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Optimal oral dose of VP24 dsRNA enhances survival and immunity in *Penaeus monodon* during WSSV challenge

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Abstract

The application of dsRNA was commonly conducted by injection and oral administration. Due to the limitations of muscular injection, delivering dsRNA by oral administration has been proven to be an effective approach for use in shrimp. One of the key factors in improving the effectiveness of the dsRNA vaccination is the concentration of dsRNA in the shrimp diet. This study was conducted to evaluate the influence of different dsRNA concentrations enriched in shrimp feed via oral vaccination on tiger shrimp. The dsRNA VP24 vaccine was produced by inactivating recombinant bacteria *Escherichia coli* DH5α using the heat-killed bacteria by the immersion technique. The density of inactivated bacteria (cells) was a reference to the dsRNA concentration in 0.02 g of shrimp feed, as the treatments, such as (A) control without dsRNA. (B) 10^7 cells, (C) 10^8 cells, and (D) 10^9 cells. The shrimp (n=160) were challenged with WSSV by intramuscular injection with 50 µL/shrimp after 15 days of vaccination. The present study results showed that the different concentrations of dsRNA had a significant survival rate, proPO activity, and THC ($P<0.05$) in tiger shrimp. The highest survival rate (33.3%), proPO activity (0.054), and THC (5.39×10^6 cells/mL) were obtained in the concentration of 10^8 cells. The proPO gene also showed a higher expression in 10^8 cells than in the other treatments. Hyaline cells had the highest average percentage of DHC, while semi-granular cells had the lowest. This finding suggested that the optimal concentration of 10^8 inactivated bacteria cells per 0.02 g of shrimp feed for the VP24 dsRNA vaccination was recommended to be applied at a feed frequency of three times a day at 3.5% of body weight.

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Introduction

Application of biotechnology tools in solving crustacean disease problems has been carried out with several approaches to produce a specific pathogen resistance (SPR) shrimp (1). Induction of shrimp immunity by the vaccination method has been reported by using the heat-killed bacteria vaccine in *Litopenaeus vannamei* (2) as well as using recombinant white spot syndrome virus (WSSV) protein in the shrimp *Penaeus chinensis* (3) and *L. vannamei* (4), and double-stranded RNA (dsRNA) in white shrimp *L. vannamei* (5–7) and in the kuruma shrimp *P. japonicus* (8,9). Enhancing the immune response in shrimp has been successfully carried out in crustacean species by applying the technology of RNA interference (RNAi) (10–14). Therefore, the RNAi technology of the dsRNA vaccine will be potentially applied to the tiger shrimp *P. monodon* using the viral protein (VP) genes. Some VPs have been identified as virulence genes and are involved in the process of WSSV infection.

The utilization of RNAi technology for disease prevention presents a novel strategy for the oral dsRNA vaccine administration to shrimp (15). The VP24, a main structural viral protein of WSSV, plays an essential role in the shrimp infection process (16,17). A gene encoding VP24 was successfully characterized (18), and a VP24 dsRNA vaccine has been applied to the tiger shrimp by intramuscular injection to improve survival rate, total haemocyte count (THC), and prophenoloxidase (proPO) activity (19). The tiger shrimp treated with VP15 dsRNA showed a survival rate 75% higher compared to shrimp without vaccination (20). The effect of different doses of VP24 and VP15 dsRNA by intramuscular injection has been evaluated on the immune response and survival rate of tiger shrimp (19,21). Due to the limitations related to difficulties in their application to the population, delivering dsRNA by oral administration seems to be an effective strategy for applying to a large individual of the population (22).

The delivery of dsRNA vaccine through feeding is necessary for the efficient and environmentally friendly application of RNAi as a molecular assay for controlling viral shrimp diseases (23,24). Because of its more accessible and applicable nature on a large scale, RNAi research has been focused on the application of dsRNA produced in vivo to enrich tiger shrimp feed (25,26). The concentration of dsRNA in terms of recombinant bacteria quantity in the shrimp feed enrichment is one of the main points to be assessed to develop the dsRNA vaccine performance.

Different concentrations may trigger different responses of dsRNA cellular uptake, so it is crucial to determine the optimal concentration that allows for effective delivery into cells or organisms under investigation. Therefore, the present study was conducted to assess the effect of the different dsRNA concentrations in the shrimp feed and applied as an orally administered vaccine on *P. monodon*.

