Effectivity of immunostimulant from *Zoothamnium penaei* protein membrane for decreasing the mortality rate of white shrimp (*Litopenaeus vannamei*) in traditional plus pond

G Mahasri¹*, R Kusdarwati¹, Kismiyati¹, Rozi¹, H Gustrifandi²

¹Department of Fish Health Management and Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Campus C Mulyorejo Surabaya, Indonesia
²Fish Quarantine Center and Quality Control of Fishery Products, Class I, Surabaya, Indonesia

E-mail: mahasritot@gmail.com

Abstract. The purpose of this research was to analyse immunogenic membrane protein as immunostimulant development material to control the mortality of white shrimp in traditional plus pond. This research was designed to use explorative experiment and experimental laboratory methods which used completed random sampling design. Collected data was analyzed with analysis of variance for examination of survival rate (SR), total haemocyte count (THC) and differential haemocyte Count (DHC). The research divided into 2 part of riset: (1) Identification, cultivation *Zoothamnium penaei*, analysed of membrane protein by SDS-PAGE, (2) Field test protein membran on Survival Rate level, immune response (THC and/or DHC level) and infestation of *Zoothamnium penaei* in traditional plus pond. The result showed that there were seven bands membrane protein of *Zoothamnium penaei* with molecular weight 38 kDa, 48 kDa, 67 kDa, 71 kDa, 77 kDa, 98 kDa dan 104 kDa by using SDS-PAGE. Immunogenicity test decrease by using ELISA and western blotting there are only found three bands with molecular weight 38 kDa, 48 kDa dan 67 kDa. The membrane protein could increase the immune resons and decrease the mortality, by subsequently, it could increase the survival rate from 17% until 68% and pressured the parasite infestation of white shrimp.

1. Introduction

The vaname shrimp (*Litopenaeus vannamei*) is one of imported species from America entering Indonesia in 1998. After various technological applications and trials, in 2002 the Indonesian Government legalised that vannamei shrimp could be developed in Indonesia. The Government expected that the emergence of shrimp vannamei would replace the position of tiger prawns, which were still one of the main non-oil export commodities from the fishery sector until 1993. The value of vannamei shrimp exports in 2006 reached 6000 billion US Dollars. Vannamei shrimp also has a big role in the fulfillment of animal protein from fish, because shrimp has a high nutritional value [1].

The production of vaname shrimp has been increasing, meeting the needs of the market especially for export. One of the main constraints that is difficult to control is the presence of disease, which causes the death of shrimp up to 100 % [2]. Furthermore, it is also stated that in general the losses caused by the disease reach billions of rupiah. This is caused by a very sudden onset of illness and the total death will occur in just 2 to 3 days of the post infection. The attack of this disease had caused
many shrimp-culture farmers to close their business and 80 % of shrimp farms and shrimp hatcheries not to operate.

One of the diseases that can cause the death of shrimp both in pond and hatchery is Zoothamniosis. This disease is one of the parasitic diseases found in vannamei shrimp caused by Zoothamnium penaei. This disease causes the shrimp to breathe hard, difficult to move and cannot find food [3, 4], difficult to moult, inhibit growth, reduced economic value and cause death to 91 % [5]. In addition, this disease is a predisposing factor of secondary infection by bacteria and viruses.

Prevention efforts have been done, such as using the circulatory system, formaline treatment, and antibiotics. According to Chamratchakool [6], the treatment of Zoothanmiosis with 30 ppm of formalin could suppress the incidence of this disease, 2 ppt effective acetic acid for larvae [5]. Many also used chemicals such as malacyst green, and methylene blue. Mahasri [7] emphasized Zoothamnium penaei infection using aeration and stocking solid arrangement, which showed that Zoozid of Zoothamnium penaei infection decreased at high aeration level and low to medium density of stocking. The use of biological filters of milkfish could also suppress Zoothamniosis attacks from 89 % to 14 % [8].

