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Abstract. Ectoparasite infestation and *Vibrio* sp. bacterial infection are a major factor causing the death of pacific white shrimp (*Litopenaeus vannamei*), which can cause an increased mortality rate up to 100% only three days after infection, especially in hatcheries. Ectoparasites that are often found in shrimp culture include *Zoothamnium* sp., *Epistylis* sp. and *Vorticella* sp. Meanwhile, the *vibrio* bacteria that are often found in the same setting are *Vibrio alginolitycus*, *Vibrio harveyii*, *Vibrio parahaemoliticus* and *Vibrio fulvinicus*. The aim of this research was to analyze the correlation between ectoparasite infestation and the total plate count of *Vibrio* sp. in white shrimp (*Litopenaeus vannamei*) during a culture of up to 90 days in ponds. The method used in the research study was an experimental design with a sampling time spaced out over 0, 30, 60 and 90 days. The results showed that the ectoparasites found to be infecting the white shrimp were *Zoothamnium* sp. and *Vorticella* sp. with the lowest number of ectoparasites being 4 individuals found on the 90th day. The highest number was 63 parasites on the 60th day. The lowest *Vibrio* sp. total plate count was 2.9 x 10^4 CFU/g and the highest was 5.55 x 10^4 CFU/g.

1. Introduction

Tiger shrimp (*Penaeus monodon* Fab.) are a type of marine shrimp that can develop within pond culture; the survival rate can reach up to 90%. In Indonesia, shrimp were once the best non-oil and gas export commodity from the fisheries sector and once made Indonesia the world's fourth major shrimp exporting country with an export value of $1.9 US dollars. However, since the beginning of 1994, shrimp production from the pond culture sector has tended to continue to decline until it has reached a stationary point. The production decline is due to the cases of shrimp death in ponds that are mainly caused by disease and the decreasing water quality [1]. In order to increase shrimp production, in 2002, the Indonesian Government legalized white shrimp (*Litopenaeus vannamei*) to be developed in Indonesia through aquaculture in ponds.

Many problems have emerged in the development of white shrimp culture, where the outbreak of disease is a factor that causes a harvest failure and therefore needs serious attention. One of the diseases that can cause the death of shrimp in both ponds and hatcheries is Zoothamniosis. This disease is one of the parasitic diseases of white shrimp caused by *Zoothamnium penaeid*. Zoothamniosis causes the shrimp to find it hard to breathe and difficult to move, and thus they are unable to find food [2]. The shrimp also find it difficult to change their skin (molting), which inhibits growth, reduces their economic value and causes death by up to 91% [3]. The prevention of

Zoothamniosis using immunostimulants made from protein membranes has never been done before, even though the isolation and identification of suitable proteins from several *Zoothamnium* species has been carried out. Meanwhile, the treatment that has been done includes the use of chemicals and antibiotics, which can cause residue in the shrimp meat.

Improving the shrimp's immune response both in hatcheries and in ponds can be done using immunostimulants. Furthermore, immunization with *Zoothamnium penaei* immunogenic membrane protein can increase shrimp survival rates by 93% [4]. Furthermore, the isolation of the immunogenic membrane protein was carried out using SDS-PAGE, ELISA and Western Blotting. The results of the analysis showed that 7 proteins were found and that 3 proteins were immunogenic, namely protein membranes MP38, MP48, and MP67.

The invertebrates' (including shrimp) immune system, which has a role in the body's defense mechanisms due to haemocytes, is where the spread and increase in the number of haemocytes is assumed to be a form of cellular immune response in the shrimp's body [5,6]. In order to carry out phagocytic activity including the encapsulation, nodulation and activation of the prophenoloxidase system, including anti-microbial and toxic compounds, the release of several proteins is needed to overcome the incoming agent [7].

The presence of an immune response in shrimp can be seen by an increase and change in the haemocytes, namely the Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC) [6]. Starting from the description above, it is very important to find an alternative effort to prevent the high mortality of white shrimp by using immunostimulants from *Zoothamnium penaei's* membrane protein, which has been tested and is ready to use in ponds and can be applied easily and widely.

