Total plate count and identification of vibrio in pacific white shrimp (*Litophenaeus vannamei*) from ponds and in those exposed to immunogenic protein membrane *Zoothamnium penaei*

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Abstract. In shrimp culture, disease is easily caused by the vibrio bacteria. One of the solutions to overcoming this problem is by using an immunogenic protein membrane from *Zoothamnium penaei*. The purpose of this study was to identify the bacteria *Vibrio* sp. and to determine the Total Plate Count (TPC) of the bacteria before and after being exposed to the immunogenic protein membrane *Zootamnium penaei*. This research is expected to resolve the problem of shrimp culture caused by disease. The method of this research was an experimental method; there was a group of shrimp that went without treatment (control) and two treatment groups of shrimp were exposed to an immunogenic protein membrane *Zoothamnium penaei* concentration of 3ppm and 5ppm. The vanamei shrimp samples were taken from their original habitat of ponds in Lamongan, East Java. The parameters in this study were the identification of conventional bacteria, the Total Plate Count (TPC) and the Survival Rate (SR). The research was held at the Wet Laboratory in the Faculty of Fisheries and Marine, Airlangga University. The results without treatment (control) showed that the bacterium found was Vibrio sp. with a TPC of 3 x 103 CFU/gr. After being exposed to the immunogenic protein membrane for 7 days, there was seen to be the presence of bacterial *vibrio sp.* The TPC count was lower than before 1 x 105 CFU/gr for the 3 ppm doses and 1 x 104 CFU/ gr for the 5ppm doses. The Survival Rate after seven days of treatment was 80%.

1. Introduction

The pacific white shrimp was officially introduced to Indonesian cultivators in 2001 after the decline in the production of tiger shrimp (*Penaeus monodon*) due to various problems such the production process, both technical and non-technical [1]. The species is relatively easy to breed and cultivate, therefore pacific white shrimp are one of the mainstay species in shrimp cultivation in several countries of the world. Shrimp production is known as the main economic source in the aquaculture sector, contributing to high-value protein in human nutrition. The pacific white shrimp (*Litopenaeus vannamei*) has become an economically important aquaculture species in Latin America and Southeast Asia, more specifically in India, China, Thailand, Indonesia and Philippines. However, inappropriate environmental conditions in shrimp farming including high density stocking and the mismanagement of daily operations can deteriorate yields in shrimp aquaculture [2].

In line with the number of enthusiasts for shrimp farming, there are also disturbing problems present, which includes the development of the cultivation businesses, namely fish diseases. One of the most common pathogenic organisms is the *Zoothamnium penaei* ectoparasite. This parasite can cause death in the seed stage by up to 86%. The occurrence of zoothamniosis in shrimp in Indonesia, both at the time of hatchery and during cultivation in ponds, still shows high numbers, so it needs serious attention [3]. According to [15], the incidence rate of zoothamniosis on the north and south coasts of West Java has reached 85%.

There are also many bacteria to be found in the context of shrimp famring, one of which is the presence of vibrio bacterial. The entry of vibrio pathogens into shrimp farming can be due to the sea water and the fries used. [4] reported that the parent shrimp originated from positive seawater and carried fluorescent bacteria, which could spread to the fries (larvae) and eventually enter the pond. If these conditions are not immediately addressed early on, shrimp farming activities will be disrupted. As a result, shrimp production will decline due to the high mortality rates. According to [5], *Vibrio* sp. is recognized as a human pathogen. It has been also reported as the cause of acute hepatopancreatic necrosis disease (AHPND) which affects multiple shrimp species, such as L. *vannamei* and P. *monodon*. The disease was first reported in 2009 in China and since then has been detected in Malaysia, Thailand, the Philippines and also in Mexico, generating important economic losses [6].

