Identification of white spot syndrome virus (WSSV) in pacific white shrimps (*Litopenaeus vannamei*) from ponds post-exposure to immunogenic membrane proteins (*Zoothamnium penaei*)

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Abstract. White spot syndrome virus (WSSV) is a pathogen that causes white spot disease; it is a major cause of decreased Pacific White Shrimp (*Litopenaeus vannamei*) production in Indonesia. The aim of this study was to identify the presence of white spot syndrome virus (WSSV) in the Pacific White Shrimps cultivated in ponds as well as the effect of exposure treatment to the immunogenic membrane protein of *Zoothamnium penaei* on the presence of the virus. The initial identification of WSSV in the field was done using the Polymerase Chain Reaction (PCR) method. Further PCR identification results indicated that the shrimp post-exposed to the 3 ppm and 5 ppm immunogenic membrane protein from *Zoothamnium penaei* for 7 days resulted in the virus being undetected (negative). These results indicate that the presence of WSSV can be triggered by environmental factors and that the shrimp’s immune system decreases during maintenance in aquaculture ponds. The exposure to the immunogenic membrane protein of *Zoothamnium penaei* has positive results in relation to preventing WSSV infection, which means that it can be developed as an immunostimulant material in Pacific White Shrimp farming.

1. Introduction
White Spot Virus Syndrome (WSSV) is a pathogen that causes “White Spot Disease”. It is also one of the main causes of various diseases in Indonesia and several other shrimp-producing countries. WSSV infection in shrimp farming cumulatively can cause deaths up to 100%, which occurs in 3 - 10 days [1]. There was a significant decline in Pacific White Shrimp production that occurred in 2015 in the first quarter; the production fell from 75,100 tons to 72,046 tons in the second quarter, and then in the third quarter, it was only 63,349 tons [2]. The presence of WSSV in the seed and brood stock of shrimps has been reported in several countries in parts of Asia [3]. The brood stock of shrimp that have been infected by WSSV can cause serious problems. This is because the transmission of the virus can occur vertically, which causes it to infect the offspring of F1 [4], and transmission can also occur horizontally through water or through animals that have been infected [5].
The main target of WSSV infection is the ectodermal and mesodermal tissue, especially in the epithelial and subcutaneous connective tissue portion of the shrimp [6]. Other clinical symptoms of WSSV infection are characterized by white spots with a diameter of 0.5-2.0 mm in the carapace, a lethargic surface and a decreased appetite [7]. Up until now, an effective method for prevention in order to control the infection of the virus has still not been found. In general, viral infections can be controlled or prevented by vaccinations. However, for the vaccinations carried out in the Crustacean group including shrimp, it cannot be said to be successful because shrimp do not have a specific immune system as they are in the vertebrate water group. The bodily defense system possessed by shrimp includes encapsulation, phagocytosis and microbicides that works by producing the cytotoxic reactive oxygen that is involved in hemocytes [8]. Efforts made to promote an immunostimulatory approach in shrimp that can be used as an alternative method to controlling WSSV infection [9].

The development of immunostimulants through molecular techniques is increasingly being carried out because it is able to provide great opportunities to study the mechanism of the Pacific white shrimp’s immune system in relation to preventing pathogen infection. According to [10], they stated that approaching immunostimulants from the protein membrane immunogenic Zoothamnium penaei can increase the shrimp’s survival rate (SR) by 93%. Furthermore, the immunogenic protein membrane isolation was explained by SDS-PAGE, ELISA and the Western Blotting methods. [11] said that partial gene isolation related to encoding the structural protein VP26 WSSV in Pacific white shrimp was successfully carried out in order to provide preliminary information on preventing WSSV disease in vaname shrimp using RNAi technology. According to [12], the administration of the VP28 envelope protein from local White Spot Baculovirus (WSBV) isolates was able to increase the survival rate by 70% (SR), phenoxoydase enzyme activity by 44.6067 ± 0.03162 units / minute / milligram and the total hemocyte count by 193.89 ± 4.314x107 cells / mL in tiger shrimp (Penaeus monodon) infected with WSBV.