Based on the research background, the objectives of this study are: 1) to conduct a protein membrane field test of *Zoothamnium penaei* immunogenic to see if it can reduce the parasitic infestation (*Zoothamnium penaei*) in white shrimp (*Litopenaeus vannamei*) in ponds and 2) to conduct a protein membrane field test of *Zoothamnium penaei* immunogenic as an immunostimulant material to decrease the mortality of white shrimp (*Litopenaeus vannamei*) in ponds.

2. Materials and methods

2.1 Materials and tools

The main materials used in this research were 10,000 healthy white shrimp post-larval at 40 days old (juvenile). *Zoothamnium penaei* whole protein was the material used for developing the immunostimulants, which had been tested previously in a laboratory setting by as much as 5 ppm [4]. The examination of the bacterial infection was done through PCR. The water quality checks were conducted using a DO meter, pH meter, thermometer, soil pH meter, secchi disk and a water sampling tool. For the immunization, a plastic tub with a 10 liters capacity filled with water was used.

2.2 Pond preparation

The pond used in this research consisted of three plots, namely a reservoir plot, recirculation plot with a biology filter against milkfish and a shrimp maintenance plot. Each plot was connected with two paralonals (8 dim) to promote recirculation flow. The recirculation will be operated by a water pump sized 20 x 8 inches.

2.3 White shrimp seed preparation

The healthy white shrimp seeds used in this study came from shrimp seedings in Tanggul Rejo Village, Ujung Pangkah District, Gresik Regency. The shrimps were in the juvenile stage; this means that they were 30 days old with a length of about 3 - 4 cm. The shrimp seeds used for the trial totaled 100,000. The shrimp were brought to the pond using plastic bags filled with oxygen. The shrimps were acclimatized by opening the plastic bag after arrival at the pond and then placing it on the surface of the pond.

2.4 Research design
This research design was an experimental study with the aim of analyzing the pathogenic infections from parasites, bacteria and viruses that often attack white shrimp in ponds with immuno-probiocirculation (SI-PBR) systems. The following treatments were implemented:
K1 → control; the group of shrimp that were kept in ponds in a semi-intensive manner without using immunostimulants from Whole Protein *Zoothamnium penaei*.
K2 → a group of shrimp kept in ponds in a Semi-Intensive system using immunostimulants from Whole Protein *Zoothamnium penaei*.

2.5 Maintenance of shrimp with immunostimulants in ponds
The shrimp were maintained for 3 months in one harvest period. The seed used was made up of white shrimp in the juvenile stage (40 days old). We used 4 pieces of plot with an area of 500 square meters each; reservoirs, biological filter plots and 2 shrimp maintenance plots. The stocking density of milkfish in the biological filter plots totaled as many as 2000 tails, while the stocking density of the white shrimp was 5,000 shrimp in one plot of maintenance. Circulation was carried out at 06.00 - 08.00 (for two hours) and 24.00 - 02.00 (for 2 hours).

2.6 Ectoparasite infestation examination of white shrimp
The observation of the ectoparasite infestation was done through the native method namely by scrapping the entire surface of the shrimp's body [8]. The results of the scraping were placed on a glass object, given one drop of water and observed with a binocular microscope on 100x magnification. The parasitic infestation was calculated through the positive infested shrimp percentage that was being examined. For the examination and characterization of the *Vibrio* sp bacteria, we performed PCR according to the modification of the Anderson method [9]. The stages of this method included the extraction of bacterial DNA, electrophoresis, documentation and reading the results with a digital camera.

2.7 Survival rate calculation
The survival rate calculation of the white shrimp was carried out at the harvest, which was after one month of maintenance in the pond. This was expressed by the percentage of the total number of shrimp that lived in the overall pond population.

2.8 Data analysis
The data was analyzed descriptively and presented in the form of a table and images. In order to find out the correlation between the ectoparasite infestation and *Vibrio* sp. bacterial infection, we used a t-test [10].

3. Results and Discussion

3.1. Zoothamnium penaei Infestation Examination in White Shrimp
The results showed that all of the shrimp samples were positive for infection by zoothamniosis caused by *Zoothamnium penaei*. Based on the identification of the parasite, the white shrimp were found to be positively infested with *Zoothamnium penaei*. All of the shrimp infected with zoothamniosis showed clinical symptoms over their entire bodily surface, and they also had attached parasites on the gills and were a brown-white color. In addition, it also appeared that they had an empty digestive tract, lackluster body surface and their gills were cloudy and dirty. Some of the shrimps showed damage to organs such as the antenna, rostrum, tail, pleopod and periopod. An illustration of a shrimp infested with *Zoothamnium penaei* has been presented in Figure 1.