Rukyani [9], argued that the resilience improvement of shrimp bodies both in hatcheries and ponds could be done by using the immunostimulant. Furthermore, Mahasri [8] added that the immunization with immunogenic membrane protein isolated from Zoothamnium penaei could increase the shrimp life rate up to 93%. It is also stated that the isolation of immunogenic membrane proteins could be done with SDS-PAGE, ELISA and Western Blotting. The results showed that found 3 proteins found were immunogenic, i.e. MP38, MP48 and MP67 membrane proteins.

The isolation of protein has been performed by Amos et al. [10] and Routledge [11], who characterized the calcium bond with proteins using the SDS-PAGE method. The synthesis of Telotroch proteins (taken from the part of Zoothamnium arbuscula during free swim stadium) Pylawka et al. [12] and characterization of Zoothamnium arbuscula spore protein with SDS-PAGE [13]. Itabashi et al [13] also stated that the spore had spasmin-1 protein in its spasmonema. The analysis of immunoblotting results showed that the antigen protein had a molecular weight of 68 kDa, 55 kDa and 71 kDa.

Spasmin protein is a Zoothamnium arbuscula trophozooid protein presented in spasmonema and contractile stalk. The genome length of this spasmin protein is 531 bp, which is predicted to consist of 177 amino acids with a molecular weight of 19.659 kDa [13]. The characterization of spasmin protein from Zoothamnium arbuscula (Familia Vorticellidae) using SDS-PAGE indicated that 60% protein bands were spread over a molecular weight of about 20,000 kDa. The rich amino acid contents found were glysin and serine, but were poor in aromatic amino acids and had no cysteine and methionine [10]. Routledge [11] has isolated proteins from contractile spasmony from Vorticella convollaria, Carchesium polypinum, and Zoothamnium geniculatum extracted in detergent, Sodium Dedocyl Sulfate (SDS), such as urea and Guanidine Hydrochloride (GuCl). After extracting with SDS, the molecular weight distribution of proteins was identified with SDS-PAGE. Clearly, there were no actine and tubuline proteins. The molecular weight of the contractile organ protein was closed to 20,000 kDal. Moriyama et al. [14], had isolated proteins from Zoothamnium sp. spasmonema and stated that the molecular weight of the protein chain was estimated to be closed to 50 kDal and 20 kDal synthesized from sponical protein binding [15]. The results show that 1 spasmin gene has no intron and 531 bp genome length. It is predicted to produce 177 amino acids with molecular weight approaching 19659 Da (19,659 kDa). The acid sequence has two calcium bonds.

The body's defense system in invertebrates (including shrimp) plays a role in the body's defense mechanism by haemocytes, where the spread and increased number of haemocytes is assumed to be a form of cellular immune response in the shrimp body [16, 17]. To perform phagocytic activity, encapsulation, nodulation, activation of prophenoloxidase system, anti microbial or toxic compound, it is necessary to release some proteins to overcome the foreign object or the incoming agent [18]. Johanson et al. [19] mentioned that hyalin cells also play a role in the shrimp body's defense system. This hyalin cell is activated by Opsonin Factor that results from proPO activation into PO in granular
cells. So, it can phagocytosis both bacteria and virus, but the most important role in shrimp body defense system is granulocyte.

Increased body resistance can be known from the increased activity of phagocyte cells from hemoocyte. These phagocytic cells function to perform phagocytosis of foreign objects entering the host's body. Phagocytosis is a non-specific defense mechanism that generally protects against disease. Hemocytes are known to be very important factors in non-specific cellular defense systems. To know that the hemoglobin is a cellular defensive body, it can be seen from its ability in phagocytosis activity that can be increased in the incidence of infection. The presence of three forms of mechanisms of observable hemocyte activity, namely (1) the mechanism of entrapment (encapsulation) of a foreign material, (2) the joint phagocytic mechanism of some hemorrhoid which form larger aggregates, and (3) aggregates of many hemoglysins forming a pigmented layer [20].