Analogous to the above problems, the diagnosis of the disease strategies in Pacific white shrimp culture with intensive systems in ponds is necessary in order to prevent transmission of pathogens to the cultivation system. Cases of viral pathogen infection in aquaculture ponds and Pacific white shrimp hatcheries in East Java need to be known in order to increase local fishery production. The purpose of this study was to identify the infection of White Spot Syndrome Virus (WSSV) using the Polymerase Chain Reaction (PCR) method in intensive ponds and to conduct prevention efforts with protein membrane exposure to Immunogenic Zoothamnium penaei in the laboratory.

2. Material and methods

2.1 Sampling location
Shrimp samples that were thought to be positive for WSSV were obtained from shrimp ponds in the area of Lamongan Regency, East Java. The shrimp samples were obtained from the ponds and then pre-screened using PCR to determine the presence of WSSV infection.

2.2 Container preparation and shrimp maintenance
Shrimp preparation and maintenance was carried out in the Fisheries Laboratory in the Faculty of Fisheries and Maritime Affairs, Universitas Airlangga, Surabaya. The shrimp samples were maintained in a maintenance tank consisting of a 60 x 30 x 35 cm³ reservoir and a shrimp rearing tank. The maintenance tanks and reservoirs were cleaned using 30 ppm chlorine, and then dried for 24 hours. The dried aquarium was then filled with water and aerated to one point per aquarium in order to provide an oxygen supply to the maintenance medium. The water used came directly from the ponds.

The test shrimps used in this study were 40 day old juvenile Pacific white shrimps from ponds in Lamongan Regency, East Java. Before the maintenance was conducted for 7 days and before they were given the immunostimulant exposure to the protein membrane from Zoothamnium penaei, the test shrimp were first acclimatized for 1 hour in an aquarium with 15 tails / container and fed until satiation with the frequency of feeding being 3 times a day. The water quality of the media was maintained by siphoning, every morning and evening, as much as 10% of the total body volume.
2.3 Preparation of the immunostimulant from the membrane protein of Zoothamnium penaei
The immunostimulant material used in this study was the immunostimulant material produced by [10]. This study was used to evaluate the ability of the immunostimulants in relation to WSSV infection control. According to [10], the stages of making the immunostimulatory materials included cultivation and the isolation of the Zoothamnium penaei immunogenic protein in vitro, followed by the characterization and purification of the immunogenic proteins through the SDS-PAGE method, the determination of the protein concentration and the testing of the immunostimulant material on pacific white shrimp (Litopenaeus vannamei) in vitro.

2.4 Test of immunogenic membrane protein zoothamnium penaei protection capability of in pacific white shrimp (Litopenaeus vannamei) age 40 days in vitro
The protective ability of the immunogenic membrane proteins that was found was then tested in vitro. The shrimp were divided into 3 containers to be immunized with the immunogenic membrane protein Zoothamnium penaei through being dipped for 15 minutes and then returned to the maintenance tank. The treatment was carried out with 2 different doses, namely 3 ppm/L and 5 ppm/L, in addition to the control.

The measurement of the protection capability was determined by the Survival Rate of up to 7 days. The water quality checks were carried out during the maintenance of the shrimp used for supporting the data. The survival rate was determined by the percentage of the shrimp that lived for the 7 days of maintenance compared to the total number of shrimp kept.

2.5 Confirm WSSV using polymerase chain reaction (PCR)
Viral identification was carried out using PCR method to evaluate the existence of WSSV virus in pacific white shrimp using the method developed by [7] by continuing to optimize according to the conditions. We primarily used WSSV-F 5 ‘ATT-GAA ACT GAA AAG GCT TTC CCT C-3’ WSSV-R 5 ‘-GTT CCT TAT TTA CTA CTA CGG CAA-3’. The required PCR concentration was a 1x PCR buffer containing MgCl2, 250 µM dNTPmix, 1.25 U Ex Taq Polymerase, each of 0.4 µM primers F and R, and 5 µL samples. The PCR reaction was carried out in a 0.2 mL tube with a total concentration made to 20 µL with the addition of distilled water. The conditions of the PCR were as follows: initial denaturation of 95 °C for 5 minutes, followed by 35 denaturation cycles of 95 °C for 30 seconds, annealing of 60 °C for 1 minute, an extension of 72 °C for 1 minute and with the final extension at 72 °C for 1 minute.