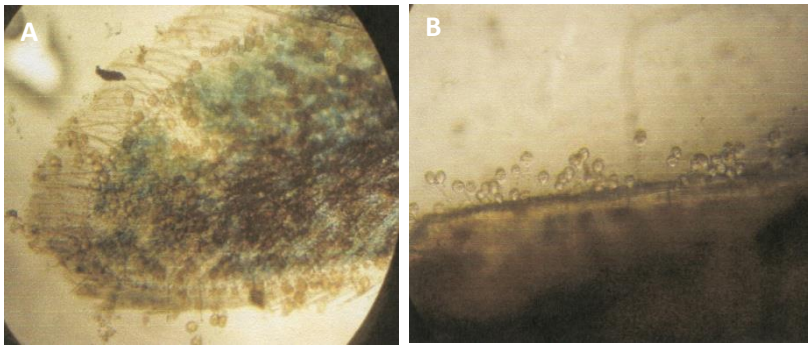

Figure 1. Illustration of *Zoothamnium penaei* infestation on a few organs, A: Tail and B: Dorsal (100x magnification)

The results of the *Zoothamnium penaei* infestation examination conducted on the white shrimp at the end of the test have been presented in Table 1, which shows that the highest level of parasitic infestation in white shrimp occurred in those that were not immunized with whole protein (K1); these showed as being 68% positive for parasitic infestation. The lowest parasitic infestation was in a group of shrimps that were given protein of 2.8% (K2) after maintenance for 90 days.

Table 1. Results of the Parasite Infestation Examination on White Shrimp

Maintenance Days (Days)	Shrimp Ectoparasites Examination Result (%)	
	Maintenance without Whole Protein (K1)	Maintenance using Whole Protein (K2)
1	2,3	2,2
30	16,5	11,3
60	38.3	7,3
90	68,2	2,8

3.2. Bacterial infection examination in white shrimp

Bacterial disease in shrimp is often caused by the *Vibrio* bacteria, especially *Vibrio harveyii, Vibrio parahaemoliticus* and *Vibrio alginolitic*us. The results can be seen in Table 2**.**

Table 2. Bacterial Infection Examination in White Shrimp

Maintenance Days (Days)	Total Plate *Vibrio* sp (CFU/ml)	
	Maintenance without Whole Protein (K1)	Maintenance using Whole Protein (K2)
1	$1,2 \times 10^5$	1.2×10^4
30	$9,6 \times 10^5$	$7,4 \times 10^3$
60	$14,7 \times 10^5$	$5,6 \times 10^3$
90	19.2×10^5	$4,8 \times 10^3$

Table 2 shows the white shrimp seeds that were stocked in ponds with SI-PBR or that were not using SI-PBR that showed as positive for *Vibrio alginoliticus* bacteria. There was an increase after this setting was maintained for the 30th day without SI-PBR as it reached 18.2×10^4 CFU/ml, while in the shrimp that were kept in ponds with SI-PBR, the number of colonies also increased**.**

3.3. White shrimp survival rate calculation result from the challenge test

The white shrimp survival rate calculation results after 30 days of maintenance showed that there were significant ($p<0.05$) differences between the white shrimp that had been given a membrane protein and those that had not given a membrane protein. The highest survival rate occurred in the white shrimp that were given the MP38 immunogenic membrane protein by 94% and this was followed by MP48 and MP67, which were 84% and 72% (Table 3).

Table 3. White Shrimp Survival Rate when Immunized by MP38, MP48 and MP67 membrane proteins

Treatment Category	White Shrimp Survival Rate (%)
Maintenance without Whole Protein (K1)	$38,00^{bc} \pm 8,37$
Maintenance using Whole Protein (K2)	$86,00^{d} \pm 4,47$

Information: The different superscripts in each column and row showed that there was a significant different ($p<0,05$**)**

3.4. Discussion

The results showed that there was a decrease in the infestation of the Zoo*thamnium penaei* parasite and the *Vibrio parahaemoliticus* bacterial infection along with an increase in the age of the shrimp in the pond (Table 1 and 2). *Zoothamnium penaei* infestation and *Vibrio parahaemoliticus* bacterial infection had begun to be found in 30 day old shrimp that were maintained using immunostimulants. However, the highest infestation rate occurred in white shrimp that were not exposed to whole protein, which amounted to 68,2%. The infestations in shrimp exposed to whole protein, at 30, 60 and 90 days, all showed lower numbers when compared with those who had not been exposed to protein.