Kondo et al. [21] found 5 types of phagocytes in kuruma shrimp (Penaeus japonicus); two of which are phagocyte cells and the rest are hemocytes. The first two types of phagocytes are found in the heart of the basal lamina covering the sarcolemma of the heart muscle and its cells have lysosomal granules of 0.1 μm diameter, the second type of phagocytic cell found in lymphoid organs. In the five types of phagocytes were found lysosomal enzymes such as acid phosphate, β-glukronidase and non-specific esterase. Very high estersae activity was found in granular cells. Phenoloxidase (PO) activity was also found in all phagocyte cells but it was very weak, whereas prophenoloxidase (proPO) activity was found only in granular cells and semigranular cells.

Humoral body defense systems include phenoloxidase (PO), prophenoloxidase (proPO), lectin and aglutinin. Both of these defense systems work together to provide body protection against infection of pathogenic organisms from the environment [16]. ProPO is activated by enzyme prophenoloxidase actuating enzyme (PPA). Meanwhile, the prophenoloxidase actuating enzyme can be activated by lipopolysaccharide. ProPO and PPA are proteins located in granular hemocyte. As a result of the activation of proPO, into Protein was produced as Opsonin Factor that stimulates phagocytosis of hyalin cells (agranular cell) [19].

Van de Braak [17] proposed that the immune response of shrimp among others can be seen with the increase and change of hemocytes, namely Total Haemocite Count (THC) and Differential Haemocite Count (DHC). He further explained that THC and DHC will increase in shrimp in the affected area, resulting in a decrease in the flow of blood circulation. This increase of THC and DHC signifies an increase in the body's defense of shrimp, thus reducing the pathogen infection and consequently increasing the survival rate of shrimp.

McKay et al. [22] said that immunization in crayfish (Paracharaps bicarinatus) 4 times with endotoxin 2.5 μg and 2.5 X 107 vaccine from bacteria Pseudomonas sp. with 4 injections resulted in very significant differences between the amount of hemocytes in the control group and the immunized ones. Proteins released during vaccination are used for immune responses such as phagocytosis, encapsulation, melanization, coagulation, peroxidase activity, opsonization, anti-microbial activity and other humoral and cellular activity processes [17].

Giving immunostimulants to crustaceans, which is also called vaccination, has no side effects and is very good to be applied to organisms that do not have memory cells in the immune system, so as to stimulate and or maximize non-specific immune responses [23]. The provision of Lipopolysaccharide (LPS) from the Vibrio harveyi bacterial cell wall orally to the vannamei shrimp (Litopenaeus vannamei Fab.) within 42 days can increase the total amount of hemocytes and the granulocyte cell type, while the phagostic activity of the hemoite cells has shown an increase on day 28 and higher on day 42. The occurrence of increased phagocytic activity of hemoite cells along with the increasing total hemocytes and granulocyte cell types is due to Lipopolisaccharide.

Based on the above background, it is necessary to examine the immune response (THC and DHC) and the survival and parasitic infestations of vannamei shrimps given immunogenic membrane proteins MP38, MP48 and MP67 orally and infested with Zoothamnium penaei.
2. Methodology
2.1. Materials Research
The materials used in this research were Zoothamnium penaei from Gresik and Lamongan, East Java. The species sample then was cultivated by means of cohabitation at the Aquarium in order to obtain the amount of zooid in large quantities planted in the vaname shrimp seed aged PL30-PL40 as much as 10,000 shrimps.

The materials used for the isolation of whole protein using the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) were Acrylamide (Sigma Chemical Company 3050, PO BOX Spruce Street 14508, St Louise, MO 63178, USA), bis- (N, N ‘-Methylene-bis-Acrylamide) (Sigma Chemical Company 3050, PO BOX Spruce Street 14508, St. Louis, MO 63178, USA), Sodium Dodecylsulfate or SDS (Merck, Postfach 4119, D-6100, 64271 Dannstadt 1 Germany), PMSF, TPCK, TLCK and EDTA.

