Immune Response of White Shrimp (*Litopenaeus vannamei*) to Different Density and IMNV Challenge

Nur Komariah Baladrat¹*, Moch Nurhudah², and Heny Budi Utari³

¹Postgraduate Program Study of Industrial Aquaculture, Faculty of Fisheries Resources Utilization, Jakarta Technical University of Fisheries, South Jakarta, DKI Jakarta, 16421. Indonesia
²Polytechnic of Marine and Fisheries Karawang, Karawang, West Jawa. Indonesia
³Animal Health Service, Central Proteina Prima Company of Jakarta, DKI Jakarta. Indonesia

Abstract

Increasing in stocking density of shrimp affects the physiology and behaviour of their moving space. The health condition of shrimp is influenced by feeding, growth, and its susceptibility on disease. The aim of this study was to determine the development of immune response in relation to density and the presence of IMNV infection. This study used a completely randomized design (CRD) at density of 100 shrimp.m⁻², 200 shrimp.m⁻², and 400 shrimp.m⁻², with three replications in each treatment. The shrimp used was 5.02±0.26 g and the virus infection was exposed orally. This research was facilitated at the Disease Research Centre Laboratory of Central Proteina Prima Company, Pasar Kemis, Tangerang for 30 days. The results showed that the Total Hemocyte Count (THC) in hemolymph of shrimp had different values between negative controls and challenged IMNV. The lowest THC value was found at a density of 400 shrimp.m⁻² (3.00x10⁶ ml⁻¹). While the highest THC value was at a density of 100 shrimp.m⁻² (4.75x10⁶ ml⁻¹). This result is supported by the increasing value of water quality parameters along with the increasing density of shrimp. Histopathology changes on skeletal muscle and lymphoid organs confirmed that the development of IMNV infection was faster at high shrimp densities.

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1. Introduction

*Litopenaeus vannamei* farming is one of the priority commodities aquaculture production (Tang et al., 2019). On the world market it is estimated that the value from marine shrimp production reaches USD 40 billion (FAO, 2016). Shrimp farming around the world is currently being affected by outbreaks of infectious disease (Apún-molina et al., 2017). One of the diseases that attack white shrimp culture is Infectious Myonecrosis Virus (IMNV) in aquaculture ponds (Sarah et al., 2018). Several factors that influence the incidence of IMNV are low water quality, stocking density, shrimp stress, and the impact of climate change Kusumaningrum et al. (2012) and Tang et al. (2019). In addition, stressful environmental conditions increase susceptibility to pathogens and decreases shrimp immunity (Song et al., 2003). Shrimp infected with IMNV disease will reduce the shrimp’s immune system (Yudiati, 2016). Stressful environmental conditions increase infectivity of pathogens, because of a reduced capacity of immune response (Tang et al., 2005). The immune system in shrimp does not have memory cells, unlike vertebrates, which have specific antibodies and complements. The shrimp immune system does not have immunoglobulins that play important role in the immune mechanism, shrimp only have a natural immune system (Kurniawan et al., 2018). The first defence against disease in shrimp is carried out by haemocytes. Haemocytes are a non-specific factor in the cellular defence system (Ridlo and Pramesti, 2009).

In principle, increasing the number of shrimp stocking density increases the risk of disease spreading (Aguilar et al., 2011). At very high stocking density, the shrimp are more aggressive and attack each other, resulting in increasing cannibalism and mortality (Miranti, 2016). The increase in density also affects the physiological processes and their movement behavior. This will reduce their health and physiological conditions that affect feed consumption, growth, and survival decreases (Purnamasari et al., 2017).

Shrimp farming at high densities provides advantages, although it presents slower growth and even lower survival are observed (Sookying et al., 2011). Although high density induces a condition of water quality stress, the effect on shrimp immune with IMNV challenge are not well established (Apún-molina et al., 2017). In one experiment, no clear influence of high density of 50, 200, and 600 shrimp m$^{-2}$ on several metabolic and immunological indicators was observed (Li et al., 2006). However, other studies have shown that high density affects several immune parameters (Lin et al., 2015). On a research by Molina et al., (2017), the measured response of shrimp immune with immune parameters did not change at high density.

