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Effect of Stocking Density on Stress-Related Gene Expression of Pacific White Shrimp (*Litopenaeus vannamei*) Infected with Infectious Myonecrosis Virus (IMNV)

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Abstract

The stress level of vannamei shrimp (*Litopenaeus vannamei*) is affected by increased density and several genes are expressed under the condition. This study aimed to determine the expression of genes encoding white shrimp stress after density treatment and the infectious myonecrosis virus (IMNV) challenge test. A completely randomized design (CRD) was carried out with 6 treatment groups, i.e. 3 different stocking density groups without IMNV infection (100, 200, and 400 shrimp/m^2) and 3 different stocking density groups + IMNV infection (100, 200, and 400 shrimp/m^2). In addition, a shrimp density of 400 shrimp/m^2 reported the fastest rate of developing the IMNV virus as seen from the clinical symptoms. The lowest cumulative number of shrimp deaths was at a density of 100 shrimp/m^2 and was caused by the IMNV virus confirmed through RT-PCR. Expression of stress-coding genes was divided into upregulated and downregulated characteristics. The upregulated genes were lectin and translationally controlled tumor protein (TCTP), while the downregulated gene was Toll Receptor. The results showed that the expression of genes related to immunity in *L. vannamei* was upregulated after pathogen challenges such as lectin and TCTP, meanwhile, the Toll receptor gene was downregulated. Further study should also be performed to measure the expression of the three genes in revealing the immune pathways.

Keywords: aquaculture, gene expression, infectious myonecrosis virus, *Litopenaeus vannamei*, stocking density

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INTRODUCTION

Litopenaeus vannamei, or Pacific white shrimp, is an important component of contemporary aquaculture efforts (Fan *et al*., 2019). Even though the species originates from the East Pacific between Mexico and Peru, it is widely cultivated in most tropical and subtropical regions. In several countries such as Brazil, *L. vannamei* was first introduced in 1983. In Indonesia, the specie replaced cultivated tiger prawns (*Penaeus monodon*) which were more susceptible to disease (Baladrat *et al*., 2022; Rahardjo *et al*., 2022; Wang *et al*., 2022). *L. vannamei* has been successfully cultivated in at least 17 provinces and the productivity has reached more than 856,753 tons in 2020 (Li *et al*., 2021).

In line with other aquaculture commodities, *L. vannamei* is very susceptible to infectious disease attacks which can impact aquaculture productivity (Zhang *et al*., 2016; Kenconojati *et al*., 2023). The development of infectious diseases such as bacteria, viruses, and parasites cannot be separated from management systems that are not optimal and include intensive cultivation systems (Tanjung *et al*., 2022). The White spot syndrome virus (WSSV) was initially documented over two decades ago and has subsequently proliferated globally. The pathogenicity is evidenced by the capacity to induce mortality rates nearing 100% within a week of infection (Wiradana *et al*., 2019). In addition, Taura syndrome virus (TSV), yellow-head disease, hypodermis necrosis, and infectious hematopoietic virus (IHHNV) cause large losses

in shrimp farming (Hou *et al*., 2023; Ochoa *et al*., 2020; Qin *et al*., 2023). The recently identified disease is infectious myonecrosis virus (IMNV) which belongs to the double-strand (ds) RNA virus, family Totiviridae (Hou *et al*., 2021; Thamizhvanan *et al*., 2019).

The IMNV often manifests the virulence acutely in ponds, with high mortality rates and severe clinical symptoms among younger shrimp, but develops chronically with persistent mortality of 40 to 70% (Prasad *et al*., 2017). Furthermore, lymphoid organs in shrimp infected with IMNV experience hypertrophy of 2 to 4 times the normal size consisting of only two similar lobes in the ventral-anterior hepatopancreas (Wan *et al*., 2023). The size is influenced by many factors including developmental stage, size, type, and health status of shrimp (Rusaini and Owens, 2010; Zakwan *et al*., 2023; Syahbirin *et al*., 2024). In 2002 and 2006, IMNV was first discovered in *L. vannamei* cultivated in Brazil (Andrade *et al*., 2022) and Indonesia (Widanarni *et al*., 2015). The virus was designated as the main infectious disease in fisheries quarantine in the same year (Lee *et al*., 2022). Furthermore, the transmission of IMNV has spread and is prevalent in more countries in Asia and Africa, as well as in sea waters around Thailand (Srisala *et al*., 2021) and Egypt in 2018 (Aly *et al*., 2021).