Materials and methods

Ethical approval

This study conformed to the guidelines of animal ethical treatment for the care and use of experimental animals for crustacean (invertebrate) species. This study has followed the standard operating procedure of the Marine and Fisheries Research and Human Resources Agency, Ministry of Marine Affairs and Fisheries of the Republic of Indonesia, and the National Research and Innovation Agency (BRIN), Indonesia.

Production of VP24 dsRNA

The dsRNA vaccine was prepared by growing the recombinant *Escherichia coli* strain DH5α carrying the dsRNA gene construct in LB liquid medium using eight 250 mL tubes, each filled with 100 mL of culture medium, followed by overnight incubation on a shaker at 120 rpm at 37˚C. The bacteria were counted according to the method of Pangloli et al. (27). The bacterial sample was diluted in a saline solution to achieve a range of concentrations, and individual colonies were well-separated and countable. A small measured volume (approximately 100 µL) of each dilution was transferred onto separate agar plates, using the spread plate method to evenly distribute the sample across the surface. The plates were incubated under appropriate conditions of 37°C for 24 hours to enable bacterial growth. After incubation, the number of colonies on this plate was counted, and then the bacterial colony density (CFU) was calculated using the formula:

$$\text{CFU/mL} = \frac{\text{Number of colonies} \times \text{Total dilution factor}}{\text{Volume of cultured plates in mL}}$$

The pellet collected by centrifuging bacteria for five minutes at 5,000 rpm was used as a PCR template and vaccine material. The dsRNA vaccine was applied by inactivating bacteria with a heating system (heat-killed bacteria) by the immersion method for 5 minutes at 80ºC (28). The successful inactivation of bacteria by the heating process was verified by re-growing bacteria and determining the presence of dsRNA gene constructs of T7 promoter and VP24 (T7-VP24) in bacterial plasmids through a PCR technique (29).

Enrichment of shrimp feed by dsRNA

The commercial shrimp feed was enriched with dsRNA according to the method developed by Puneeth et al. (30). The harvested bacteria were then dissolved in a saline solution. The dsRNA in the form of inactive bacteria with the appropriate density in terms of dsRNA concentration as the treatments, namely: (A) control without dsRNA, (B) 10^7 cells, (C) 10^8 cells, and (D) 10^9 cells. The different concentrations (based on the treatments) of bacterial density was mixed with 0.02 g of shrimp feed (equivalent to 10 µg, 100 µg, and 1,000 µg bacteria in 0.02 g feed). The feeds containing dsRNA were then incubated for 15 minutes on ice and then coated with fish oil (15 mL for 1,000 g of feed) to avoid the dispersal of dsRNA into the rearing water.

Animal testing and challenged test

The animal testing in this study (n=160) was the tiger shrimp of a size of 15.28±0.82 cm in length and 34.76±5.72 g in weight. The shrimp were tested for free WSSV infection using the method of Parenrengi et al. (29), and then acclimatized in tanks for one week before being given the feed based on the treatments in four replicates for each (three for survival observation and one for immune response sampling). The vaccinated shrimps were reared in 250 L of 32 ppt seawater at a density of 10 shrimp/tank. The shrimp were fed at a frequency of three times a day at 3.5% of their body weight. After 15 days of vaccination, the shrimp were

challenged with WSSV by intramuscular injection with 50 µL/shrimp, based on our preliminary study (29). After that, the shrimp were fed with normal commercial feed at the same dose for the study's challenging test duration. The dead shrimp after the challenge test were morphologically and molecularly assessed to determine the effect of virus exposure. This analysis was carried out only to validate the cause of shrimp death, but not to determine the quantity of virus attacks on shrimp.

Observation of survival rate and immune responses

The observation of the survival rate was conducted daily during the challenge test. The immune responses, such as total hemocyte count (THC), prophenoloxidase (proPO), and differential hemocyte count (DHC) of three randomly representative samples were performed before (initial) and on the 1st, 3rd, and 5th day post-challenged test (dpc). The expression of the proPO gene was analysed at the end of the study.