However, all previous studies were focused on the effect of stocking density on shrimp immune level. To our knowledge, very few studies have been conducted concerning the stocking density (high and low) and IMNV challenge on the immune response of *vannamei*. Determining stocking densities is a basic procedure in shrimp culture, and the IMNV is the cause of outbreaks of infectious disease.

The purpose of this present study is to assess the effect of the *vannamei* shrimp immune response observed for fifteen days on immunity (total haemocyte count), conditions of water quality parameters, and the level of viral infection (histopathology) after different density treatments and IMNV challenge.

2. Materials and Methods

2.1 Materials

This research was conducted from November 2020 to January 2021 at the Disease Research Center (DRC) laboratory of PT. Central Proteina Prima, Pasar Kemis, Tangerang. The shrimp used was 488 juvenile *Litopenaeus vannamei*, with average mean body weight of 5.02±0.26 g. Myonecrosis virus inoculum was obtained from DRC with a virus copy number of 5.97x10$^3$. The aquarium was measured at 60 x 40 x 50 cm and filled with approximately 80L of clean seawater. Water quality measuring instruments consist of thermometer, pH, DO, heater, test kit of TAN, TOM, and TOC.

2.2 Methods

2.2.1 Experimental design

The experimental design in this study was a completely randomized design (CRD) with 6 treatments with 3 replications. The treatment carried out consisted of 3 treatments with IMNV challenge test and 3 treatments were negative controls. The treatment was different stocking densities; 100 shrimp m$^{-2}$, 200 shrimp m$^{-2}$, and 400 shrimp m$^{-2}$. During the research activity, both IMNV challenged and control without IMNV challenged treatments were carried out.

2.2.2 Viral challenge

Stock of IMNV virus has been prepared by reinfection of some isolates directly into the shrimp to increase their efficacy. The IMNV inoculum which obtained from the DRC archives of PT. CP Prima, Tan-
gerang was stored at -80°C. A total of 50 shrimps with average mean body weight from 6-8 g were stocked in the aquarium with size of 80L. The inoculum that has been prepared was injected with intra muscularly as much as 0.1 ml shrimp\(^1\) into the body of shrimp (Yudianti, 2016). Then the shrimp was cultured and observed. The dead shrimp will be stored in the freezer. Observation were conducted for 14 days, at the end of the observation, both live and dead shrimp in the freezer were mashed. Infected shrimps were dismantled from its carapace, head, and tail; leaving the muscle part of the shrimp and then crushed and homogenized (Tang et al., 2005). To confirm the number of copies of the IMNV virus, Real Time-PCR was performed.

The IMNV infected tissue then was fed orally into the shrimp. The number of virus copies of infected tissue was 5.97 x 10\(^3\). Shrimp that have been mashed and then weighed was then fed to the tested shrimp for 10% of its biomass. Infected tissue was spread in the aquarium with a frequency of 3 times at 07.00 am, 01.00 pm, and 05.00 pm for 3 days (Umiliana et al., 2016).

2.2.3 Water quality

Water quality parameters of temperature and pH were observed every day at 8 a.m. and 4 p.m. Weekly measurements of Total Amonia Nitrogen (TAN), Total Organic Meter (TOM), and Total Organic Carbon (TOC) were tested by taking samples of 2 water samples in each treatment and would be duplicated when testing in the laboratory. Water samples are taken using a sample bottle and will be directly tested in the laboratory. Water quality parameters such as temperature that ranging from 29.7°C to 30.5°C and Dissolved oxygen (DO) > 4.0 mg/L were not significantly different (Table 1). The heater installation in the aquarium was set at 30°C during the observation. At this temperature level, the spread of the IMNV virus for challenge test can be evenly distributed. This level would also trigger the development of the IMNV (Disease Research Centre, CP Prima-Company, Unpublished data). According to Tobing (2019), the optimum temperature for rearing shrimp ranges from 22-32°C. While the temperature triggers the development of IMNV is >28°C (Silva et al., 2015). As explained by Sulmartiwi et al. (2013), the water temperature that accelerates the spread of IMNV is around 30°C.