2.2. Research instruments
The instruments used in this research were micro pipettes (Gilson), J-6B centrifuge (Beckman), microfugeTM11 (Beckman), stereo type 102 (Nixon) microscope. The immunostimulatory apparatus equipments were 3 plot ponds, 2 sets of saprodi, and a genset for water pump.

2.3. Research procedure
2.3.1. Zoothamnium penaei protein membrane isolation
6 x 108 Zooid/mL result of in vitro cultivation was resused with 10 mM PBS to obtain final concentration of 6 x 108 Zooid/mL, then added with protease inhibitor (for 100 mL PBS plus 100 μl 40 mM PMSF, 7.3 mg TLCK, 7 mg TPCK and 7.5 mg EDTA). The suspension was synchronized with 4 x 0.5 minutes in ice. The suspension was then centrifuged at 4000 rpm for 1 hour. The supernatant was taken. The cytoplasmic protein (soluble protein) and pellets contain whole protein. The pellet was further resolved with 0.5 % Nonidet P40 in a protease inhibitor solution with the same composition as in the previous composition. Sonication was performed with 4 x 0.5 min in the subsequent ice incubated overnight at 4°C while stirring with stirrer at medium speed. The suspension was then centrifuged at 4,000 rpm for 30 min at 4 °C and the supernatant was taken for measurement of its protein concentration.

2.3.2. Protein concentrations determine
To determine the concentration of homogeneous protein, the Bio-Rad Protein Assay Method was used. It was read using UV-Visible Spectrophotometer with 595 nm wavelength.

2.3.3. The characterization and purification of protein membranes with SDS-PAGE
The purpose of this activity is to look at the molecular weight pattern of the whole protein fraction. Running gel was made and inserted into the glass plate, after hardening the prepared stacking gel was inserted at the top. The composition of running gel and stacking gel was made by mixing Acrylamid, Tris, SDS 0.8 %, Temed, APS and aquades in a glass beaker. A total of 10 μg samples added with laemly buffers with a 2: 1 ratio were boiled at 100°C for 5 min, then put into a molding column located on the stacking gel and performed running on a chamber that had been filled Electrode Buffers 1X with 100 volt, 40 mA. After running, the gel was introduced into a washing solution consisting of 25 mL methanol, acetic acid 3.7 mL, and Aquades ad 100 mL. It was then stirred over sacker for 30 minutes. Repeat washing was done with the same solution with a reduction of the ethanol composition and the addition of acetic acid from the previous half for 30 minutes. Next, it was washed with 10 % glutaraldehyde and Aquades for 30 minutes. After washing, the gel was stained with AgNO3 for 15 minutes, then washed with aquades twice, each for 2 minutes. A color development solution was given comprising formaldehyde 3.7 %, zitronsauce 5 % and aquades. After the protein bands were seen, then the reaction was stopped by adding 10 % acetic acid. The resultant gel of the protein bands
was stored in a 10% glycerol solution, ready for documentation. Calculation of molecular weight was done by comparing the standard marker.

2.3.4. Inspection of zoanthinnium penaei infestation in shrimp
Observation of ectoparasite infestation was done natively using Jhonson’s method (1986) that is by doing scrapping on the entire surface of the shrimp body. The result of the scrap was placed on top of the glass of the object, given water and observed with a microscope with 100X magnification. Parasitic infestation was calculated with a positive shrimp percentage on the number of shrimp examined.

2.3.5. Total haemocyte count (THC)
Haemocytes were taken in the ventral portion of the second abdominal segment by using a 25 G needle and a mL syringe which had been inserted 0.2 milliliters of cold Alsever modified solution (AS 19.3 mM, 239.8 mM Na citrate, NaCl 182.5 glucose and 6.2 mM EDTA, pH 7.2) as an anticoagulant (Van de Braak, et al., 2000). The calculation of the number of haemocytes was done by the method of Van de Braak, et al. [17] using a bright microscope (LM) with 1000 magnification, then calculated by ZM model counter (Counter Electronic Ltd). The size of the hemosite particle ranged from 0.4 to 800 μm. As the supporting data, it can also be observed with an electron microscope (EM) with first centrifuge 700 X gravity at 40 C for five minutes.