Environmental factors such as temperature, as well as decreased salinity and pH, are known as stressors influencing the incidence of disease outbreaks (Riandi *et al*., 2021; Sani *et al*., 2020; Wiradana *et al*., 2022) such as IMNV. In addition, horizontal transmission of IMNV has been proven through cannibalism and is thought to also occur through water. Vertical transmission from broodstock to offspring is also considered a possible factor in the infection (Naim *et al*., 2014). However, environmental stress is the main factor causing a decrease in shrimp immunity due to oxidative stress (Chen and He, 2019). Measurement of metabolic markers corresponding to physiological stress responses is used to detect stress conditions in aquatic organisms. Several genes related to hemocyte defense against pathogen infection in shrimp bodies have different properties from vertebrate animals (Cui *et al*., 2020; He *et al*., 2021).

Many immunological and stress indicators, such as hematologic, serological, molecular, and histomorphological can provide an accurate health condition of aquatic animals (Hu *et al*., 2024; Viana *et al*., 2024). Several genes including lectin, TCTP, and Toll Receptor are related to the shrimp immune system (Niu *et al*., 2023; Rajesh *et al*., 2014; Viana *et al*., 2024). Lectin is a mannose-binding lectin (MBL) derived from granular hemocytes and has a vital role in pathogen recognition, the innate immune system, and contact between cells (Wiradana *et al*., 2019). Meanwhile, TCTP is a gene found in cell proliferation, cycle progression, and anti-apoptosis (Graidist *et al*., 2006). In an infected shrimp, TCTP responds in the process of programmed cell death through apoptotic activity. Toll Receptor functions to identify bacteria in the digestive glands right after the pathogen enters the body of shrimp (Qiu *et al*., 2021).

Some stress-related genes may vary due to different stocking densities in *L. vannamei* to understand the mechanisms of producing adverse outcomes due to infections (Lusková *et al*., 2002) such as IMNV. Previous study has established the impact of stocking density variation on shrimp affected with IMNV, characterized by a low total hemocyte count (THC). Specifically, at a density of 400 shrimps/m^2, shrimp reported a THC value of 4.75×10^6 mL. This condition corresponded with diminished water quality and histopathological alterations in skeletal muscle and lymphoid organs when compared to control groups (Baladrat *et al*., 2022). However, the mechanism of stress-related gene expression affecting shrimp physiology has not been reported. For example, high upregulation of stress genes is considered a valid biomarker for toxic damage (Singh *et al*., 2019). Cellular responses to important immune and stress genes expressed in animal tissues are useful to describe changes at the tissue level (Majhi *et al*., 2023). Therefore, this study aimed to examine variations in stocking density on the physiological function of *L. vannamei* by measuring the expression of stress-

related genes such as lectin, TCTP, and Toll Receptor.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Polytechnic of Marine and Fisheries Karawang, West Java, Indonesia, through a feasibility and qualification examination.

Study Period and Location

This study was performed at PT. Central Proteina Prima (CPP) Disease Research Center (DRC), Pasar Kemis, Tangerang from November 2020 to January 2021.

Experimental Design

This study used a completely randomized design (CRD) which included 6 treatment groups with 3 replications. The group treatments differed in stocking density of *L. vannamei*, comprising 100 shrimps/m^2, 200 shrimps/m^2, as well as 400 shrimps/m^2 and infected with IMNV isolates. The control group had the same density but without IMNV infection (Table 1).

Media Preparation

The maintenance medium used was an aquarium with dimensions of 60 × 40 × 50 cm and a water level of 33.5 cm. The volume of water obtained for study was 80,400 cm^3 or the equivalent of 80 L. The number of aquariums used was 18 aquariums (9 density aquariums with IMNV challenge tests and 9 aquariums for control). Before treatment, the aquarium was washed thoroughly using calcium hypochlorite at a dose of 20 mg/L for 24 hours. Free chlorine residue will decompose into chloride and water when the aquarium is exposed to sunlight. After 24 hours, the aquarium was drained and filled with clean sea water with a salinity of 15 g/L. A heater is installed in the aquarium with a set temperature of 30℃ and a thermometer. Each aquarium is equipped with an aerator as an oxygen supply (Marwiyah *et al*., 2019; Wiradana *et al*., 2019).

Animal Assay

The assay organism used was *L. vannamei* with specific pathogen free (SPF) status originating from the Disease Research Center Laboratory of PT. Central Proteina Prima Tangerang. According to Coelho *et al*., (2009), *L. vannamei* with a size of 5 g/ind was prepared and placed in a rearing container. Shrimp was fed three times daily with a feeding rate (FR) of 5% of biomass/day. Feeding was carried out at 07.00 a.m., 1.00 p.m. and 6.00 p.m. (Cokrowati *et al*., 2012).