Observation of the pH value carried out in the morning (8 a.m.) and afternoon (4 p.m.) did not affect the pH value. Changes in the pH value in observations were influenced by the amount of stocking density and IMNV. As the number of stockings increases, the pH value will decrease. In IMNV challenge, the density of 400 shrimp m\(^2\) decreased to pH value <7 at dpi 8 (Table 1). While in other treatments, the pH value was >7. This is due to the deteriorating condition of water

2.2.4 Haemocyte analysis

Sampling haemocyte analysis was taken at day post infection (dpi) 0, 5, 10, and 15. Approximately 50 μL of haemolymph were taken in each test shrimp, 3 pieces per treatment. It was performed on the ventral sinus of shrimp using a 1 ml syringe and then inserted into a microtube which was already filled with 50 μL of 10% formalin as anticoagulant. Then let it stand for 10 minutes and add 100 μL of rose Bengal for cell colouring. THC was carried out as described by Wang and Chen (2005). The solution mixture was then dripped as much as 10 μL on a haemocytometer and then covered with a cover glass. Total haemocytes were observed and the number of cells was counted under a microscope. Count were made on 5 of the 25 small squares in the centre of the haemocytometer.

2.2.5 Histopathology

For confirmation of IMNV virus infection, histopathology has carried out conventionally (Lightner, 1996). The number of shrimp samples were 2 from each density. Observation of histopathological parameters was carried out on skeletal muscles and lymphoid organs of the tested shrimp. Histopathological sampling was carried out on 5\(^{th}\), 10\(^{th}\) and 15\(^{th}\) day post-infection (dpi). The process of tissue preparations included: fixation, trimming (preparation), processing, embedding, rough, and fine sectioning, staining, respectively.

2.3 Data Analysis

Microsoft Excel 2013 was used for analysing water quality data descriptively after comparing it with water quality standards and other relevant research. THC data was analysed through two-way analysis of variance (ANOVA) with 95% confidence level. Then proceeded with Duncan’s test to determine the effect of various treatments. The results of histopathology parameters were descriptively analysed by describing the existing results.

3. Results and Discussion

3.1 Water Quality

The results of this study showed that daily water quality parameter such as temperature that ranging from 29.7°C to 30.5°C and Dissolved oxygen (DO) > 4.0 mg/L were not significantly different (Table 1). The heater installation in the aquarium was set at 30°C during the observation. At this temperature level, the spread of the IMNV virus for challenge test can be evenly distributed. This level would also trigger the development of the IMNV (Disease Research Centre, CP Prima-Company, Unpublished data). According to Tobing (2019), the optimum temperature for rearing shrimp ranges from 22-32°C. While the temperature triggers the development of IMNV is >28°C (Silva et al., 2015). As explained by Sulmartiwi et al. (2013), the water temperature that accelerates the spread of IMNV is around 30°C.
quality during the observation. As the density increases, the amount of feed remains and metabolism in the water increases. High organic matter causes acidification in the waters, so the pH becomes low. The decreasing pH value was caused by the decomposition of organic matter by microorganisms (Supriatna et al., 2020). Reaction of decreasing pH can increase the total organic matter content in the water (Hendrawati et al., 2008). The optimal pH value range is 6.8-7.8 (Tobing, 2019). However in other studies, Supriatna et al. (2020) conveyed that a good pH for ponds is in the range of 7.6-8.4.

