

## Effect of Maintenance at Different Salinity against White Spot Syndrome Virus (WSSV) Infection Level in Post Larvae *Litopenaeus vannamei* Shrimp

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### Abstract

White spot syndrome virus (WSSV) is a highly contagious disease in shrimp culture and causing 100% death within 3-10 days of clinical symptoms. WSSV can infect shrimp in post larvae stage (PL) to a size of 40 g. This study was aimed to determine the effect of different salinity against WSSV infection level in post larvae vannamei shrimp (*Litopenaeus vannamei*). In this study PL 40 size of vannamei shrimp infected with WSSV in virus concentration 20  $\mu\text{g}.\text{mL}^{-1}$  in three different salinity is 5 ppt, 15 ppt, 25 ppt for 4 hours using the soaking method then maintained for 7 days post-infection and then infection level and water quality observed. Data analysis used simple linear regression and F test with a confidence level 95% ( $P < 0.05$ ). The results indicate changes in behavior between healthy and infected shrimp in three levels (mild, moderate, severe). The highest total mild infection in salinity treatment of 25 ppt by 30%, the highest total medium infection at salinity treatment 25 ppt by 37%, the highest total severe infection at salinity treatment 5 ppt by 57%. Shrimp that have mild level of infection are increasing in concomitant higher salinity, while the severe infection level has decreases with increasing salinity.

**Keywords:** Infection Level, *Litopenaeus vannamei*, Post Larvae, Salinity, WSSV

### INTRODUCTION

Vannamei shrimp demand is very large, both local and international markets. It has any advantage like high nutrition and high economic value [1]. Vannamei shrimp can growth rapidly, can be cultured with high density, relatively high market prices [2] and shorter maintenance time which is about 90-100 days for each cycle [3].

However, the level of vannamei shrimp farms productivity became uncertain, even failed [4]. In culture, there are various problem, one of which is the disease. Vannamei shrimp are very susceptible to disease caused by parasites, fungi, protozoa, bacteria and viruses. As well as in black tiger shrimp, a disease caused by a virus is also a major problem in the vannamei shrimp culture [5].

White spot syndrome virus infection mechanism initially are intracytoplasmic, entry into host cells and then at a higher infection rate of viral DNA into the host DNA and take over the process of transcription and translation processes to the corresponding viral DNA [6].

There is sufficient evidence to support that environmental changes caused the modification of the immune system of shrimp that cause increased susceptibility to disease agents [7]. It has been reported also that manipulate environmental factors can cause the shrimp have been infected with WSSV to survive during the period of culture takes place [8]. Salinity is the most important environmental variables that can directly affect the physiology and ecology of shrimp farming. Significantly decreased salinity can cause stress on the shrimp, making shrimp susceptible to disease such as WSSV [9]. At salinity of 30 ppt, WSSV infection in black tiger shrimp is lower than the salinity of 10 ppt, 15 ppt, 20 ppt and 25 ppt [10].

The effect of salinity on WSSV infection level have not been studied, particularly in vannamei shrimp culture in Indonesia. Thus it is necessary to study the effects of salinity differences on the level of WSSV infection in vannamei shrimp.

### MATERIALS AND METHODS

The research activities were carried out in January - March 2015. Laboratory analysis used laboratory of Aquatic Sciences, Faculty of Fisheries and Marine Sciences, University of Brawijaya and Central Laboratory of Life Science, University of Brawijaya, Malang.

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This study consisted of two variables: the independent variables, i.e. seawater different salinity and the dependent variable, i.e. clinical symptoms and the infection level.

#### Preparation and Sea Water Sterilization

Sea water used in this study has taken from Sendang Biru beach, Malang. The sea water stored in large tub with volume 100 liters. Before the sea water used as culture media, sterilized using  $\text{Ca}(\text{ClO})_2$  1  $\text{mL}\cdot\text{L}^{-1}$ , and  $\text{Na}_2\text{S}_2\text{O}_3$  1  $\text{mL}\cdot\text{L}^{-1}$ , 2 drops of chlorine and give strong aeration.

#### Containers Sterilization

Disinfection of jar that used in maintenance process, WSSV infections process and observation using  $\text{KMnO}_4$  10 ppm for 24 hours, then all the equipment that has been disinfected flushed with sterile water and dried.

