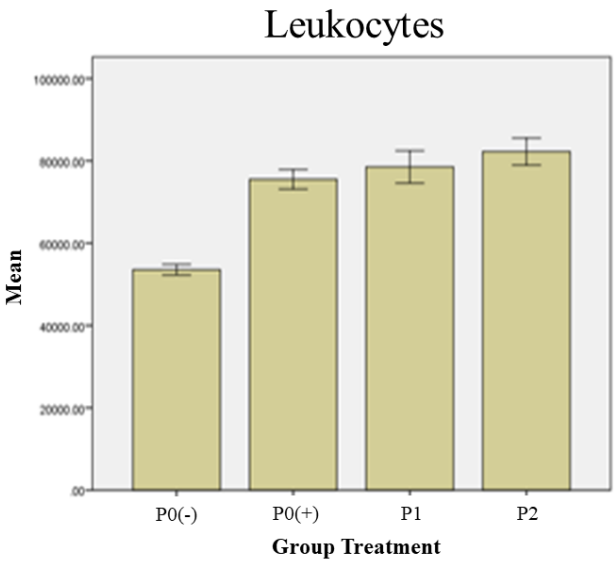

Figure 5. Leukocyte count post-treatment.

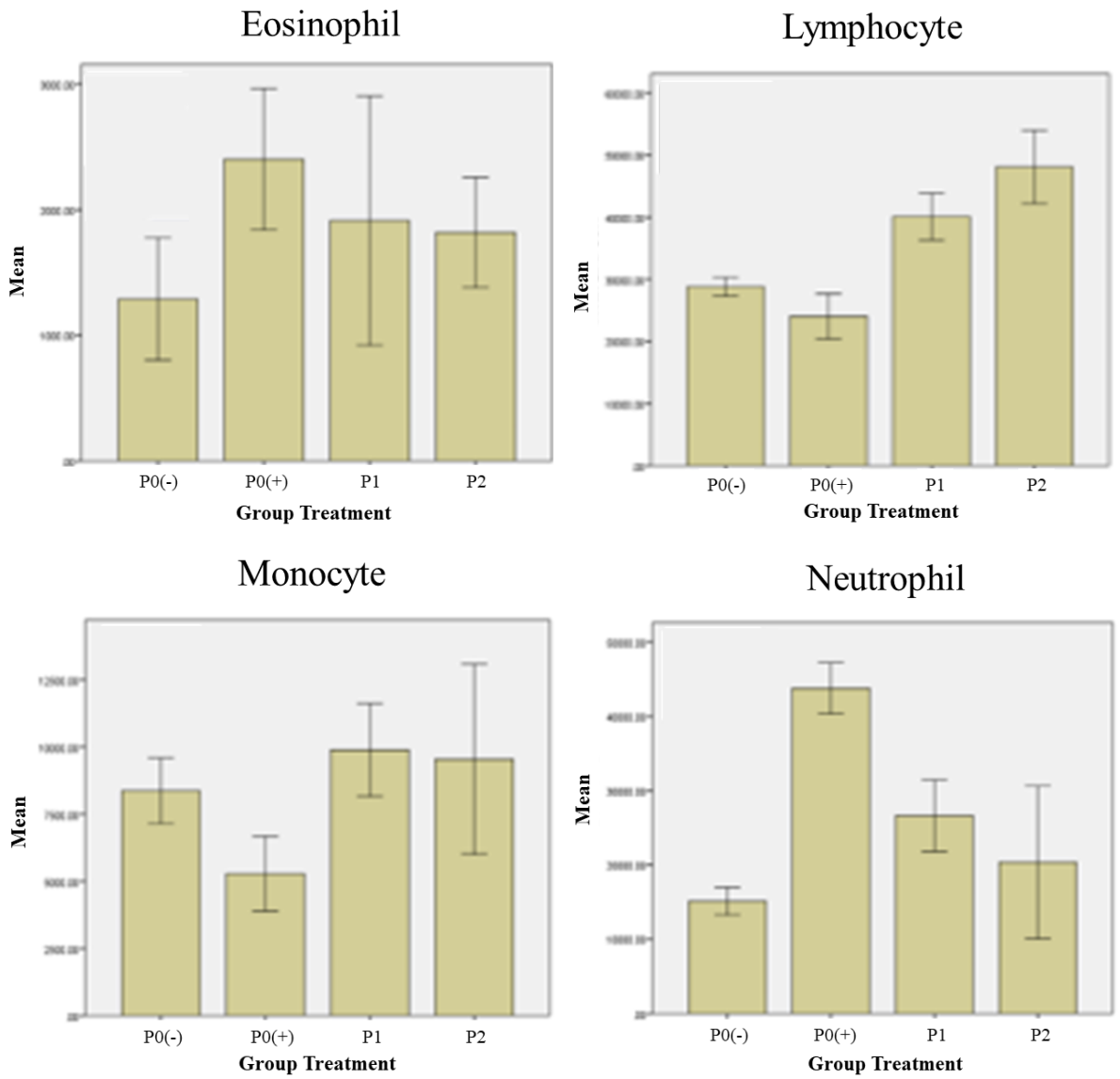

Figure 6. Leukocyte count post-vaccinated and infected with *Aeromonas hydrophila*.

4. Discussions

Tilapia treatment P0(-), which was not vaccinated and injected with 0.9% physiological NaCl, did not cause clinical symptomatology. Clinical signs such as bleeding, exophthalmia, and the appearance of fins and tails growing thinner were present in the P0 (+) tilapia that were not vaccinated but infected with *Aeromonas hydrophila*. Clinical signs such as bleeding, exophthalmia, and the appearance of fins and tails growing thinner were present in the P0 (+) tilapia that were not vaccinated but infected with *Aeromonas hydrophila*. This is because the invasion process of pathogenic bacteria into the body begins with the attachment of bacteria to the surface of the skin by utilizing pili, flagella, and hooks to move and firmly attach to the outermost layer of the fish body, namely scales protected by chitin substances. During the invasion process, *Aeromonas hydrophila* produces several enzymes, namely chitinase, lecithinase, and protease. The chitinase enzyme degrades the chitin layer on tilapia fish scales so bacteria easily penetrate it. Then, the lecithinase enzyme is produced in the fish body to enter the bloodstream quickly. Protease enzymes also play a role in damaging blood vessel channels that contain a lot of protein content. This damage causes blood to leak from the blood vessels, causing the surface of the fish's body to bleed.