The THC and DHC observations were carried out by collecting 0.1 mL hemolymph from the second segment using a syringe of 1 mL containing 0.3 mL of anticoagulant sodium citrate 3.8% (31). The mixture was homogenized by shaking hands to form a figure eight. The first drop was discarded and then dripped into the hemocytometer. The THC was observed using a hemocytometer under a binocular microscope with a magnification of 100 times, according to the formula Braak (31). THC was calculated using the formula:

$$N = \frac{n1 + n2 + n3 + n4 + n5}{5} \times 25 \times 10^4$$

where: N = Number of haemocyte cells (cells/mL), n1, n2, n3, n4, n5 = Number of haemocyte cells in a haemocytometer small box (cell).

The DHC was counted based on the morphological identification of the cell types (hyaline, semi-granular, and granular) (32) and presented in the hemocyte cell type composition. Haemocyte number was counted up to 100 cells to determine the percentage of each type, using the formula:

$$H = \frac{N}{\text{Total haemocyte (cell)}} \times 100\ \%$$

Where: H = Percentage of haemocyte cell types (%), N = Number of each type of haemocyte cell (cell).

The observation of proPO activity was performed according to the formation of dopachrome produced by L-dihydroxyphenylalanine (L-DOPA) and measured in a spectrophotometer with an absorbance wavelength of 490 nm. The measurement of optical density (OD) activity refers to the procedure of Liu and Chen (33) by using a negative control (haemolymph+L-DOPA+buffer) and a positive control (haemolymph+L-DOPA+trypsin). The OD of proPO activity under all test conditions was expressed as dopachrome formation in 50 µL of haemolymph.

The gene expression and viral detection were performed in a Gene Amp PCR System 2700 (Applied Biosystems, USA). The amplification process of the gene expression was conducted based on our previous standard PCR program (20). The proPO and gene β-actin expression in tiger shrimp was carried out by isolating RNA using an RNA isolation kit (Geneaid Biotech Ltd., New Taipei), and then cDNA synthesis was carried out to serve as a DNA template for the process. Total RNA was extracted from 10 mg of shrimp hepatopancreas using an RNA isolation kit, followed by cDNA synthesis using the Ready-To-Go You-Prime First Strand Beads kit (Sigma-Aldrich, Germany). The cDNA results were used as DNA templates in the PCR amplification process using the proPO-1F: 5'-gat gca gga cgc caa gta c-3' and proPO-1R: 5'-gta gtc ggc agt cgt gtt g-3". PCR conditions were an initial denaturation at 95°C for 1 min, followed by 30 cycles of (denaturation at 95 °C for 15 s, annealing at 58°C for 15 s, and extension at 72 °C for 10 s), and a final extension at 72°C for 5 min. The β-actin gene expression, applied as an internal control using the primers (F: 5′-gag cga gaa atc gtt cgt gac-3′ and R: 5′-gga agg aag gct gga aga gag-3′), was carried out in the PCR conditions of an initial denaturation at 95°C for 1 min, followed by 35 cycles of (denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s, and extension at 72 °C for 10 s), and followed by 72 °C for 5 min. The PCR results were run on a 1.0% electrophoresis agarose gel and visualized using a gel documentation system. The presence of a DNA fragment in positions of approximately 450 bp and 106 bp indicated the gene expression of the β-actin and the proPO, respectively.

The tiger shrimp were screened using the PCR technique before being used in animal testing, and WSSV detection on the dead shrimp after the challenge test was also performed to verify the white spot disease infection, based on the method of Parenrengi et al. (29). The DNA genome was isolated using the commercial kit (Geneaid Biotech Ltd., New Taipei) and used as a DNA template in the PCR amplification process using the Ready To Go (RTG) You-Prime First Strand Beads (Sigma-Aldrich, Germany) and primers of WS-93 F: 5'- aga tag cga aac aac atc caa c -3' and WS-93 R: 5'- gtc gcc ttg ccg gaa att a -3'. The PCR was programmed to a pre-denaturation at 95°C for 1 min, 30 cycles of (denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 10 s), and then final extension at 72°C for 5 s. To confirm the successful amplification of the target gene, PCR results were run on a 1.0% agarose gel and visualized using a gel documentation

system. The presence of a band in size of 93 bp indicated a positive infected-WSSV.