2.3.6. Differential Haemocyte Count (DHC)
Blood was dripped on a glass of the object and a blood pillow with giemsa staining was made (Nash et al., 1993). Then, the cell type was identified. This hemositic differential was aimed at identifying the number, type and percentage of hemosite cells. The analyzed hemocytes were classified according to the instructions of Owens and O’Neill [25]. The amount of hemosite was calculated to 100 cells. The percentages of each species was identified, performed at the beginning of the study.

2.3.7. Vaname shrimp survival rate (SR) calculation
The survival rate (SR) was calculated by the percentage of shrimp that lived during the maintenance of the total shrimp kept, which was done at the end of maintenance, ie at harvest time of 90 days in pond. The formula used to measure according to Effendi [26] was:

\[
SR = \frac{\text{Total shrimp were alive}}{\text{Total shrimp stocked}} \times 100\% 
\]

Information: 
SR = Survival rate / Kelangsungan hidup (%) 
Nt = The number of shrimp lives at the end of the study 
No = The number of shrimp lived at the beginning of the study

2.4. Data Collection and Analysis
The data obtained were divided into two kinds, namely qualitative and quantitative data. The qualitative data were the data of isolation and molecular characterization, namely Spasmin Performin with SDS-PAGE. On the other hand, the quantitative data were protein data of vannamei shrimp on Zoanthinnium penaei at a certain time, including: the amount of hemosite (THC), differential hemosit (DHC), parasitic infestation and number of survival of vaname shrimp used by ANOVA test if there is difference followed by Duncan [27].
3. Results And Discussion

3.1. Infestation in Vaname shrimp

The results of the parasite infestation examination on vaname shrimp were presented in table 1. Meanwhile, the prawn image infested with Zoothamnium penaei was presented in figure 1.

![Figure 1](image-url)

**Figure 1.** Description of the Zoothamnium Penaei Infestation on Some Organs. A: Tail and B: Back (100x Magnified Microscope Lens).

The results of examination of samples in the form of immunized and un-immunized vaname shrimp showed positively infested by Zoothamnium penaei. Clinical symptoms of shrimp infested with this parasite are similar to the shrimp attacked by zoanthamniosis; the shrimp tail does not look like a fan if swimming, swimming on the surface and clustered. On the entire body surface and gills, there are parasites stick to the white brownish, empty digestive tract, the surface of the body and gills are muddy like moss. Moderate healthy shrimp (not attacked by zoanthamniosis) looks clear, transparent, clean and there is no color change on the entire surface of the body and gills, active swimming prawns and the tail opening like a fan. Zoothamnium identification results found align with the results of identification [28], which has a circular peristome surrounded by cilia. In the inside, there were a contractive vacuola and some feed vacuola, nucleus. It has a stem on the posterior part of the spasmonema. Vaname shrimp had already started to infest at the age of 30 days, both immunized and unimmunized, but the prevalence value indicated the value in unimmunized shrimp. The highest prevalence was found in non-immunized shrimp at the age of 90 days, on average 67.22 % and the lowest at the age of 90 days. The lowest occurred in 30-day immunized shrimp ie 5.55 %. The result of zoanthamniosis prevalence on vaname shrimp can be seen in table 1.