One effort to prevent and overcome *Vibrio* sp. is by using immunostimulants. [7] states that efforts to improve the resistance of the shrimp's body, both in the hatchery and maintenance stages of the pond, can be done using immunostimulants, but up until now, it has been said not to be successful in the target population. [8] stated that the cellular and humoral immune systems in shrimp function synergistically to protect the shrimp and to eliminate any pathogens that enter the body of the shrimp. Laboratory prevention efforts against zoothamniosis in tiger shrimp using the immunostimulant immunogenic protein membrane of *Zoothamnium penaei* have been carried out by [9]. The results reported indicate that the immunostimulants can improve the bodily defenses of the tiger shrimp and can improve the survival of the tiger shrimp by up to 86%. So, in this study, the researchers tried to give the treatment to pacific white shrimp. This study aims to determine the relationship between the vanamei shrimp infected with *Vibrio* sp. treated using the immunostimulatory immunogenic protein membranes of *Zoothamnium penaei* and the number of bacterial colonies (Total Plate Count) after seven days of treatment.

2. Material and methods

2.1. Sample collection animal maintenance and challenge test

The *L. vannamei* (weight 10 g, length 10-12 cm, as many 75 shrimp) shrimp were obtained from Instalasi Budidaya Air Payau (IBAP) Lamongan. Each container of 20 L of water was filled with as many as 15 shrimp, brackish water with 18 ppt (habitat original) and aeration. This research was conducted from April to June 2018 in the Wet Laboratory of the Faculty of Fisheries and Marine, Airlangga University. The shrimp were fed as much as 2% of their body weight. The Total Plate Count (TPC) testing was carried out at Balai Karantina Ikan (BKI) Juanda.

2.2. Preparation of the tools and materials

The tools used included 5 containers able to hold 20 L, 2 large plastic tubs, nets, filters, and digital scales and water quality measuring devices (pH pen, thermometer, DO meter, ammonia test kit). There was also the TPC tools (electric autoclave, thermometer gun, pH meter, plastic gloves, spatula or spoon, sterile plastic, stomacher, petri dish, volume pipette, test tube, incubator, colony counter, schootduran bottle, digital scales, and laminar air flow) and the immunostimulatory device (syringe 1 ml). The material used consisted of pacific white shrimp (*L. vannamei*), aquades, BPW (Buffered Pepton Water), PCA (Plate Count Agar), the TSA media (Trypticase Soy Agar), 2.5% NaCl (Sodium Chloride), the TSB medium (Tryptic Soy Broth), the TCBS (Thiosulfate Citrate Bile Salts Sucrose) medium, the GSP (Glutamate Starch Phenol) medium, and 70% alcohol. The preparation of the research began by cleaning the maintenance tub. The tub was then made sterile using chlorine, soaking it for 24 hours and rinsing it with water until it was clean. After drying, the maintenance tub was filled with pond water suitable

for the shrimp habitat. To maintain the oxygen demand for shrimp, each maintenance tank was given aeration with high oxygen levels. The vanamei shrimp were acclimatized first, and then divided equally between each maintenance tank, up to as many as 15 shrimp.

2.3. Immunostimulant treatment

The immunostimulant consisting of the immunogenic protein membrane *Zoothamnium penaei* was carried out after the 7^{th} and 8^{th} day, and then the shrimp were tested after treatment on day 14. The administration of the immunostimulants was given in different treatments; P1 = 100% (Control) artificial feed and P = immunostimulant administration (immunogenic protein membrane *Zoothamnium penaei* with 3ppm and 5 ppm doses respectively).

2.4. Identification of the ectoparasites – Zoothamnium penaei

The identification of the ectoparasites in the pacific white shrimp begins with taking the parts of the body of the shrimp that best allow for the identification of the presence of *Zoothamnium penaei*. The parts of the body include the gills, swimming legs and feet. The parts were taken and then cut into small pieces, before being added to a little water. The prepared body part was placed on the glass object and covered with a cover glass. The preparations were then observed using a binocular microscope. Observations came in the form of parasitic acid and calculating the number of parasites in each part of the body.

2.5.TPC of Vibrio sp.

2.5.1. Observation - external examination

Observations were made of the symptoms that arose on the outside of the body. The signs or symptoms of illness that can be seen vary greatly, depending on the type or intensity of the disease.