#### Virus Preparation

The procedure of making virus inoculum was as follows [11]. Total of 1 grams of WSSV infected shrimp crushed with mortar. Then suspended in 9 ml sterile sea water. Suspension of organ centrifuged at 3,000 rpm at 4°C for 20 minutes, then centrifuged at 8,000 rpm for 30 minutes at 4°C. Supernatant filtered by using filter milipore 0.45  $\mu\text{m}$  and obtained a suspension of the virus by concentration 10% (w/v) which is equivalent to the concentration of 20  $\text{mg}\cdot\text{mL}^{-1}$  virus.

#### Virus Dilution

The procedure of making virus dilution following a method from Supriatna [12]. Total of 20  $\mu\text{g}\cdot\text{mL}^{-1}$  virus was obtained by preparing dilution tubes. First tube contained 1 mL solution of the virus with concentration 20  $\text{mg}\cdot\text{mL}^{-1}$  and added 9 ml sea water and homogenized then the tube shaken. Total of 10 mL of the 2  $\text{mg}\cdot\text{mL}^{-1}$  were taken into second tube and 90 mL sterile sea water were added. Last, 100 mL of the 0.2  $\text{mg}\cdot\text{mL}^{-1}$  were taken and 900 mL sterile sea water was added. Total 1,000 mL viruses 0.02  $\text{mg}\cdot\text{mL}^{-1}$  used for infection test.

#### WSSV infection test

After obtained 20  $\mu\text{g}\cdot\text{mL}^{-1}$  virus concentration and then it was put in a jar that had contained 2 liters of sterile sea water and aerated with a given density of 63 shrimps on each jar. Shrimp put into the jar with 3 different salinity (5 ppt, 15 ppt, 25 ppt) treatments with 3 repetition.

#### Post-Infection Maintenance

Shrimp that have been infected was cultured in container with aeration. Each jar contained 21 infected shrimps and maintained post-infection shrimp for seven days. During the maintenance, shrimp fed with pellet powder with the frequency of feeding 3 times a day. Control was not infected with WSSV and given the optimal salinity level. Water quality and clinical symptoms that arise due to WSSV infection has been observed during maintenance.

#### Clinical Symptoms Observation

Salinity changes can cause changes in haemolymph metabolism during the process of viral infection. Thus the role of immuno-competence can be reduced and the vulnerability of shrimp to other pathogens increases [13].

Shrimp in this study grouped according to the level of infection which based on morphology and behavioral changes of vannamei shrimp, i.e. mild, moderate, and severe infections [14]. Explanation of the category described as follows (Table 1).

Table 1. WSSV Infection Level of Vannamei Shrimp

Infection Level	Morphology
Mild	<ul style="list-style-type: none"><li>• No visible changes to discoloration</li><li>• Abnormal behaviour</li></ul>
Moderate	<ul style="list-style-type: none"><li>• Change of body color</li><li>• White spots occurred (1-3) on the carapace</li><li>• Broken tail</li></ul>
Severe	<ul style="list-style-type: none"><li>• White spots spread on the body</li><li>• Body and tail color changes into red</li><li>• Broken tail</li><li>• Broken antenna</li><li>• Damaged eye</li></ul>

#### Water Quality Measurement

Water quality measurement conducted during the research. The parameters that observed was dissolved oxygen (DO), salinity, pH and temperature. Measurement performed on each experiment jar about three times a day, at 09.00, 14.00, 20.00 WIB. Salinity measured using refractometer, while temperature and DO was measured using DO meter, and pH using pH meter.

#### WSSV PCR Analysis using Primer ICP11

Shrimp DNA isolation [15] was done by taking a sample of shrimp meat and gill that have been infected by WSSV on each level of infection (mild, moderate, severe), then purity of the isolated DNA was measured. PCR test used primer ICP 11,

**Wsv230\_19F22:** 5'GACGATTGCCCTGCTGGTGG 3' and **Wsv230\_202R24:** 5'GGGAATTGCTCATCCA GCGTTGA 3' with following PCR program: Hot start at 95°C for 3 min; Denaturation at 94°C for 1 min, Annealing at 59°C for 1 min; Extension at 72°C for 1 min. Denaturation process, annealing and extension carried out for 35 cycles and Post extensions at 72°C for 7 min.