**Table 1. Zoothamnium Penaei infestation determination in vaname shrimp.**

| Age of Maintenance (Day) | Infestation Zoothamnium penaei Shrimp Vaname (%) |
|--------------------------|-------------------------------------------------|
|                          | Not Immunization | Immunization           |
| 30                       | 19.99±2.9829     | 5.55±1.7196            |
| 60                       | 38.20±1.9839     | 14.44±2.7189           |
| 90                       | 67.22±5.3395     | 15.18±8.6482           |

Different superscripts on different columns and rows shows a very real difference (p<0.01)

Anova results showed that the prevalence of zoanthamniosis in vaname shrimp immunized before being stocked was very significantly different (P <0.01), when compared to the prevalence of unimmunized shrimp (table 1).
3.2. **Immune response (THC and DHC) of vaname shrimp seeds**

The immune response of seed (PL 30) was not measured, because the seed was still too small and had difficulties in taking the blood sample. Table 2 shows the calculated total haemocyte count (THC) of vaname shrimp seeds that are not immunized and immunized at 30, 60 and 90 days on the pond maintenance, while the Differential Haemocyte Count (DHC) count can be seen in table 3. The results of anava showed that there was a significant difference between THC in immunized and unimmunized vaname shrimp (p <0.01). The highest THC was found in 60-day immunized shrimp, 54.08 x 106 cells/mL, while the lowest was in 30-day shrimp, 25.27 x 106 cells/mL.

| Age of maintenance (Day) | Total Haemocyte Count (THC) (10^6 Sel/mL) Shrimp | Not Immunization | Immunization |
|--------------------------|-----------------------------------------------|-----------------|-------------|
| 30                       | 25.27±0.4001                                  | 39.14±0.2984    |
| 60                       | 26.95±0.3345                                  | 54.08±1.1259    |
| 90                       | 27.08±0.4334                                  | 41.55±3.7776    |

Different superscripts on different columns and rows shows a very real difference (p<0.01)

Immunization of immunogenic membrane protein *Zoothamnium penaei* gave different effects on vaname shrimp immune response (DHC). There was a very significant effect difference between treatments (p<0.01).

| Age of Maintenance (Day) | Differential Haemocyte Count (DHC) (%) Shrimp | Not Immunization | Immunization |
|--------------------------|-----------------------------------------------|-----------------|-------------|
| 30                       | 13.09±0.2256                                  | 15.73±0.4658    |
| 60                       | 14.20±0.1989                                  | 17.20±0.1150    |
| 90                       | 12.99±0.2267                                  | 24.03±0.3135    |

Different superscripts on different columns and rows shows a very real difference (p<0.01).

Table 3 also shows that the highest DHC occurred in 90-day immunized vaname shrimp, while the lowest was found in unimmunized shrimp at 90 days, i.e. 24.03 % and 12.99 %.

3.3. **Survival rate (SR) of vaname shrimp**

The result of determining the survival rate of vaname shrimp can be seen in table 4.

| Treatment       | SR (%) |
|-----------------|--------|
| Not Immunization| 17     |
| Immunization    | 68     |

The survived vaname shrimp immunized before being stocked and maintained in ponds reached 68%, higher than the unimmunized which was only 17% at the end of the maintenance period of 90 days.
3.4. Water quality

The water quality check results for 90 days of maintenance can be seen in Table 5.

| Parameter          | Average Parameters / Water Quality During Shrimp Maintenance | Range Normal Value |
|--------------------|-------------------------------------------------------------|--------------------|
| Suhu (°C)          | 27 – 29                                                     | 27 – 32            |
| Salinitas (%/oo)   | 21 – 24                                                     | 16 – 30            |
| pH                 | 7.6 – 8.8                                                   | 7.5 – 8.5          |
| Disolvet Oxygen (ppm) | 3.4 – 6.2                                                 | >3 – 7             |
| Amoniak (ppm)      | 11 -13                                                      | <15                |

Table 5 demonstrates that pond water quality during 90 days of vaname shrimp using average immunostimulant was still within the normal range. So, it is in accordance with vaname shrimp maintenance requirements.