Data analysis

The data on tiger shrimp survival and immune responses (THC and proPO activity) were statistically analyzed using the one-way ANOVA of SPSS at a significance level of 0.05. The results of DHC and gene expression observations were descriptively discussed.

Results

Production of VP24 dsRNA

According to the results of the experimental inactivation of bacteria, the recombinant bacteria containing the VP24-WSSV construct grew normally without heating, but they died after 5 minutes at 80˚C. (Figure 1A). The presence of DNA fragments at the appropriate position of VP24 indicated that a heating process did not damage the DNA construct in the plasmid (Figure 1B).

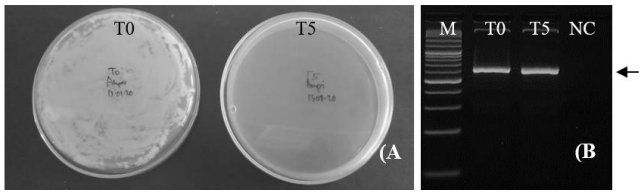

Figure 1. Verification of the dsRNA VP24 construct by re-growing the bacteria on the agar plate (A) and plasmid isolation on the agarose gel (B). T0 = without heating, T5 = heating with 80˚C for 5 minutes, M=100bp DNA marker, NC=negative control, and arrow=position of DNA plasmid of VP24.

Survival rate

A result of a challenge test on vaccinated tiger shrimp fed with a diet containing dsRNA at different concentrations showed that relatively large mortality occurred on the 3rd dpc, in which the survival rate showed differences between treatments up to the 5th dpc (Figure 2). At the 5th dpc, the highest survival rate was obtained at a concentration of 10^8 cells (33.3%), followed by a concentration of 10^7 cells (13.3%), and the lowest (3.3%) at the control and 10^9 cells. The use of bacteria-containing dsRNA with a concentration of 10^8 cells in the shrimp feed increased the survival of tiger shrimp by 30%, higher than other treatments. The statistical analysis on the 5th dpc indicated that different concentrations of dsRNA had a significant effect ($P<0.05$) on tiger shrimp survival.

Activity of proPO

The proPO activity tended to decrease with increasing exposure to WSSV until the 3rd dpc, and after that, proPO activity increased on the 5th dpc. The proPO activity in the concentration of 10^8 cells showed the highest average value, reaching 0.054, compared to other treatments (0.011-0.019) (Figure 3). The different concentrations of dsRNA showed a significant difference ($P<0.05$) in the average value of proPO activity.

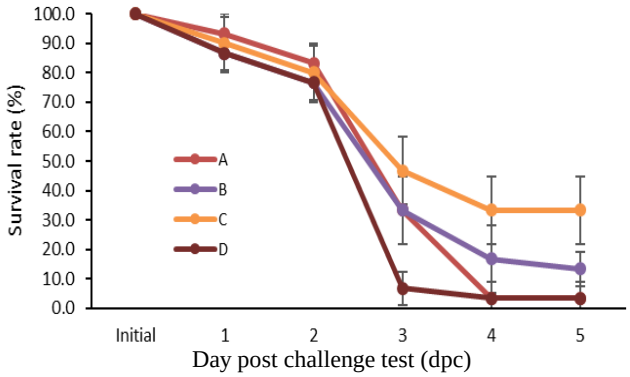

Figure 2: The cumulative survival rate of tiger shrimp after the WSSV challenge test (A=control without dsRNA, B=10^7 cells, C=10^8 cells, and D=10^9 cells).

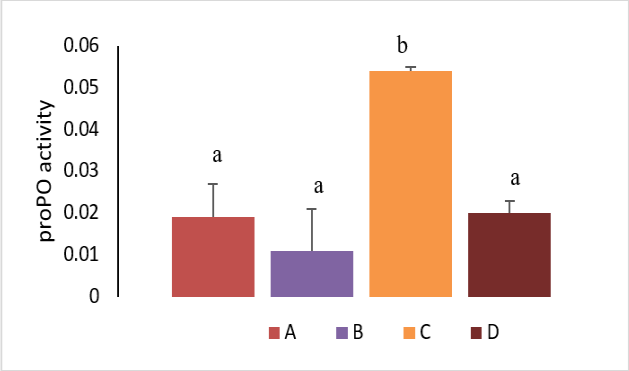

Figure 3. The proPO activity profile during the study of different concentrations of dsRNA post-challenge test (A=control without dsRNA, B=10^7 cells, C=10^8 cells, and D=10^9 cells). The significant difference ($P<0.05$) in average proPO activity was indicated by different small letters.