The use of immunostimulants in white shrimp culture can increase the immune response characterized by the decrease in parasite infestation and in the infection rate of *Vibrio* sp bacteria. The immunostimulant's ability in the culture system can also be seen in the survival rate of white shrimp that increased from 38% to 86%. This means that immunostimulants from the *Zoothamnium penaei* protein were able to provide protection to the white shrimp maintained in the ponds. Whole protein that enters the body of the shrimp will stimulate the activity of the haemocyte cells in an effort to fight the pathogens that entered the body of the shrimp during maintenance. Haemocytes that are activated by crude protein will carry out phagocytic activity in the shrimp through their hyalin (granular) and semi-granular cells [4,11].

The immune system in shrimp is still primitive and unlike that of fish and mammals that contain immunoglobulins. The immunoglobulins in shrimp are replaced by a Prophenoloxidase Activating Enzyme (PPA) [5]. PPA is a protein located in the granular hemocytes. PPA can be activated by lipopolysaccharide and β 1,3-Glucan, which will stimulate prophenoloxidase to become phenoloxidase. As a result of these changes, a kind of Opsonin Factor protein can be produced which can induce the hyalin cells to phagocytosis. The haemocyte cells will degranulate and some of the proteins will be released for the benefit of the immune response, such as increased haemocyte cells, andentrapment and phagocytosis activities [8]. In addition, immunogenic membrane proteins will stimulate haemocytes to release proPO and protein-binding PPA, resulting in haemocytes increasing their activity to trap and go into phagocytosis against any disease agents, which in this case, is *Zoothamnium penaei*. This is evidence that the prevalence of Zoothamniosis in immunized shrimp is lower and very significantly different from the prevalence in the unimmunized shrimp. There were still shrimp infested with *Zoothamnium penaei* because this parasite is opportunistic, so in normal water conditions, it still grows but develops over a long time and does not cause pain in the shrimp.

However, the condition of these waters has not been able to cause increased activity in the *Vibrio parahaemoliticus* bacteria, therefore it has not caused pain in the shrimp.

If whole protein, as an immunostimulant ingredient, enters the body of the shrimp, then it will cause an increase in the total number of haemocytes (THC) and differential haemocyte cells (DHC). This indicates the increase in the body's shrimp defenses against pathogenic infections [4]. Furthermore, the administration of vaccines can prevent infection and lead to the increased activity of the phagocytes related to the haemocytes and proPO enzymes [12]. Vaccine ingredients that enter the body of the shrimp will cause antibodies that can neutralize the *Zoothamnium penaei* infestation [6].

Immunogenic membrane proteins that enter the body can increase the survival rate of white shrimp from 38% to 86% in 90 day old shrimp (end of maintenance) [4]. Furthermore, it was also said that the immune response of the tiger shrimp also increased.

An increased immune response can be used as an indicator or sign of pathogenic infection in the host's body. This infection will cause inflammation, which is a non-specific bodily defense characteristic due to influencing factors such as parasites, bacteria, fungi, viruses and non-living agents [13,14].

The increase in the immune response is because the shrimp do not have memory cells in their immune system, so they are unable to detect pathogenic substances that they have been exposed to. Thus it can be argued that immunostimulant material from crude protein can induce the body's shrimp defense mechanism. However, it takes time to stimulate the hematopoietic organs to produce granulocytes to fight zoothamniosis attacks [4]. These granulocytes will destroy pathogens by ingesting them, so the granulocytes will migrate to organs that have parasitic and bacterial infection.

5. Conclusion

The conclusion that can be drawn from in this study is that in white shrimp, there is a correlation between ectoparasite infestation and *Vibrio* sp bacterial infection. The higher presence of *Vibrio* sp bacterial infection was indicated by the increase in the *Vibrio* bacteria Total Plate Count (TPC)**.** The shrimp were maintained in semi-intensive ponds infested with ectoparasites and infected with *Vibrio* bacteria. There were populations that were both exposed and not exposed to *Zoothamnium* whole protein.

6. References

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