Virus Reinfection

Virus reinfection was carried out to increase IMNV virus stock given to the test shrimp from the inoculum. IMNV inoculum was obtained from DRC PT. Laboratory collection and CP. Prima Tangerang stored at -80℃. A total of 50 shrimp with sizes ranging from 6–8 g/ind were placed in the aquarium and 0.1 mL/shrimp prepared inoculum was injected intramuscularly (Yudiati, 2016). During the observation, shrimp was stored in the freezer when death was reported after injection. Observations were carried out for 14 days before grounding the live and dead shrimps in the freezer. Shrimp were cleaned from the carapace and tail, leaving the muscle, then crushed and homogenized (Tang *et al*., 2005). Meanwhile, the IMNV virus copy number confirmation test was carried out using real-time PCR.

Infection of IMNV Isolates

IMNV virus was infected with *L. vannamei* orally. Shrimp used were reinfected positively at the previous stage with a virus copy number of 5.97×10^3. Subsequently, ground shrimp biomass equivalent to 10% of the experimental biomass was weighed and introduced into the aquarium. The dosage was repeated three times daily, at 07:00 a.m., 1:00 p.m., and 5:00 p.m., over three days (Umiliana *et al*., 2016).

Post-infection Observation Procedures

Observations were performed two days after oral administration of the virus and observed for 15 days. During the study, aquarium observations

were conducted every 1 hour to ensure shrimp deaths. Furthermore, shrimps were stored in a freezer at -80℃ to be confirmed for virus using PCR.

Table 1. *L. vannamei* stocking density treatment group and IMNV challenge test

Treatments	Description
A	A density of 100 shrimps/m^2 + IMNV
B	A density of 200 shrimps/m^2 + IMNV
C	A density of 400 shrimps/m^2 + IMNV
Control A	Density 100 shrimps/m^2
Control B	Density 200 shrimps/m^2
Control C	Density 400 shrimps/m^2

Table 2. Observation level of clinical symptoms of IMNV disease in *L. vannamei* (Hasan, 2011)

Level	Clinical symptom	Symbols	Category
1	Infected without symptoms	+	Mild
2	Slight white bruising in the tissue in several abdominal segments	++	Intermediate
3	Most of the abdominal tissue is white and bruised	+++	Severe
4	The abdomen from the tail is red (dead tissue)	++++	Chronic

Table 3. Specific primers and nucleotide sequences

Gen	Function	Sequences (5'-3')	Size (bp)	References
Lectin	Pathogen recognition	F: TTTGTAAACAACAGGGCAGTTCCAC R: CTGTCTTTCATCAGAATGCTACCTC	210	Zhang *et al*., (2009)
TCTP	Anti-apoptotic (cell death) activity	F: CAATGGACCCTGATGGC R: GCTTCTCCTCTGTTAGACCGTAT	195	Wu *et al*., (2013)
Toll Receptor	Pathogen recognition and immune cell activation	F: ATGTGCGTGCGGATACATTA R: GGGTGTTGGATGTCGAGAGT	298	Wang *et al*., (2010)

Total RNA Extraction

Total RNA extraction was obtained from *L. vannamei* hemolymph using the High Pure Viral mRNA Extraction Kit (Roche, Germany). The hemolymph was mixed with 500 µL of RNA Extraction, crushed with a grinder, and incubated at room temperature for 5 minutes (Subaidah, 2013). In addition, 100 µL of chloroform ($CHCl_3$) was added and centrifuged at 12,000 rpm for 15 minutes. The solution was divided into 3 layers, and 200 µL of the supernatant layer was mixed with 200 µL isopropanol and centrifuged at 12,000 rpm for 10 minutes. Meanwhile, the resulting hemocyte pellet was rinsed with 500 µL ethanol and centrifuged at 9,000 rpm for 5 minutes. The pellets were rinsed with 50–100 µL of DEPC ddH_2O Buffer solution.

Confirmation using PCR

Confirmation of the IMNV challenge test was carried out using Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) IQ-2000 (IQ-IMNV, Genereach, Taiwan). This test was conducted twice with the first-stage primers, namely IMNV-F (5'-CGACGCTGCTAACCATACAA-3') and IMNV-R (5'-ACTCGGCTGTTCGATCAAGT-3'). For the subsequent step (nested step), the primers used were IMNV-NF (5'-GGCACATGCTCAGAGACA) and IMNV-NR (5'-AGCGCTGAGTCCAGTCTTG-3') (Poulos and Lightner, 2006).

Dead shrimps were removed from the muscle and placed in a PCR tube stored in a freezer at -80℃ for IMNV PCR assay. Furthermore, shrimps were subjected to RNA

extraction before conducting amplification. The reagent mix used was 8 µL/reaction (RT-PCR Premix 7 µL, Iqzyme DNA Polymerase 0.5 µL and RT Enzyme Mix 0.5 µL). The profile for RT-PCR was 42℃ 30 min, 94℃ 2 min, 94℃ 20 sec, 62℃ 20 sec (15 cycles), 72℃ 30 sec, and 20℃ 30 sec. The results of electrophoresis for the IMNV virus appeared in a band at 139 base pair (bp).