In the P0(+) therapy group, additional symptoms included a change in eye colour to grey and protrusion of the eyeball, often known as exophthalmia. Hemolysin toxin produced by *Aeromonas hydrophila* causes damage to the eye's choroid. Choroidal damage to the eye causes fluid accumulation in the eye so that the eye becomes oversized and cloudy in colour. This statement is by Asniatih et al. Excessive fluid accumulation in the eye causes the eyeball to become sunken and bulge [11].

In the P1 treatment group of Tilapia, vaccinated with commercial, then infected with Aeromonas hydrophila bacteria caused clinical symptoms such as haemorrhage and exophthalmia. This is by the P1 study, which has been treated with commercial vaccines, showing a survival rate of 40%, and still causes clinical symptoms in the form of hemorrhage, exophthalmia, and thinning of the fins and tail. The states that inactive vaccines do not always provide immunity at the first vaccination [23].

In the P2 treatment group, tilapia vaccinated with 52 kDa outer membrane protein and *Aeromonas hydrophila* infection, rather than bacteria, did not cause clinical symptoms. This is because the outer membrane protein vaccine is immunogenic and protective. This statement is based on the research of Yadav et al. OMP induces a potent innate immune response, regulates gene expression, and protects fish from *Aeromonas hydrophila* infection [12].

4.1. Relative Percent Survival (RPS)

According to Table 5, the survival of fish from vaccination observation and challenge test for four days shows the highest results in the P2 treatment, which is 80%, and the lowest value is in the P0(+) treatment, which is 0% due to infection with lethal doses of *Aeromonas hydrophila* bacteria.