### Data Analysis

All the level of infection data obtained from the control and treatment of salinity (5 ppt, ppt, 15 ppt, 25 ppt) were tabulated in Microsoft Excel. We also performed a simple linear regression and F test with 95% confidence level (P < 0.05).

## RESULT AND DISCUSSION

### Morphology of Healthy Vannamei Shrimp

Behavior and morphological observation of healthy vannamei shrimp showed normal behavior, e.g. fresh body color and complete body parts, shrimp move in the bottom of waters column and motionless on the bottom without raises to water surface at noon. Otherwise, at night shrimp had seen moving for eating the given feed. Normal condition is shown in the control treatment (without giving the virus). The behavior of the other shrimp shown as response to stimulus such as light and touch. This can be seen at night when the light of flashlight given, the shrimp moved closer to the light source. It was imiliar with the touch stimulation. Shrimp immediately swam away in the opposite direction to the existing stimuli. This is consistent with the statement of Kilawati [16], the characteristic of healthy shrimp has bright body colors, there are no white spots on the shrimp body, actively move and quickly response.

**Table 2.** Behavioral changes in infected vannamei shrimp, comparing between control group (healty shrimp) and treatment group (infected shrimp). **Control:** healty shrimp, **5ppt, 15ppt, 25ppt:** salinity treatment and infected shrimp

Behavior	Salinity Treatment							
	Control	%	5 ppt	%	15 ppt	%	25 ppt	%
<b>Shrimp movement</b>	Active	100	Slow, motionless at the bottom, flounder	90	Slow	85	Not too active	65
<b>Appetite</b>	Normal	90	Decline	80	Slightly decreased	70	Slightly decreased	40
<b>Response to stimulation</b>	Normal and fast	100	Decline, weak	90	Less fast	70	Fast	65
<b>Body color</b>	Fresh white	100	White	95	White	80	Bright white	65
<b>Body condition</b>	Normal	100	Tail, foot path, swimming legs, and reddish tail	90	reddish tail	75	reddish tail	60

### Behavioral Change of WSSV-Infected Vannamei Shrimp

The results of salinity differences in WSSV infection againts behavioral change in vannamei shrimp showed different behavior in each treatment. Behavioral changes in vannamei shrimp post-infection are presented in Table 2.

### WSSV Infection Level

The highest total of WSSV infected shrimp at 5 ppt salinity treatment with severe infection is 57%, followed by 15 ppt salinity treatment by moderate infection for 37%, and 25 ppt salinity treatment by severe infection for 30%. From data analysis, we obtained the average number of infected shrimp (Figure 1).

### Relationship of Salinity and Infection Level

This study consisted of independent variables (seawater with different salinity) and dependent variable (clinical symptoms and infection level). The results revealed that there was a relationship between independent variables and the dependent variable (Fig. 2). The relationship between salinity comparison with infection level in mild infection showed in the average number of shrimp with the characteristics of vannamei shrimp morphology. No morphological changes were visible, in addition to changes in behavior that is not normal and discoloration on the body. If the shrimp is WSSV-infected but there has been no sign of white spot, it is categorized as mild infection [17]. The infection occurred in low tissue thus white spots and redness did not appear. Figure 2 show a positive correlation where the higher salinity range, the higher number of shrimp that has scoring 1.

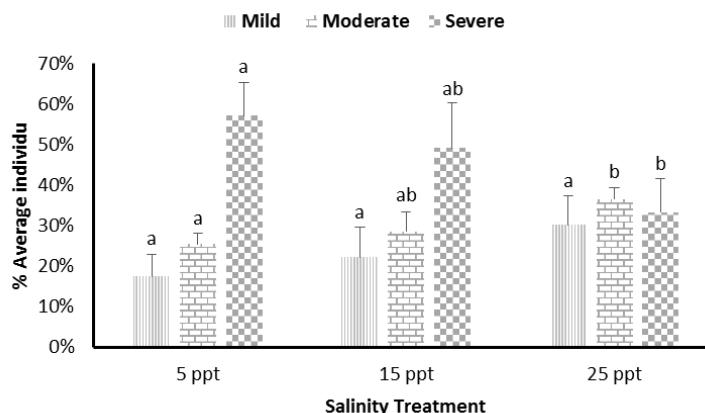


Figure 1. The average scoring level graph of WSSV infection level in infected shrimp