Clinical Symptoms of IMNV

Observation of clinical symptoms was carried out every day for 15 days after infection including recording of mortality. Clinical symptoms were observed to determine the initial time shrimp showed symptoms of IMNV and to see the development of the level. Observations were made visually by considering physical changes and shrimp appetite (Table 2).

Gene Expression Analysis

Lectin, Toll Receptor, and TCTP gene expression were analyzed at 5 and 15 days post-infection (dpi). Gene expression was conducted qualitatively by RT-PCR. In addition, the premix for each running sample consisted of 8 µL Nuclease Free Water (NFW), Primer F and R 1 µL each, RT enzyme 0.5 µL and Master Mix 12.5 µL. Analysis was carried out by RT-PCR with the reverse process at 42℃, initial denaturation at 95℃, denaturation at 95℃, annealing process at 60℃, extension process at 72℃, final extension at 72℃, and termination at 40℃ for 30 minutes, 5 minutes, 15 seconds with 40 cycles, 30 seconds, 30 seconds, 5 minutes, and 30 minutes, respectively. To verify the target gene, the PCR product was electrophoresed on a 1% agarose gel. Specific primers and the nucleotide sequences were presented in Table 3.

Shrimp Mortality Rate

Shrimp mortality was measured to determine the number of deaths during post-infection observation. This variable is measured using the following equation (Hasan, 2011):

$$MR = \frac{Nt}{No} \times 100\%$$

Description:
MR = shrimp mortality rate (%)
Nt = number of shrimp mortality at time (t)
No = number of shrimp at the initial of rearing

Statistical Analysis

Mortality rate data was analyzed using One-way ANOVA (SPSS, IBM) with a confidence level of 95%. Subsequently, the Tukey test was continued to determine the effect of various treatments. For IMNV confirmation results data, clinical symptoms, and gene expression were analyzed descriptively based on electrophoresis results.

RESULTS AND DISCUSSION

Clinical Symptoms of IMNV in *L. vannamei*

Clinical development of IMNV disease was carried out during challenge treatment, as reported in Table 4. Clinical signs in shrimp infected with IMNV are that the two rear segments of the abdomen and the base of the tail change color from white to reddish. Pathologically, IMNV infections can be detected by necrosis, which appears white in the muscle but proceeds to a reddish color in the fan tail and the final two abdominal segments (Figure 1).

The development of IMNV clinical symptoms was observed with each treatment of the infection. On visual observation, the appearance of clinical symptoms was different. The appearance takes longer in treatments with lower density. The first symptoms of necrosis were observed at dpi-5 at the level of mild necrosis, while clinical symptom level 4 was reported at dpi-12. The clinical symptoms in the form of necrosis were seen on day 5 post-infection and severe necrosis occurred on day 14 (Hasan, 2011; Tang *et al*., 2005). The first level of clinical symptoms in shrimp infected with IMNV was reduced appetite. Shrimp experienced disturbances in the motor system and reduced appetite (Rekasana *et al*., 2013).

Figure 1. Clinical symptoms in shrimp according to the level of infection. (A) Normal shrimp without symptoms; (B) Grade 2, necrosis initiate to occur in shrimp abdominal muscles 6 and the base of the tail; (C) Grade 3, necrosis in shrimp spreads to abdominal segment 3; (D) Grade 4, shrimp necrosis has caused a reddish color at the base of the tail and abdomen 6.

Table 4. Development of clinical symptoms during the observation period in IMNV challenge treatment

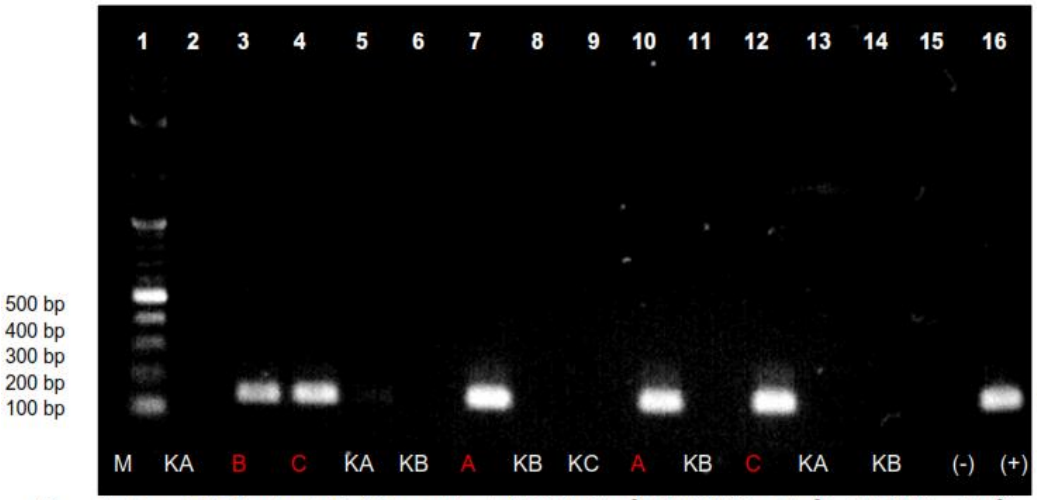
Replication	Days of cultivation															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Treatment A (100 shrimp/m^2)																
1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	++	++
2	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
3	-	-	-	-	-	-	-	-	-	-	+	+	+	++	++	++
Treatment B (200 shrimp/m^2)																
1	-	-	-	-	-	+	+	+	+	+	++	++	++	+++	+++	+++
2	-	-	-	-	+	+	+	+	+	++	++	++	++	++	++	+++
3	-	-	-	-	-	+	+	+	+	++	++	++	+++	+++	+++	++++
Treatment C (400 shrimp/m^2)																
1	-	-	+	+	+	+	+	+	+	+	+++	++++	++++	++++	++++	++++
2	-	-	-	+	+	+	+	+	+	+	+++	++++	++++	++++	++++	++++
3	-	-	+	+	++	++	++	++	+++	+++	+++	+++	+++	++++	++++	++++