2.6 Statistical analysis
The data that was obtained was divided into two types, namely the qualitative data and the quantitative data. The qualitative data consisted of the results of the confirmation of the presence of WSSV through the Polymerase Chain Reaction (PCR) method before and after exposure to the protein membrane of Immunogenic Zoothamnium penaei. The quantitative data consisted of the results of the determination of the survival rate using ANOVA with three treatments.

3. Result and discussion
3.1 Determination of the survival rate (SR) of pacific white shrimp (Litopenaeus vannamei)
The determination of the Survival Rate in pacific white shrimp aimed to determine the best dose range from the immunostimulant protein membrane of Zoothamnium penaei used in this study on the presence of the WSSV virus. The results of seeking to determine the survival rate of pacific white shrimp can be seen in Table 1.

| Treatment               | Survival Rate (%) |
|-------------------------|-------------------|
| Not immunized (control) | 53.3 %            |
| Immunized 3 ppm         | 86.6 %            |
Based on Table 1, the survival rate of the pacific white shrimp at 40 days was maintained by the immunostimulant protein membrane with a dose of 3 ppm reached 86.6%, higher than the treatment with a 5 ppm dose of 60%. The lowest results were found in the non-immunized pacific white shrimp, which was 53.3%.

3.2 Confirm WSSV presence using polymerase chain reaction (PCR)

The confirmation of the existence of WSSV was done using the PCR method. The tests conducted on the shrimp before and after being kept for 7 days showed that the shrimp treated with immunostimulant protein membrane at a dose of 3 ppm and 5 ppm was not infected by WSSV after 7 days of maintenance (Figure 2). This was compared to the test shrimp before the WSSV infection was treated, indicated by the DNA band around 941 bp (Figure 1).

Figure 1. Confirmation of WSSV infection in the pacific white shrimp using the PCR method before treatment. 1. Marker; 2. Positive control of WSSV; 3. Negative control of WSSV; 4. Shrimp samples did not have DNA fragments at 941 bp; 5. Shrimp samples contained DNA fragments at 941 bp.

Figure 2. Confirmation of WSSV infection in pacific white shrimp using the PCR method after the administration of the immunostimulant protein membrane.
eliminary data state, he amplified DNA band at a length of 941 bp (Figure 1.). According to organic matter ording to Lightner (2011) - y shrimp. White d, clinical symptoms lity suggest that the fact that White Spot Disease infection is one of the main causes of crop failure to bacterial and viral infections. In addition, predisposing factors can also be one of the causes of super intensive). This can reduce the immune system of the shrimp so then the shrimp are susceptible to bacterial and viral infections. In addition, predisposing factors can be caused by infection from ectoparasites such as Zoothamnium penaei, which causes Zoothamniosis as described by [10]. Other studies suggest that the fact that White Spot Disease infection is one of the main causes of crop failure.

3.3 Water quality parameter
The results of the water quality inspection can be seen in Table 2.

| Parameter               | Average results of Water Quality | Normal Value Range |
|-------------------------|----------------------------------|--------------------|
| Temperature (°C)        | 27 – 29                          | 27 – 32            |
| Salinity (‰)           | 16 – 20                          | 16 – 30            |
| pH                     | 7.5 – 8.8                        | 7.5 – 8.5          |
| Dissolved oxygen (ppm) | 4.3 – 6.2                        | >3 – 7             |
| Ammonia (ppm)          | 11 – 15                          | <15                |

The results of the measurements conducted to determine the water quality during the study indicate that the water quality conditions are still in the normal range, although the ammonia parameter figures are close to 15 ppm which can be caused by the decomposition of feed residues (organic matter) containing protein and Pacific white shrimp excretion residue [13].