The results showed that immunization with immunogenic membrane proteins *Zoothamnium penaei* on shrimp before stocking could decrease the prevalence of zoothamniosis and increase shrimp survival rate as well as increase immune response of THC and DHC. Table 1 shows that *Zoothamnium penaei* infestation had begun to be found in 30-day-old shrimp whether immunized or not immunized. The prevalence of non-immunized vaname shrimp always increases with the increasing maintenance period, where the lowest prevalence occurred in shrimp with a 30-day maintenance period of 19.99 % followed by maintenance period of 60 and 90 days, with prevalence values of 38.20 % and 67.22 %. In vaname shrimp immunized with immunogenic membrane protein *Zoothamnium penaei*, which showed that prevalence increased during the 60 days maintenance period from 5.55 % to 14.44 % and 15.18 % on day 90 (end of maintenance).

Immune responses (THC and DHC) of vaname shrimp immunized with immunogenic membrane proteins *Zoothamnium penaei* were increased and from day 30 maintenance from 25.27 x 106 se / mL to 39.14 x 106 se / mL. While at shrimp aged 60 days THC shrimp increased from 26.95 x 106 se / mL to 54.08 x 106 se / mL and at age 90 day increase from 27.08 x 106 se / mL become 41.55 x 106 se / mL. When viewed as a whole that the immature vaname shrimp always increased during the 90 days maintenance (harvest). Meanwhile, in the non-immunized shrimp THC increased up to 60 days maintenance, however there was a decrease on day 90 with not much different value, although the result of anava was different (Table 2). Table 2 also shows that immunized shrimp THC increased up to day 60 and decreased on day 90.

The immunized DHC shrimp vaccine (Table 3) also increased from 13.09 % to 15.73 % in 30-day shrimp, and from 14.20 % to 17.20 % in shrimp aged 60 days and from 13.99 % to 24.03 % at age 90 days. Table 3 also shows that immunized vaname DHC shrimp also increased during maintenance ie 15.73 %, 17.20 % and 24.03 % at 30, 60 and 90 days day, while those not immunized ranged from 13 %.

The ability of immunogenic membrane proteins *Zoothamnium penaei* as immunostimulant could also be seen from the survival rate of vaname shrimp immunized before being dispersed higher when compared with that not immunized, i.e. between 68 % and 17 %. This could mean that the immunostilant was able to provide protection on shrimp that are maintained in the pond, especially against zoothamniosis. The immunostimulants that entered the body of the shrimp would stimulate the haemocytes activity cells in shrimp, attempting to fight the pathogens entered the body of the shrimp during maintenance. This is in accordance with Van de Braak [17], which stated that hemocyte-activated immunostimulant cells would perform phagocytic activity on shrimp by hyalin (granular) and semi granular cells.
According to Soderhall and Cerenius [18], the immune system in shrimp was still primitive and unlike that in fish and mammals that has immunoglobulins. So, immunoglobulin in shrimp was replaced by Prophenoloxidase Activating Enzyme (PPA). The PPA is a protein located in granular haemocytes. This PPA can be activated by lipopolysaccharides and β 1.3-Glucan, which will stimulate prophenoloxidase into phenoloxidase. As the result of the activation, it will then produce a Opsonin Factor protein that can induce hyalin cells to perform the process of phagocytosis. Van de Braak [17] and Rukyani [9], also supported the statement above that the haemolytic cells will degranulate, and some proteins would be released for immune response, such as increased haemocytes, increased trapping activity, and phagocytosis. In addition, the immunogenic membrane proteins would stimulate haemocytes to release proPO and protein-binding PPA, thereby causing hemocyte cells to increase their activity for trapping and phagocytosis against disease agents, which related in this case was the Zoothamnium penaei. It is evident that the prevalence of zoothamniosis in immunized and different immunized shrimp was very marked with the prevalence of unimunized shrimp. The presence of infested shrimp Zoothamnium penaei, because this parasite was opportunistic, so that in normal water condition, this parasite still grows but it develops long, which does not cause disease in the shrimp.