Total haemocyte count and differential haemocyte cell

The tiger shrimp resistance pattern through observation of the THC showed a tendency to decrease on the 1st day to the 3rd day after the WSSV challenge test, and then increase with a longer time of pathogen exposure. After the 3rd dpc, there was a tendency for THC to rise again, which was seen in the 5th dpc. The increase in THC, especially in the treatment of bacteria carrying dsRNA 10^7 and 10^8 cells. This indicated the effort to fight against pathogen infection by increasing the amount of THC in tiger shrimp haemolymp.

Observation of THC after the challenge test revealed that the feed containing dsRNA with a concentration of 10^8 cells showed a higher THC average of 5.39 x 10^6 cells/mL than the 10^9 cells (3.00 x 10^6 cells/mL), 10^7 cells (2.48 x 10^6 cells/mL and the lowest was in the control treatment of 1.30 x 10^6 cells/mL (Figure 4). The statistical analysis showed that the use of dsRNA vaccine was significantly different ($P<0.05$) in the amount of THC in tiger shrimp hemolymph.

The result of DHC observations on tiger shrimp showed that the highest percentage of hyaline cells was obtained for all treatments, while the lowest was for semi-granular cells. There was a tendency for all treatments to decrease the percentage of hyaline cells from the 1st dpc to the 3rd dpc, except for the treatment with a concentration of 10^8 cells, where there was an increase in the percentage of hyaline cells. This finding illustrated that the formation of new cells (hyaline) is a form of tiger shrimp resistance that increases the number of blood cells against pathogens. In general, a relatively high composition of hyaline blood cells was found in the dsRNA treatment with a concentration of 10^8 cells (Figure 5).

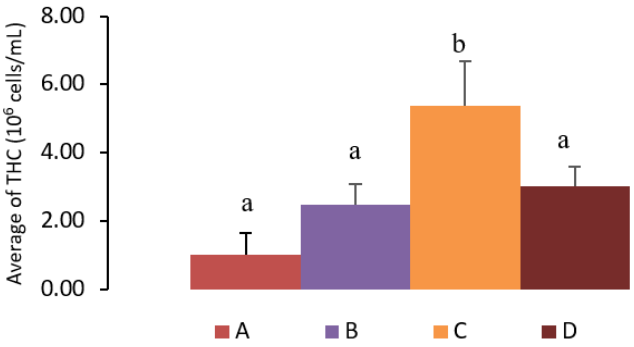

Figure 4. THC profile during the study of different concentrations of dsRNA after the WSSV challenge test. A=control without dsRNA, B=10^7 cells, C=10^8 cells, and D=10^9 cells, and the values with different letters were significantly different ($P<0.05$).

Expression of proPO gene

The successful isolation of total RNA, cDNA synthesis from tiger shrimp hemolymph, and the PCR process were indicated by the similar expression profile of the β-actin gene as an internal control (Figure 6A). The higher proPO expression was observed at 10^8 cells with the dsRNA concentration than the other treatments, and this treatment also showed the high proPO expression post-vaccination before the challenge test. Nonetheless, the proPO gene showed a relatively similar expression pattern between treatments, where there was a tendency for expression to increase on 1st dpc and then decrease on 3rd and 5th dpc.

White spot disease detection

The tiger shrimps were morphologically and molecularly observed to declare a negative or free from WSSV infection before they were used in the study. Figure 7 showed that the shrimp used for animal testing were free of the WSSV infection or had negative PCR detection. The shrimp that died after the WSSV challenge test showed disease signs of pathogen infection and positive PCR detection (Figure 7).