3.4 Discussion
The initial identification results using PCR showed that the shrimp samples obtained from the ponds in Lamongan Regency, East Java were positively infected by White Spot Syndrome Virus (WSSV). The confirmation and identification of the presence of the WSSV virus was carried out to prove the cause of shrimp death before and after administering the immunostimulant protein membrane. The results of the PCR examination on the shrimp samples before being treated were proven to be positively infected with WSSV as indicated by the amplified DNA band at a length of 941 bp (Figure 1.). According to [14], the use of PCR in the early detection of the presence of a virus or other pathogen is very helpful because PCR properties has a high sensitivity and is disease-specific. It also only requires a little of the virion (101 copies of DNA) to work. The preliminary data stated that continuous identification is needed in vaname shrimp farming ponds to determine the presence of pathogenic infections, including WSSV. Research on the prevalence of viral infections other than WSSV in vaname shrimp farms in Lamongan still needs to be done. This is due to the spread and development of viruses that require living cells from the host to metabolize and multiply themselves, which makes matters very difficult to determine empirically [15].

The clinical symptoms in the infected shrimp include discoloration, empty intestines, pale hepatopancreas and lesions with white spots, which are one of the particular characteristics of WSSV infection [16]. In addition, according to Lightner (2011), he stated that an acute infection of WSSV can be characterized by occlusions or white spots with a diameter of 0.5-2 mm generally found on the surface of the carapace. There is also a rapid decrease in appetite compared to healthy shrimp. White spots on the carapace are the result of the occurrence of deviations in calcium metabolism that accumulate in the epithelial layer of the shrimp [7].

The existence of WSSV in the shrimp samples obtained from Lamongan Regency, East Java, can be caused by environmental factors such as decreasing water quality and high stock density (intensive / super intensive). The results of the water quality inspection can be seen in Table 2.
in West Bengal, Sundarban, India, where crop failure is known to be caused by high levels of ammonium (TAN) nitrogen due to poor water quality [17]. This causes various kinds of infections such as bacteria and viruses.

One treatment that can be undertaken to prevent pathogenic infections such as WSSV is the use of immunostimulants. According to [18], immunostimulants are usually carried out by giving microbial components such as β-glucans and lipopolysaccharides or those derived from bacterial cells that have been killed. According to [10], the use of immunostimulants using proteins membrane immunogenic Zoothamnium penaei can be used as an alternative. The WSSV confirmation results after being given immunization showed that no DNA bands were found in the shrimp samples at 3 ppm or 5 ppm doses (Figure 2). It can be suspected that there is an increase in the shrimp’s bodily defense system due to exposure to the protein membrane given through the dipping. Another factor is an influence is the presence of active ingredients contained in the immunostimulant which is able to improve the defense system, inhibit viral transcription and reduce the replication process in the host cells, increasing the nonspecific level of immunity in the body of the shrimp [19].

Table 1 showed the highest survival rate (SR) of the shrimp as being associated with the 3 ppm dose treatment, which was 86.6%. This can be caused by the active compound found in the immunostimulant protein membrane Zoothamnium penaei. The determination of the best dose is needed in the development of immunostimulants. When compared with a 5 ppm dose of 60%, this is lower than the survival rate at 3 ppm. This is not in line with the statement from [20], which states that high doses of immunostimulants can suppress the defense mechanism while low doses are less effective for increasing the immune response. In addition, according to [21], proteins that have immunogenic properties have molecular weights greater than 1000 Dalton and have complex structures. Furthermore, [22] states that aromatic amino acids have high antigenicity compared to other types of amino acids.

Based on the results of this preliminary research, further research is needed on the effect of the immunostimulant protein membrane of Zoothamnium penaei on the pacific white shrimp immunity system. Keep in mind that shrimp only have a nonspecific immune system, which means that they do not have the ability to produce antibodies to preventing pathogens entering the body, so the role of hemocytes is very important as a cellular immune response which includes phagocytosis, melanization, encapsulation, cytotoxins and inter-communication cells [23].

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