The P0(-) treatment had a 100% survival rate, while the control group did not get treatment. In comparison, P1, treated with a commercial vaccination, had a 40% survival rate and caused clinical signs such as bleeding, exophthalmia, and fin and tail weakening, while P2 had an 80% survival rate with no clinical symptoms.

The Ministry of Marine Affairs and Fisheries claims that the requirements for producing fish vaccines that can be said to be good and can be circulated to farmers must have an RPS> 50% [8]. According to Sudirman et al. a vaccination is considered acceptable if its relative protection rate (RPS) is more than 50% [13].

4.2. Profile of leukocyte

According to the findings, the proportion of leukocyte counts differed significantly across treatments. The Duncan Multiple Range Test revealed an increase in the number of leukocytes in the P0(+) treatment ($75520.00^{b} \pm 2363.42$), P1 ($78520.00^{bc} \pm 3934.40$) P2 ($82250.00^{c} \pm 3278.53$), which was significantly different (<0.05) from the standard control P0(-) ($53560.00^{a} \pm 1296.34$).

Leukocytes are immune-system cells found in the blood. The response of fish to increase their immune system is to increase the quantity of leukocytes, which are immune cells. Through the immune system and other mechanisms, leukocytes assist in purging the body of foreign substances, including pathogen invasion [14]. The state and health of the fish body are factors that influence the number of leukocytes. The number of leukocytes present in a given type of fish can alter depending on the fish's health; when pathogenic bacteria infect the fish, the total number of leukocytes will increase or decrease [15]. The normal leukocyte range in tilapia is 20,000-150,000 cells/mm^3 [14].

As illustrated in Figure 5, group treatment P2, P1, and P0(+) had larger total leukocytes than P0(-). Vaccine delivery increased P1 and P2 leukocyte counts. P2 was inoculated with the 52 kDa. *Aeromonas hydrophila* outer membrane protein vaccination increases more than P1, which is inoculated with a commercial whole-cell protein vaccine.

Vaccine delivery increased P1 and P2 leukocyte counts. P2, who received an *Aeromonas hydrophila* 52 kDa outer membrane protein vaccination, had a more notable increase than P1, who received a commercial whole-cell protein vaccine.

Leukocyte cell expansion indicates the immune system's ability to generate a non-specific immunological response as a trigger for the immune response. Immunogenic vaccinations can increase total leukocytes [16]. High leukocyte counts reflect the fish's immune system's ability to establish cellular (non-specific) immune responses to trigger an immunological response, including phagocytose infections, and create antibodies [17]. Group treatment P2 leukocyte increase indicates a response to Outer Membrane Protein, a vaccine-specific protein. Immunogenic proteins have molecular weights more significant than 20 kDa [18].

A non-specific immune response to bacterial infection with *Aeromonas hydrophila* induces an increase in leukocytes in P0+. With increased leukocyte counts, the fish mounts an immunological response to foreign objects in the body, as evidenced by leukocytosis. An increase in the total value of leukocytes signifies an enhanced immune response, characterized by heightened activity of phagocytic cells that engulf foreign bodies entering the fish's body. As the infection dose exposure increases, the number of leukocytes becomes more pronounced [19].

4.3. Neutrophils

After analysis with ANOVA, the proportion of neutrophils showed a significant difference between treatments. The increase in neutrophils in the P0(+) treatment was detected using Duncan's Multiple Range Test ($43784.40^{c} \pm 3421.47$) and was significantly different from P1 ($26610.00^{b} \pm 4819.32$), P2 ($20363.60^{ab} \pm 10258.13$) and P0(-) ($15112.40^{a} \pm 1834.35$).

As illustrated in Figure 6, a rise in neutrophils occurred in the P0(+), P1, and P2 treatments, demonstrating the ability of neutrophil cells to locate and combat antigens that are ingested, indicating the phagocytosis process. The immunological process that responds to infection in the body causes a rise in neutrophil numbers. White blood cells, called neutrophils, support the immune system [16]. Chemotaxis and phagocytosis processes protect neutrophils against pathogens, particularly bacteria. Neutrophils depart from the peripheral group and infiltrate the site of infection due to the infection in the fish body. The thymus will activate its reserve capabilities, leading to an increase in granulopoiesis. Phagocytosis involves neutrophil cells accessing and attacking antigens (foreign particles) that enter the body, causing the neutrophil count to rise.