2.5.2. Observation - internal examination

The examination of the internal symptoms was done by dissecting (sectio) the goldfish and observing the symptoms that were present in the internal organs such as the gills, digestive devices, liver, and kidneys.

2.5.3. Isolation, purification, and examination of the bacteria

The isolation of bacteria from the samples was carried out from the gill organs, kidney, liver, and digestive devices using sterile oil on the TSA medium with the addition of 2.5% NaCl, GSP 2.5% NaCl and TCBS media. Incubation was conducted at room temperature for 18 - 24 hours. Bacterial purification was done by separating out the bacteria that have different colony morphologies. The colony morphological observations included colony shape, elevation, shape, edge and colony color. The observation of the bacterial colonies also included the form of the bacterial cells and Gram staining. The pure bacterial isolates were then stored in a slanted agar and given sterile liquid paraffin. The examination of the vibrio pathogenic bacteria that cause disease in pacific white shrimp examined the colony morphology, bacterial cell morphology and biochemical testing**.**

2.5.4. Total plate count

The procedure involved in the Total plate count of the *Vibrio* sp. included preparing the tools and materials to be used. The work table and researcher's hands were sprayed with alcohol up to 80%, before wrapping the petri dish, test tube and measuring pipette with opaque paper (each test tube was filled with 9 mL Buffered Peptone Water (BPW) and the test tube hole was covered with cotton wool). The Potatoes Dextrose Agar (PDA) was used as a medium. All of the tools were sterilized after they had been wrapped in opaque paper in the autoclave for 15 minutes at 121˚C. We then cooled the tools and materials that had been sterilized. To perform the dilution, the sample was put into a test tube containing 9 mL of sterile BPW (10^1) aseptically, before shaking it 25 times using the vortex.

Performing the next dilution required 1 mL from the test tube of 10^1, before placing it into a dilution test 10^2 tube. This was followed by vortexing and piping 1 mL into the petri dish aseptically. The next dilution was performed until the dilution reached 10^5. The filled petri dish was shaken. The media was poured into each dish. The petri dish was put into the incubator in an upside-down position at a temperature of 35 ± 1˚C for 24 - 48 hours. We recorded the growth of the colonies in each cup containing 25 - 250 colonies to determine the shape using the colony counter.

2.6. Survival rate

The survival rate is a supporting parameter used to analyze the response of the pacific white shrimp to each treatment. The level of survival was expressed as a percentage of the number of shrimps that lived up to the 7th day after the experimental treatment of the total number of shrimp that were kept. The survival rate was calculated using the following formula:

$$SR = \frac{Nt}{N} \text{ x } 100\%$$

Information:
SR = Survival Rate (level of survival)
Nt = Number of shrimps that lived at the end of observation (shrimp)
No = Number of shrimps that were alive at the beginning of the challenge test (shrimp)

2.7. Water quality

The measurement of the water quality (temperature, pH, salinity, dissolved oxygen (DO) was conducted every day in the morning, afternoon, evening and night. This supporting parameter data was used as complementary data to the main parameters.

3. Results and discussion

3.1. Pathogens and TPC of Vibrio sp. *on pacific white shrimp*

The pathogens that were identified in the pacific white shrimp were before (day 0) and after treatment (day 7). The pathogens examined included *Zoothamnium penaei* ectoparasites and *Vibrio* sp. The bacterial examination conducted looked at the colony morphology, bacterial cell morphology and biochemical testing. The parasitic examination was conducted, and then we measured its intensity. The results of the Total Plate Count of *Vibrio* sp. in the shrimp have been shown in Table 1.

Table 1. Number of TPC *Vibrio* sp. in the pacific white shrimp.