**Table 1.** Average observations of water quality variables according to different densities

| Water Quality variables | Density (shrimp m⁻²) | Reference |
|-------------------------|----------------------|-----------|
|                         | 100                  | 200       | 400       |
| Temperature (°C)        | A 29.98±0.37         | 30.01±0.48| 30.06±0.33| 26.00 – 32.00 |
|                         | B 30.01±0.41         | 30.25±0.59| 30.04±0.42| (Tobing, 2019) |
| pH                      | A 7.31±0.19          | 7.03±0.32 | 6.52±0.15 | 7.60-8.45 (Supriatna et al., 2020) |
|                         | B 7.54±0.20          | 7.15±0.22 | 7.15±0.22 |
| Dissolved oxygen (mg L⁻¹) | A 5.25±0.09         | 5.19±0.08 | 5.14±0.12 | 4.00 – 6.00 (Tobing, 2019) |
|                         | B 5.23±0.11          | 5.28±0.09 | 5.19±0.05 |
| TAN (mg L⁻¹)            | A 0.98±0.09          | 1.03±0.04 | 2.08±0.59 | 1:50 (Fendjalang, 2016) |
|                         | B 0.84±0.02          | 0.92±0.05 | 1.21±0.25 |
| TOC (mg L⁻¹)            | A 0.71±0.04          | 1.43±0.04 | 3.71±0.91 | 2:00 (Ariadi et al., 2020) |
|                         | B 0.57±0.17          | 1.26±0.21 | 1.86±0.34 |
| TOM (mg L⁻¹)            | A 107.21±0.51        | 118.59±0.72| 124.91±1.03| <105.60 (Wafi et al., 2020) |
|                         | B 85.72±0.54         | 98.36±0.83| 116.06±0.52| |

Description: A (IMNV chellenge test), B (control). TAN (Total Ammonia-N, TOM (Total Organic Meter), TOC (Total Organic Carbon).

**Table 2.** The results of observation of the THC value in each treatment

| Treatments                  | Total Haemocyte Count (x 10⁶ mL⁻¹) |
|-----------------------------|-----------------------------------|
| (shrimp m⁻²)                | dpi 0   | dpi 5   | dpi 10  | dpi 15  |
| IMNV+100                    | 10.25±0.25ᵇ | 9.33±1.25ᵇ | 4.83±0.29ᵇ | 5.75±0.66ᵇ |
| IMNV+200                    | 10.17±0.76ᵇ | 9.50±1.32ᵇ | 5.83±0.29ᵇ | 3.67±0.29ᵇ |
| IMNV+400                    | 8.83±1.04ᵇ | 8.00±0.50ᵃ | 4.33±0.76ᵇ | 3.00±0.50ᵇ |
| 100                         | 11.50±0.50ᵇ | 13.83±0.29ᵇ | 7.83±0.29ᵇ | 10.83±0.76ᵇ |
| 200                         | 11.67±0.29ᵇ | 15.83±0.76ᵇ | 8.33±0.28ᵇ | 10.33±0.76ᵇ |
| 400                         | 10.33±0.76ᵇ | 10.17±2.08ᵇ | 8.00±0.87ᵃ | 11.83±0.73ᵇ |

Description: dpi (day post infection), IMNV+ density (treatment IMNV challenged test). Different superscripts in the same column shows that there are significant differents (p<0.05(Total Organic Carbon).
Total Ammonia Nitrogen (TAN) is toxic ammonia and can harm the condition of shrimp. The results of TAN level in this study showed that there were differences between each density in both IMNV challenge test and the control. The amount of TAN content between the IMNV challenge was higher with an average of 0.96-1.66 mg L⁻¹ compared to the control 0.698-0.89 mg L⁻¹ (Table 1). In the other observations of IMNV challenge, the number of TAN content almost reached a potentially toxic concentration at week-2 (dpi 10), which was in the range of 0.61-2.08 mg L⁻¹. Fatal TAN level that can kill shrimp is 1.5 mg L⁻¹ (Ariadi et al., 2020; Syafaat et al., 2013). Research by Aguilar et al. (2011) reported the maximum value of TAN is 2.4 mg L⁻¹. The highest TAN value was obtained from density of 400 shrimp m⁻². This was directly proportional to the increasing amount of feed input and waste produced because of shrimp stress from IMNV infection which affect their appetite. The increase in stocking density affects the physiological condition of shrimp due to infection, the shrimp begins to decrease their appetite which results in increasing the remaining feed and the amount of nitrogen released into the water as studied by Syafaat et al. (2010).