(-) No clinical symptoms; (+) Level 1 (No symptoms and reduced appetite); (++) Level 2 (Slight white bruising in the tissue); (+++) Level 3 (Most of the abdominal tissue is white bruised); (++++) Level 4 (The abdomen from the tail is red).

Table 5. Description of stress-related genes in shrimp

Treatments	Lectin		Toll Receptor		TCTP	
	dpi 5	dpi 15	dpi 5	dpi 15	dpi 5	dpi 15
100 shrimp/m^2						
Challenge assay	+	-	+	+	+	-
Control	-	-	+	-	-	-
200 shrimp/m^2						
Challenge assay	+	-	+	-	+	+
Control	-	-	+	+	-	-
400 shrimp/m^2						
Challenge assay	+	+	-	-	+	+
Control	-	-	+	-	-	-

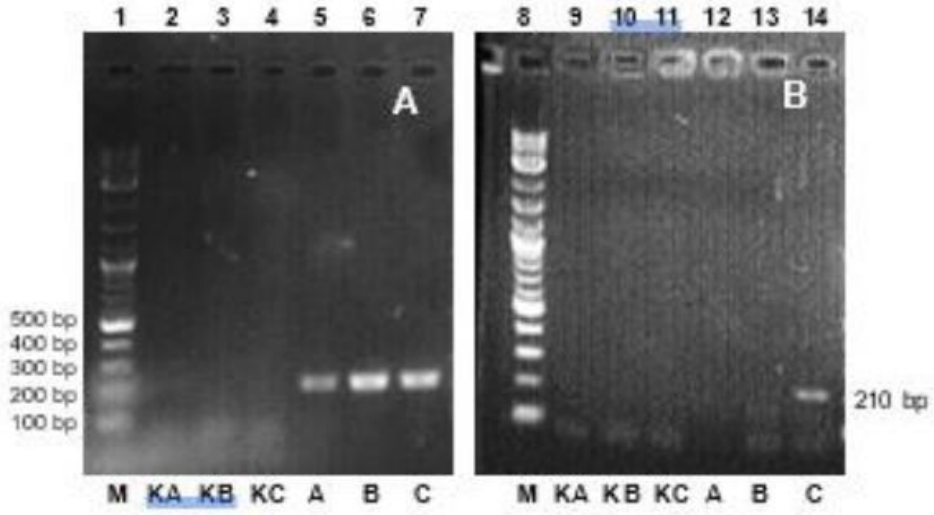
(+) upregulated; (-) downregulated.



Description: M (Marker); K (Control); A (100 ind/m^2), B (200 ind/m^2), C (400 ind/m^2)

1. Marker
2. Control 100 ind/m^2 (dpi 3)
3. Treatment 200 ind/m^2 (dpi 5)
4. Treatment 400 ind/m^2 (dpi 5)
5. Control 100 ind/m^2 (dpi 7)
6. Control 200 ind/m^2 (dpi 7)
7. Treatment 100 ind/m^2 (dpi 9)
8. Control 200 ind/m^2 (dpi 9)
9. Control 400 ind/m^2 (dpi 11)
10. Treatment 100 ind/m^2 (dpi 11)
11. Control 200 ind/m^2 (dpi 13)
12. Treatment 400 ind/m^2 (dpi 13)
13. Control 100 ind/m^2 (dpi 15)
14. Control 200 ind/m^2 (dpi 15)
15. Negative control
16. Positive control