Treatment	Bacterial colony (CFU/g)
P0 (day 0)	$3x10^3$
P2(3) (day 7)	$1x10^5$
P2(5) (day 7)	$1x10^4$

Table 1 showed that the results on the day before the treatment and after the treatment for the results of TPC *Vibrio* sp. was 3 x 10^3. After seven days, the results of the TPC of the *Vibrio* sp. was 1 x 10^5 for the treatment with immunostimulants in the 3 ppm dose and 1 x 10^4 for the treatment of immunostimulants in the 5 ppm dose. These results indicate that the number of bacterial colonies decreased after being given the immunostimulatory treatment for 7 days. In the treatment of P2 (5), the decrease in the bacterial colonies was much more than in the P2 (3) treatment. Good immunostimulants must pay attention to the optimal dose and frequency of the treatment. According to [10], high doses of immunostimulants can suppress the shrimp's defense mechanisms, while low doses are less effective at providing an immune response.

Vibrio sp. is a gram-negative bacterium, motile, positive oxidase in the form of single bent or straight short stem cells. It measures 1.4 - 5.0 μm long and 0.3 - 1.3 μm wide, is fermentative in glucose, fluorescent and has flagella on one of the poles. It does not form acid from glucose and can use sucrose

as an energy source [14]. According to [11], *Vibrio* sp. are found in almost all habitats, like fresh water, seawater, estuary and soil; it is a disease-causing agent in fish, humans and crustaceans.

The immunogenic protein membranes from *Zoothamnium penaei* were able to suppress the attack from the pathogens derived from *vibrio* sp. This can be seen in the lowest decrease in the number of bacteria before being treated with after being treated. The frequency and treatment of the sustained immunostimulants was needed to provide more of an immune ability in order to achieve optimal protection [12]. Immunogenic proteins are proteins that have a molecular weight of 20,000 - 100,000 Dalton. Most good immunogens have a molecular size <100 Kilo Dalton, while proteins with molecular sizes <5-10 Kilo Dalton are poor immunogens [13]. The mechanism of the action of the immunostimulants began when the immunostimulants entered the body. The immunostimulants stimulate the monocytes to produce cytokines such as interleukin, which will activate the lymphocytes which then divide into B lymphocytes and T lymphocytes. T lymphocytes in non-specific responses will produce interferons that are able to activate the performance of the macrophages through the mechanism of phagocytosis in the face of parasites, bacteria, viruses, and foreign particles that are considered to be antigens [7].

3.2. Survival rate

The results of the calculation of the survival/ SR of the vanamei shrimp (*L. vannamei*) over the 7 days of observation showed the survival rate of the control treatment P0 (6.6%), after the administration of immunostimulant whole protein membrane; for P2 (3) it was 93.3% and for P2 (5), it was up to 80% higher than the treatment P0 (6.6%). Low SR before the treatment can be caused by the shrimp being stressed due to the displacement of their place from the pond into the reservoir, unstable environmental conditions and due to the high number of bacterial colonies. The results showed that the treatment of the protein membrane immunostimulants was able to boost the high SR in the pacific white shrimp.

3.3. Water quality

The results of the water quality measurements for the 7-day maintenance of the vanamei shrimp on average was still in the normal condition range, with an average temperature of 28 - 29ºC, a salinity of 16 - 18 ppt, a pH of 7.6 - 8.2, dissolved oxygen of 4.6 - 7, 3 mg/ L and an ammonia level of 0.03 - 0.8 mg/ L. According to [9], water quality that is in a good condition will cause opportunistic pathogens to not cause pain in the shrimp. Therefore, the shrimp will be in good health and the body's defense system will be maintained. Maintaining the quality of the water will cause the shrimp to live well and minimize the appearance of disease attacks. The shrimp will therefore grow well.

4. Conclusion

The conclusions that can be submitted from the results of this study are as follows: the provision of whole protein membrane ectoparasite immunostimulants like *Zoothamnium penaei* can suppress the growth of *Vibrio* sp bacterial colonies. The immunostimulant dose of 5 ppm was able to suppress the bacterial colony growth over the 7 days of treatment. The highest survival rate was in the P2 (3) treatment with the *Zoothamnium penaei* dose of immunostimulant whole protein membrane ectoparasite being 3 ppm.The water quality results showed that over the 7 days maintenance, the water quality condition was both stable and normal.

5. References

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