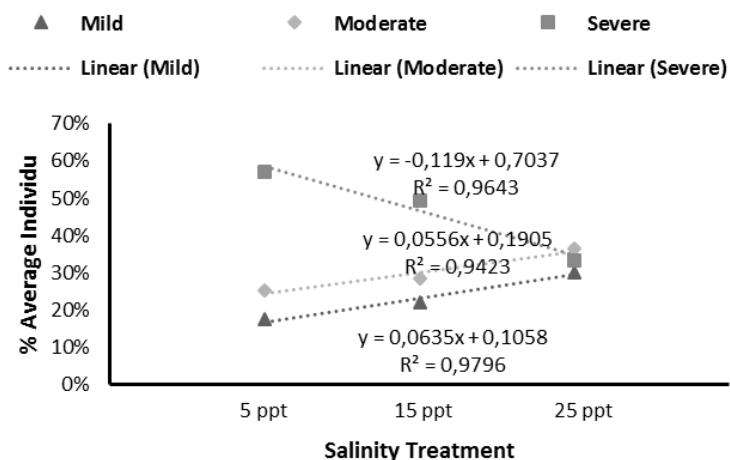


Figure 2. Correlation of salinity difference with the average of individuals (%) at the infection level (scores). Mild level have positif correlation with salinity changes; Moderate level have positif correlation with salinity changes; Severe level have positif correlation with salinity changes

Moderate infection level showed the change of color on the body and tail become red and the occurrence of white spots on the carapace between 1-3 unit. Presence of white spots on the carapace has become a common sign of WSSV [18], and also the adult shrimp has red color [19]. The Figure 2 show a positive correlation where the higher the salinity range, the higher number of shrimp that has scoring 2.

Severe infection level showed that the white spot has spread to parts of the body shrimp as well as the change in color to red on the tail and body, the antenna broken and eye damaged. Directorate General in Fisheries Farming described severe infection characterized by reddish body color changes into bolder red color on the tail [20]. The Department of Marine and Fisheries also explained that when it is severe, white spot spread to all parts of the body [21]. Figure 2 showed a negative correlation where the

higher ranges of salinity lead to the lower scoring 3. Other study showed that the shrimp at salinity 30 ppt, WSSV infection in black tiger shrimp is lower than the salinity of 10 ppt, 15 ppt, 20 ppt and 25 ppt [10].

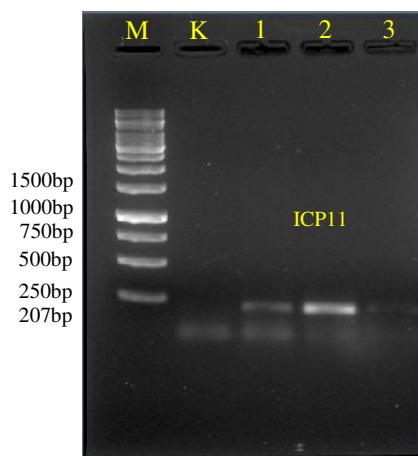
Vannamei shrimp have high osmoregulation ability, and successfully cultured in low salinity conditions (2 ppt) to high (40 ppt). The salinity changes of the water causes changes in haemolymph metabolism during viral infection process, thus reduce the role of immunocompetence and increase the vulnerability of shrimp to other pathogens [22].

Activity increase on the haemolymph metabolic for WSSV infection progresses. Increased haemolymph metabolic during early stage of infection can be caused by mobilizing energy reserves towards the hepatopancreas and muscle, to meet energy needs in order to ward off infection. A decrease in haemolymph

metabolic is significant in infected shrimp under salinity stress. It can be described as irregularities in the flow of energy to support the osmotic process under double stress due to salinity and pathogenic stress. Metabolic response variables have a correlation with some immune variables. It can be explained that bad metabolic response lead to decreased immunocompetence thus increase the amount of virus in infected shrimp [23]. Nearly optimal salinity range for vannamei shrimp life can be one of the supporting factor from the environment to the shrimp health condition.

#### WSSV Detection

Primer ICP11 [24] has chosen to be specific primers. ICP11 is a non-structural proteins encoded by genes ICP11 which allegedly role in WSSV infection. At the level of transcription and translation, host cells that have been infected by WSSV expressed a protein [25]. These proteins are nonstructural called ICP11. ICP11 gene amplification results are shown in Figure 3.