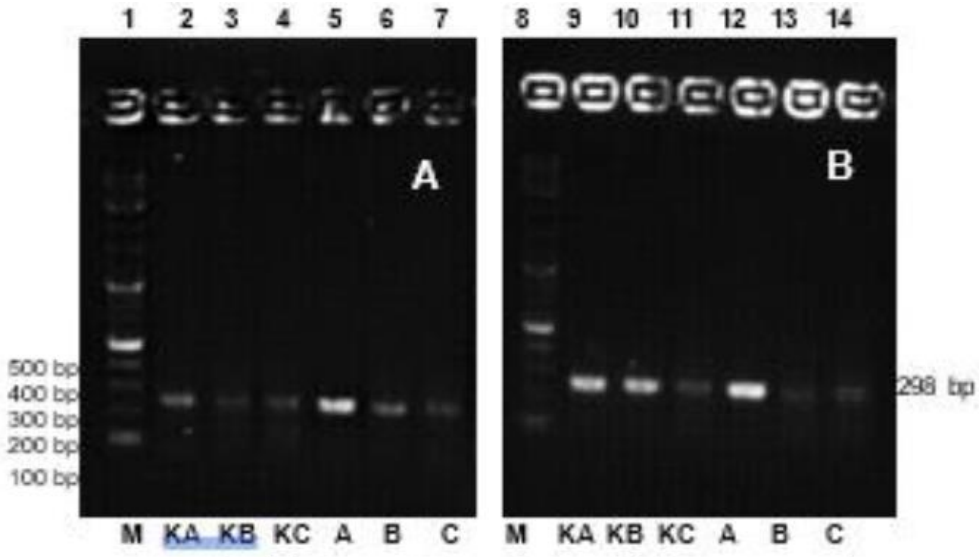
Figure 2. PCR results showed that positive values for IMNV presented in lines 3, 4, 7, 10, 12 and 16.



Ket: M (Marker); KA, KB, KC (Control); A,B,C (Challenge test treatment)

Line:
1. Marker
2. Control 100 ind/m^2
3. Control 200 ind/m^2
4. Control 400 ind/m^2
5. Treatment 100 ind/m^2
6. Treatment 200 ind/m^2
7. Treatment 400 ind/m^2
8. Marker
9. Control 100 ind/m^2
10. Control 200 ind/m^2
11. Control 400 ind/m^2
12. Treatment 100 ind/m^2
13. Treatment 200 ind/m^2
14. Treatment 400 ind/m^2

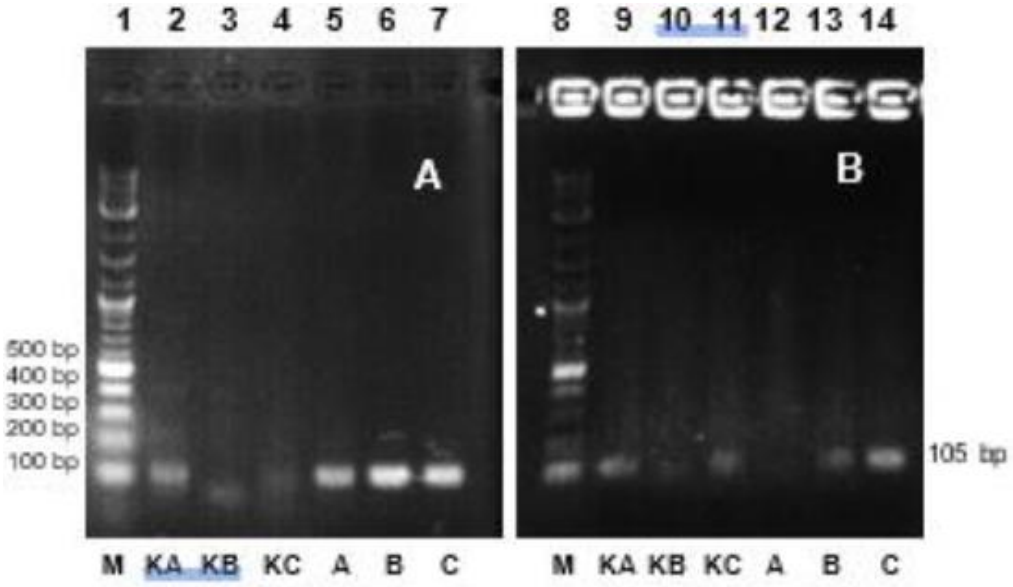
Figure 3. RT-PCR test for lectin gene expression at (A) dpi-5, (B) dpi-15.



Ket: M (Marker); KA, KB, KC (Control); A,B,C (Challenge test treatment)

Line:
1. Marker
2. Control 100 ind/m^2
3. Control 200 ind/m^2
4. Control 400 ind/m^2
5. Treatment 100 ind/m^2
6. Treatment 200 ind/m^2
7. Treatment 400 ind/m^2
8. Marker
9. Control 100 ind/m^2
10. Control 200 ind/m^2
11. Control 400 ind/m^2
12. Treatment 100 ind/m^2
13. Treatment 200 ind/m^2
14. Treatment 400 ind/m^2

Figure 4. RT-PCR test for Toll Receptor gene expression at (A) dpi-5, (B) dpi-15.