In addition to TAN, the lowest TOM content was obtained in the treatment of 100 shrimp m⁻² (A) with an average of 78.14 mg L⁻¹, while the highest TOM content was found at 400 shrimp m⁻² which was 123.64 mg L⁻¹ (Table 1). TOM shows the content of organic matter, in this study the TOM content increased according to the increase in the amount of density. High density causes the feeding to be increased, but the presence of IMNV infection causes the shrimp to become weaker. High density also disrupts the physiological process of shrimp due to stress. This increases the concentration of dissolved organic matter along with feed and metabolic waste. Increasing the amount of feed and shrimp metabolism increases the amount of decomposition carried out by microorganisms. Research by Supriatna et al. (2020) stated that TOM is a description of the concentration of total organic matter in waters consisting of dissolved, suspended, and colloidal organic matter. On research by Wafi et al. (2020), a good content for TOM is <90 mg L⁻¹. It was clarified in other studies that the optimum range of TOM values in ponds was <105.6 mg L⁻¹ (Supriatna et al., 2020).

TOC is the total organic carbon consisting of dissolved organic matter. TOC levels during the same study with other parameters increased at week 2 (dpi 10). In the observation, the highest TOC was found in the IMNV challenge test treatment with high stocking density, at density of 400 shrimp m⁻² which reached 4.143 mg L⁻¹. Poersch et al. (2020) said that the factor of shrimp density contributed to the TOC value content of the feed and manure of the reared biota. With increasing density, it increases the amount of feed and metabolic waste increasing organic carbon content (Fast and Lester, 1992).

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Table 3. Histopathology development in each treatments

| Treatment (shrimp m⁻²) | Dpi 5 | Dpi 10 | Dpi 15 |
|------------------------|-------|--------|--------|
|                        | Limfoid Organ | Muscle | Limfoid Organ | Muscle | Limfoid Organ | Muscle |
| 100                    | -     | -      | -      | -      | -              | -      |
| 200                    | -     | -      | -      | -      | -              | -      |
| 400                    | -     | -      | -      | -      | -              | -      |
| IMNV+100               | -     | -      | -      | -      | LOS            | Nec (25%) |
|                        |       |        |        |        | -25%           |         |
| IMNV+200               | -     | -      | -      | Nec    | LOS            | Nec |
|                        |       |        |        | -25%   | -60%           | -60%   |
| IMNV+400               | -     | -      | LOS    | Nec    | LOS            | Nec |
|                        |       |        | -40%   | -40%   | -80%           | -80%   |

Description:
• dpi (day post infection), Los (Limfoid Organ Spehroid), Nec (Necrosis), IMNV+density (treatment IMNV challenged test)
• The percent value indicates the infection rate in each treatment
• (-) IMNV symptoms have not appeared
did not affect THC values and did not confess susceptibility to WSSV virus. However, another study showed that the THC value of vanname shrimp infected with TSV has decreased (Song et al., 2003). The decrease in the value of THC might be due to foreign objects that enter the shrimp body will be recognized by hemocyte cells and then responded through several stages of mechanisms and various immune responses to pathogen (Muharrama, 2020). In the presence of foreign objects, it causes hemocyte cells to migrate from the shrimp body’s circulation system to tissues where many cells are infected (Widanarni et al., 2020). It is indicated that the THC value for every different virus will showed different number.

Observation of THC value in control treatment gave the same effect on increasing stocking den-

3.2 Haemocyte Analysis

Based on the results of the study, THC value was different between each treatment. THC values in each treatment generally decreased at the 10th dpi or the 2nd week. Besides this is due to deteriorating water quality conditions (Table 1), it also increasing the development of the IMNV infection in the body of shrimp. This was apparently different in IMNV challenge and control.