**Figure 3.** WSSV detection. band amplified in 207 bp indicated sample was infected by WSSV, Description: M:Marker, K:Control, 1:Mild, 2:Moderate, 3: Severe [15].

Vannamei shrimp DNA amplification results (Fig. 3) was used to ensure checks based on morphological condition. Morphological analysis results showed symptoms of being infected by the WSSV and infection level ranging from mild to severe. The results obtained by PCR amplification of gene ICP11 occurred at 207 bp, which means that there is a WSSV DNA virus in shrimp DNA samples which indicates infection by WSSV. The thickness of the band formed in the gel is

affected by the concentration of DNA possessed by each sample, in mild  $119.40 \text{ ng} \cdot \mu\text{L}^{-1}$ , moderate  $235.91 \text{ ng} \cdot \mu\text{L}^{-1}$  and severe  $1.78 \text{ ng} \cdot \mu\text{L}^{-1}$ .

WSSV infection mechanism to the body of vannamei initially intracytoplasmic into the host cell. Then at higher infection level, DNA viral entry into host DNA and takes over the transcription and translation processes in accordance with the DNA of the virus. At the stage of transcription and translation, the WSSV genes expressing a non-structural protein called ICP11, which was assumed essential in WSSV infection [10]. Exposure to stress on the shrimp can increase the risk of WSSV attack, because stress harm the shrimp immune system [26]. Consequently, under stress conditions, WSSV can proliferate rapidly and cause death [27].

#### Water Quality

Water quality measurement on the shrimp culture of this study was shown in Table 3. The lowest temperature is  $24.13^\circ\text{C}$  and the highest temperature is  $25.53^\circ\text{C}$ . Temperature greatly affects the life and growth of aquatic biota. In general, growth rate increases with increase in temperature, can suppress the animal life and even cause death if an increase in temperature is extremely occurred [28].

The lowest salinity is 4 ppt, while 25 ppt is the highest salinity. Salinity is closely related to osmoregulation of aquatic animals, in case of a sudden drop in salinity and the range is large enough, it will be difficult for the animal in osmoregulation setting which can lead to death [10].

Dissolved oxygen (DO) measurement in the seawater during the study showed the lowest value of DO is 4.9 and the highest value of DO is 8.66. The range of the oxygen in this study still support the shrimp life because the good range of DO for the shrimp life is 4-8 ppm [29].

Measurement of pH in this study showed the lowest pH for 6.65 and 8.15 as the highest pH. This value is still in the normal and optimum conditions for the life of vannamei shrimp. Budiardi [30] said waters with extreme pH depressed the shrimp, softening carapace and lowering their survival rate. High mortality in shrimp occur in waters pH below 6.0 while at pH 3.0 in the 20 hours can occurred up to 100% mortality.

**Table 3.** The water quality assessment result after 7 days of post infection culture

Code	Temperature (°C)	Salinity (ppt)	DO (mgL <sup>-1</sup> )	pH range
K	24.99± 0.55	23.95± 0.74	6.65± 0.98	7.34± 0.26
A-1	24.95± 0.79	4.80± 0.40	6.73± 0.80	7.84± 0.20
A-2	25.14± 0.49	4.95± 0.21	6.79± 0.75	7.87± 0.18
A-3	25± 0.56	4.95± 0.21	6.74± 0.77	7.83± 0.14
B-1	25± 0.54	14.85± 0.35	6.77± 0.81	7.68± 0.14
B-2	24.94± 0.59	14.95± 0.21	6.87± 0.94	7.70± 0.12
B-3	25.07± 0.53	14.95± 0.21	6.80± 0.84	7.57± 0.19
C-1	25.02 ± 0.61	24.61± 0.59	6.48± 0.73	7.44± 0.12
C-2	25.06± 0.64	24.85± 0.47	6.68± 0.79	7.47± 0.14
C-3	25.37± 0.26	24.95± 0.21	6.74± 0.89	7.57± 0.11

Description: (K) = control (A) = 5 ppt, (B) = 15 ppt, (C) = 25 ppt, (1,2,3) = repetition

## CONCLUSION

Shrimp that has score 1 (mild infection level) and Score 2 (moderate infection level) have a high number of individu with increasing salinity range. Otherwise, the shrimp that had score 3 (severe infection level) has decreased number of individu with the increasing salinity range.

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