Ket: M (Marker); KA, KB, KC (Control); A,B,C (Challenge test treatment)

Line:
1. Marker
2. Control 100 ind/m^2
3. Control 200 ind/m^2
4. Control 400 ind/m^2
5. Treatment 100 ind/m^2
6. Treatment 200 ind/m^2
7. Treatment 400 ind/m^2
8. Marker
9. Control 100 ind/m^2
10. Control 200 ind/m^2
11. Control 400 ind/m^2
12. Treatment 100 ind/m^2
13. Treatment 200 ind/m^2
14. Treatment 400 ind/m^2

Figure 5. RT-PCR test for TCTP gene expression at (A) dpi-5, (B) dpi-15.

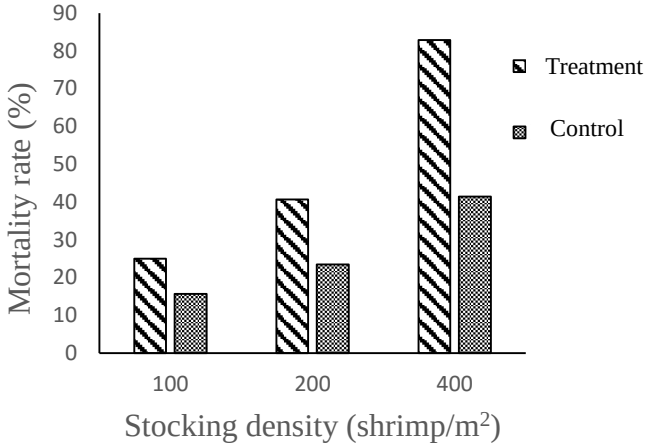


Figure 6. Mortality rate of *L. vannamei* in each treatment group with different stocking densities and observed at 15 days after IMNV infection.

Confirmation of IMNV in *L. vannamei*

Confirmation of IMNV virus in the body of the test shrimp was carried out using RT-PCR. The results of the PCR test with a PCR product target of 139 base pairs (bp) showed that treatment with the test had succeeded in infecting shrimp at dpi-5. In density treatment without challenge, death was reported but not due to IMNV. Even though quantitative PCR analysis was not carried out, the PCR results proved that there were differences in causes of death between challenge and control tests. Images of PCR results with selective samples and representing each day post-infection were presented in Figure 2.

Stress-related Genes in Shrimp

Observations were made at the beginning and end of dpi-5 and dpi-15, respectively. Gene expression results for each density treatment group, challenge test, and control were different (Table 5).

Lectin gene expression upregulated in the viral challenge treatment, specifically when the first clinical symptoms appeared (dpi-5). Figure 3 reported that the lectin gene in dpi-5 only appears in UT IMNV treatment at all densities. The expression shows that shrimp are experiencing stress due to the injection of IMNV virus. At dpi-15, the lectin band only appeared in UT IMNV treatment with a density of 400 shrimps/m^2. In other densities of 100 shrimps/m^2 and 200 shrimps/m^2, no lectin gene expression occurred.

Lectin genes increased during virus treatment, specifically when the first clinical symptoms were reported (dpi-5). The results showed that expression was upregulated in the IMNV challenge treatment. At dpi-15, almost all lectins did not appear, except in density treatment and UT IMNV 400 shrimps/m^2 because shrimp experienced stress due to crowding and viruses. Lectins are proteins capable of recognizing and binding certain carbohydrates or glycoproteins (Kurniawan *et al*., 2018). Due to this ability, several previous study reported the inclusion of the protein in immune signaling pathways in crustaceans. These included opsonization, phagocytosis, activation of the proPO cascade, and regulation of expression of other immune genes, such as antimicrobial peptides (AMPs) (Viana *et al*., 2022; Fikri *et al*., 2022). Lectins played a role in recognition patterns and defense responses against infections (Zhang *et al*., 2009). However, study from Yudiati (2016) stated that lectins decreased or were downregulated due to WSSV invasion.

Toll receptor is shrimp's innate immune system downregulated after IMNV virus infection. The penetration of pathogens through the skin or gills is recognized by the Toll receptor, as presented in Figure 4.