4.4. Eosinophils

Due to the lack of an allergic reaction, this research found no substantial differences between therapies. The second core cell of the myeloid system, eosinophils, is linked to allergic reactions to environmental stimuli. Eosinophil granules contain antihistamines that actively participate in the hypersensitivity process by specializing in detoxifying histamine.

4.5. Lymphocytes

After variance analysis, the data revealed a significant difference in the percentage of lymphocytes between treatments. Duncan's Multiple Range Test demonstrated increased lymphocytes in the P0(-) treatment ($24057.00^{a} \pm 1469.49$) and P0(+) ($28794.00^{a} \pm 3644.65$) had no significant difference. However, it significantly differs from P1 ($40108.40^{b} \pm 3762.19$) and P2 ($48092.80^{c} \pm 5880.23$).

Vaccination increased lymphocytes in the P1 and P2 treatments. The proportion of lymphocytes in the P1 and P2 vaccine groups following vaccination and challenge tests indicates that adding vaccinations as a trigger for immunity in tilapia is responsible for the body's defence mechanism. The rise in lymphocytes in tilapia reflects the fish body's immune response to illness infection [14]. The increase in lymphocytes produced plays a crucial part in strengthening the fish body's immunological response or resistance to sickness and virus attacks. Lymphocytes are not phagocytic, yet they are necessary for antibody production. Furthermore, the study's findings indicate that lymphocyte cells have

the highest presentation compared to other cells. This is related to the activity of lymphocyte cells as immunological substance makers. Fish have more lymphocyte cells than other vertebrates, more than neutrophils and monocytes [3]. The lymphocytes will enlarge, forming specific antibodies according to the antigen providing stimulation.

The mechanism of action of lymphocytes is in their role in identifying antigens through specialized receptors on the cell membrane. The immune system delivers immunological components for the body's defence. Lymphocyte cells divide and differentiate into effector cells and memory cells after the antigen is bound to the lymphocyte cell antigen receptor. T-lymphocyte cells with cytoplasmic granules contain many proteins responsible for lysing antigens [14].

When T cells are exposed to antigens, they cannot detect them without the help of particular receptors. These specific receptors enable T cells to recognize existing antigens more quickly, resulting in prompt immunological response and stimulation of B cells to make antibodies. When a vaccine is administered, the body produces more lysozymes and complements, which causes B cells to become more active and specialize in creating certain antibodies [20].

Group treatment P0(-) dropped due to the decline in lymphocyte cells brought on by the rise in resistance activity, according to Rustikawati, who asserts that as the severity of an infection with a particular pathogen rises, lymphocytes, the body's providers of immunological material, will become less plentiful as the body's requirement for white blood cells (lymphocytes) increases [21].

4.6. Monocytes

Analysis within ANOVA revealed a significant difference in the proportion of monocytes between treatments. Duncan's Multiple Range Test has increased the number of monocytes in the patient population P0(-) ($8366.00^{b} \pm 1221.26$), P2 ($9890.20^{b} \pm 3535.10$), P1 ($9548.80^{b} \pm 1731.80$) was significantly different from P0(+) ($5277.80^{a} \pm 1389.70$), but not significantly different from each other.

There was a decrease in monocytes in P0(+). Lymphocytes, the body's providers of immunological material, will become less plentiful as the body's requirement for white blood cells (lymphocytes) increases; hence, the fish body does not create monocytes [22]. This is in response to Utami et al. claim that the low proportion of monocytes is connected to their activity as macrophages [16]. Monocytes, which function like macrophages, are frequently seen in sites of inflammation or infection. In P1 and P2, there was an increase in the number of monocytes.

5. Conclusion

Based on research findings, the *Aeromonas hydrophila* of 52 kDa outer membrane protein administration is recommended. The *Aeromonas hydrophila* vaccine can increase leukocyte production and the number of lymphocytes.

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Conflict of Interest

Regarding this inquiry, there are no conflicts of interest for the author.

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