If the immunostimulant goes into the body of the shrimp then it will cause an increase in the number of haemocytes (THC) and haemocyte cell differential (DHC). This is an indication of increased shrimp body defense against zoothamniosis attacks [8]. Furthermore Itami et al. [16] supports the basic theory, which says that vaccine administration can prevent infectious diseases in the host body and lead to increased phagocyte activity of haemocytes and proPO enzymes. This statement is reinforced by Soderhall et al. [30] and Van de Braak [17], that membrane proteins entering the shrimp body will induce antibodies capable of neutralizing Zoothamnium penaei infestation, thus unable to infest the shrimp.

According to Mahasri [8], the immunogenic membrane proteins which entered the body could increase the tiger shrimp survival rate from 17 % to 68 % in 90 days old shrimp (the end of maintenance). Furthermore, it was also said that the immune response of tiger shrimp also increased as indicated by the increase of THC and DHC, because immunogenic membrane protein Zoothamnium penaei had a large molecular weight that was greater than 1000 Dalton, thus making it immunogenic. Proteins that had high molecular weight and had a high level of immunogenity, the protein must have a complex structure. In the opinion of Tizard [31] and Baratawidjaja [32], proteins that were immunogenic had a large molecular weight of more than 1000 Dalton and had a complex structure.

Increased total haemocyte (THC) and DHC cells could be used as the indicators or signs of pathogen infection in the host body. This infection would cause inflammation, which was a non-specific body defense characteristic due to influencing factors such as parasites, bacteria, fungi, viruses and nonliving agents [11, 18]. The results showed that there was an increase in THC and DHC in shrimp immunized with membrane proteins and different from those not immunized. THC in immunized shrimp increases in shrimp from 30 days to 60 days, but decreases at age 90 days. This was because the shrimp immune system would increase along with the increasing shrimp life and but at certain age would decrease again. The high THC indicated that immunogenic membrane proteins could improve the shrimp immune response, because high THC in shrimp is one indicator of increased shrimp body resistance. This is in accordance with the opinion of Soderhall et al. [30], who suggested that increased immune responses to invertebrates were indicated by an increase in THC. Increased THC and DHC could be used as the indication of shrimp body defense reaction with Z. Penaei infestation. The differences between THC and DHC among shrimp treatment groups in this protein-protective testing test, probably due to the immunogenicity of different membrane proteins. According to Baratawidjaja [32], protein immunogenity was determined by large molecular weight.

The results also showed that there was a significant difference (p <0.05) between THC and DHC shrimp immunized with immunogenic membrane proteins at different doses and infested with the same zooid Zoothamnium penaei. The main factor causing this difference is the immunogenity of the membrane proteins as immunostimulants. There is a significant increase in DHC (granular haemocytes), presumably because shrimps did not have memory cells in the immune system, which
were unable to detect pathogens that had been exposed. Thus, it can be argued that immunostimulant membrane proteins could induce the defense mechanism of the shrimp body. However, it took time to stimulate the haematopoietic organ to produce granulocytes to counteract zoothamniosis attacks [8]. These granulocytes would then destroy pathogens by swallowing the pathogens, so these granulocyte cells would migrate to parasitic infestation areas. On the other hand, the water quality maintenance also affected the infestation and immune response in the shrimp, but based on the results of research, it showed that the water quality was in the optimal conditions for shrimp life during maintenance.

4. Conclusion
The conclusions that can be drawn from the results of this study: 1) Immunization with immunostimulant from membrane Z. penaei protein could decrease Zoothamnium penaei infestation on vaname shrimp maintained in pond from 68% to 17% during 90 days of maintenance period; 2) Immunization with immunostimulant of immunogenic membrane proteins Z. penaei was able to increase the immune responses (increase THC and DHC) in the vaname shrimp, from 27.08 x 10^6 cells/mL to 41.55 x 10^6 cells/mL for THC and 12.99 % to 24.03 % for DHC; and 3) Immunization with immunostimulant from immunogenic membrane protein Z. penaei was able to increase the survival rate of vaname shrimp from 17% to 68%.

5. References
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