In IMNV challenge, the lowest THC value was $3 \times 10^6$ ml$^{-1}$, while in control was $7.75 \times 10^6$ ml$^{-1}$ (Table 2). Therefore, it can be seen that THC value in all IMNV challenge was lower than control. In other words, it has been showed that in this study, the THC value was significantly influenced by viral factors. Even though the study by Molina et al. (2017) showed that high density

Figure 1. Histopathology changes of muscles ad lymphoid organs in the IMNV challenge test. [A] Normal muscle tissue; [B] Necrotic muscle tissue; [C] Normal lymphoid tubules; [D] Lymphoid organs that form spheroids (LOS) and their comparaison with normal lymphoid tubules (LT). The arrows indicate the formation of vacoula (treatment 400 shrimp m$^{-2}$ dpi 10).
sity. The lowest THC value was observed at a density of 400 shrimp m², which reached 3.00x10⁶ cell/ml. Meanwhile, the density of 100 shrimp m² and 200 shrimp m² varied. At dpi 0 and dpi 15 THC at a density of 100 shrimp m² (10.25x10⁶ mL⁻¹ and 4.50x10⁶ mL⁻¹) was slightly lower than the density of 200 shrimp m² (10.50x10⁶ mL⁻¹ and 4.75x10⁶ mL⁻¹). This indicated that THC was slightly affected by high density in this study even more to the IMNV infection. On research by Apún-molina et al. (2017) mentioned that although high density can cause stress and suppress the immune system in shrimp, there was no significant change in THC at a high density. The normal THC value for shrimp is a minimum of 20x10⁶ – 40x10⁶ mL⁻¹ (Chang et al., 1999) or minimum is 16,4x10⁶ mL⁻¹ (Song et al., 2003), meanwhile in this study appointed that THC value was lower and corresponded to the high density.

3.3 Hystopathology

IMNV infection was confirmed by examining the histopathology of shrimp. Tissue observation was performed in skeletal muscle and lymphoid organs as described by Andrade et al. (2008) and Poulos et al. (2006). Based on histopathological results, IMNV challenge shrimp were showed abnormalities compared to normal. In IMNV challenge as well as in high density of shrimp, percentage of necrosis muscle tissue and spheroid formation in lymphoid organs are higher than normal (Figure 1).

In density of 400 shrimp m², the fastest clinical symptoms appeared at dpi 10 and the percentage of shrimp exposed to IMNV exceeded 80% of the observed shrimp (Table 3). The last clinical symptom of IMNV that appeared was at a density of 100 shrimp m² which showed necrosis of muscle tissue and spheroid at the last observation (dpi-15) and the percentage of shrimp exposed was 25% of the observed shrimp. In control, no shrimp were confirmed to be infected with IMNV. Shrimp is declared infected with IMNV if muscle necrosis is found accompanied by the formation of spheroids in lymphoid organs. Necrosis in the muscle tissue could cause loss of transparency in muscle tissue and at advanced stage, it will turn red as a sign of IMNV infection as observed by Poulos et al. (2006); Senapin et al. (2007); and Sarah et al. (2018). Similar results from a study by Sukenda et al. (2010) in IMNV infection tissue have shown the formation of tissue degeneration, necrosis, and infiltration of haemocytes in muscle tissue. Besides the muscle necrosis, the presence of lymphoid organs are used as confirmation of IMNV disease (Andrade et al., 2008). Histopathology of abnormal lymphoid organs was commonly found in cases of shrimp infected with RNA viruses. Therefore, it is generally believed that the formation of spheroids is a non-specific reaction of the shrimp immune system to viral infections (Rusaini and Owens, 2010). The abnormality of the lymphoid organs are when it cannot maintain their normal shape, formation of spheroids, is known as hypertrophy of lymphoid cells, viral inclusions, and degradation of granulocyte haemocyte (Hasan, 2011).

4. Conclusion

Based on results of the study, the change in the shrimp immune response which were observed from THC value and histopathology after IMNV challenged has significant difference for each different densities. In high stocking density, the speed and the percentage of appearance of clinical symptoms such as degeneration of muscle necrosis and spheroid in lymphoid organs had increased. The speed of water quality degradation as pH, TAN, TOC, and TOM were also high. From this study, we recommend 100 shrimp m² for the best stocking density.

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Authors’ Contributions

All authors have contributed to the final manuscript. The contributed of each author are as follows HBU; as a determinant of topic ideas, funding and critical revision of articles. NKB; collecting data, compiling manuscripts, and analyzing data. MN; provide conceptual ideas and critical revision of articles.

Conflict of Interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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