Shrimp defence mechanisms, particularly the Toll receptor, are not as well understood as those of insects and mammals. However, due of its significance in disease prevention, there is now more curiosity on prawn immunity. By

upregulating costimulatory molecules, triggering antigen presentation, and producing proinflammatory cytokines and chemokines, toll receptors can trigger antimicrobial responses (Yang *et al*., 2007). Toll receptors play a role in defense against fungi, gram-positive bacteria, and viruses. In this study, the first sampling was carried out at dpi-5, showing the results of the receptor gene expression in control and UT IMNV density treatments of 100–200 shrimps/m^2. Therefore, the expressed Toll Receptor gene does not affect IMNV infection or controls. At dpi 15 sampling, the Toll receptor gene is expressed in all IMNV UTs and controls at all densities. In this study, the protein was not influenced by IMNV challenge or density.

The Toll receptor gene is assumed to be expressed because shrimp experience stress due to crowding and IMNV challenge. During the last week of observation, the receptor gene appeared in treatments with lower density with the IMNV challenge test. This observation confirms that stressed shrimp conditions do not occur in other treatments after changing the water in the rearing media. Toll receptors include defense against fungi, gram-positive bacteria, and viruses (Yudiati, 2016). Furthermore, the protein plays an important role innately in immune and inflammatory responses (Yang *et al*., 2007). According to Feng *et al*.,(2016), Toll receptor gene expression increased in post-challenge treatment with WSSV. Another study stated that there was no significant difference in the protein tested by WSSV (Wang *et al*., 2010).

TCTP is an upregulated shrimp innate immune system. Similar to the lectin gene, TCTP will respond first when cell death occurs to stimulate anti-apoptotic activity. At the dpi 5, expression occurred during the IMNV challenge test in all treatment groups, but not in the control. At the dpi 15, TCTP gene expression appeared in the high-density treatment in stocking densities of 200 and 400 shrimps/m^2, and a clear band was reported in the control of 400 shrimps/m^2. In this study, TCTP gene expression was influenced by the IMNV challenge test and stocking density (Figure 5).

The TCTP expression increased after challenge with WSSV, and the protein played an important role in shrimp immune response. TCTP is a gene included in the response to extracellular signals and cellular conditions such as stress (Wu *et al*., 2013). In addition, it is known as a highly conserved multifunctional protein in eukaryotes that plays an important role in cell growth, cycle progression, and anti-apoptotic activity (Liu *et al*., 2005; Tahir *et al*., 2024). In this study, the stress level of shrimp was reported in each treatment group. This incident was due to worsening water quality and increased accumulation of feed waste to increase the infectious power of IMNV. At the dpi 15, the electrophoretic band appeared in the IMNV challenge treatment with a density of 200 and 400 shrimps/m^2. In this context, the results of observations from the last sampling showed that TCTP gene expression was influenced by the stocking density of *L. vannamei.*

Mortality Rate of *L. vannamei*

The observed mortality rate is the total cumulative mortality during 15 days of observation after IMNV infection. The highest result was obtained from the treatment group with a stocking density of 400 shrimps/m^2. On the 3rd dpi, shrimp died in the treatment group with a stocking density of 100 shrimps/m^2 without showing clinical symptoms, as reported in Figure 6.

The mortality rate with a density of 400 ind/m^2 and tested against IMNV reached 82.83%, while the control treatment was only 41.40%. This percentage was certainly higher than the density group with IMNV challenge tests of 200 shrimps/m^2 (40.63%) and 100 shrimps/m^2 (25%) with an infection occurring at 5 dpi. The control treatment tested at dpi-11 showed that the rate of shrimp death was not caused by disease. According to Coelho *et al*., (2009), the most severe IMNV infection was in shrimp measuring 3–12 g/shrimp with a mortality rate of more than 60%. Other study confirmed that IMNV infection resulted in 40–70% mortality, a prevalence rate of 11.1%, and an incidence of 58% (Novitasari *et al*., 2017). The recommended stocking density to

support the growth performance of *L. vannamei* on a field scale was 100–400 shrimps/m^3 for intensive and super-intensive systems, provided water quality management and early monitoring of infectious diseases were needed by farmers (Irani *et al*., 2023). This can be achieved by providing nutrition and selecting quality shrimp seeds from healthy broodstock (Rahardjo *et al*., 2022; Sookying *et al*., 2011; Wiradana *et al*., 2023).

CONCLUSION

The expression of stress-related genes such as lectin and TCTP was reported to experience upregulation after IMNV infection under the influence of stocking density of *L. vannamei*. Meanwhile, the Toll receptor gene was subjected to downregulation without stocking density. The higher density, the faster the appearance of IMNV clinical symptoms and the death rate. Stocking density influenced IMNV infections, showing the necessity for monitoring efforts regarding other diseases. In this context, farmers and relevant authorities should be provided with comprehensive insights for the effective management of *L. vannamei* cultivation.

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AUTHORS' CONTRIBUTIONS

MN: conceptualization, preparation of the study, and performed sample preparation. NKB and SR: Validation, supervision, and formal analysis. HBU and PAW: Drafted the manuscript, performed the statistical analysis, and prepared the table and figure. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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