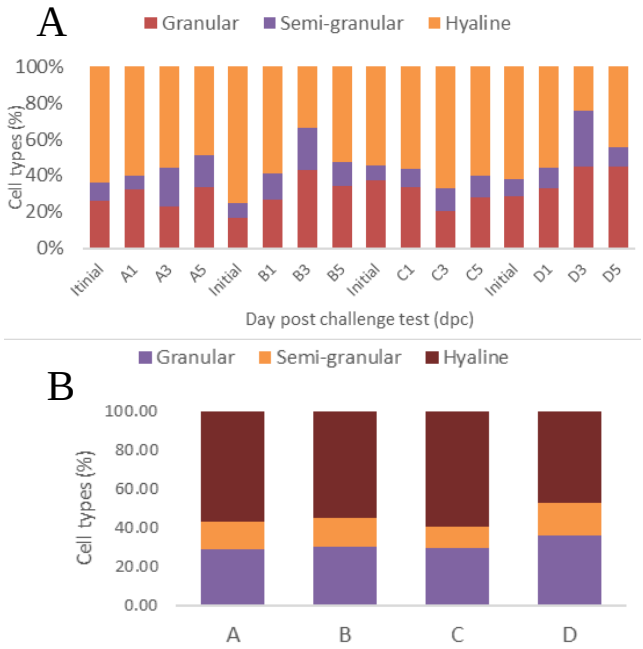

Figure 5. DHC profile during the study (A) and average value (B) of different concentrations of dsRNA after the WSSV challenge test (A=control without dsRNA, B=10^7 cells, C=10^8 cells, D=10^9 cells, 1=the 1st dpc, 3=the 3rd dpc, 5=the 5th dpc).

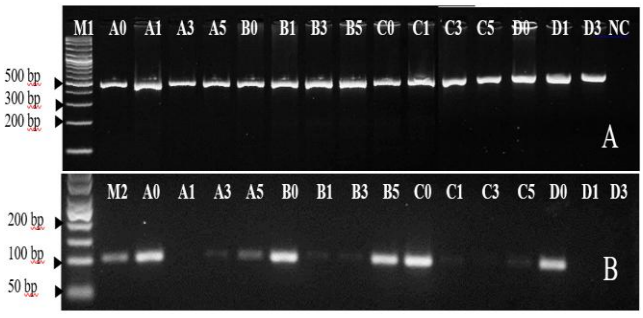

Figure 6. Expression of the β-actin (A) and proPO (B) genes at different dsRNA concentrations post-challenged test in tiger shrimp haemolymph (A=control, B=10^7 cells; C=10^8 cells; D=10^9 cells; 0=initial; 1=the 1st dpc, 3=the 3rd dpc, 5=the 5th dpc, M1=100bp DNA marker, M2=50bp DNA marker, and NC=Negative control.

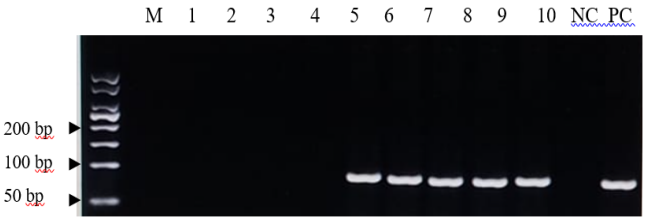

Figure 7. Verification of tiger shrimp test animals before and after the WSSV challenge test. M=50 bp DNA marker, 1- 5 = shrimp samples before the challenge test, 6- 10 = dead shrimp samples at the 3rd dpc, NC=negative control, and PC=positive control.

Discussion

The bacteria harboring the VP24 dsRNA construct demonstrated normal growth at ambient temperatures (34). However, exposure to 80°C for 5 minutes resulted in complete cell death, where high temperatures can disrupt bacterial cellular structures and functions, leading to inactivation (28). Despite the thermal inactivation of the bacteria, the dsRNA construct within the plasmid remained intact, as evidenced by the presence of DNA fragments at the expected position (35). This suggested that plasmid DNA demonstrates greater thermal stability than bacterial cells, remaining amplifiable even after heating (36,37), and the bacterial exposure to 80°C up to 15 minutes did not damage plasmids carrying dsRNA constructs (38). The bacterial inactivation step is the most crucial step before being used as a shrimp feed enrichment material for dsRNA vaccine purposes, including safety and vaccine efficacy.

Application of dsRNA in feed showed the enhancement of the survival rate of tiger shrimp when challenged with WSSV. The mechanism of dsRNA in silencing the virulence genes in shrimp viruses has been clearly described (39,40). The dsRNA-treated shrimp were found to have resisted infection by strengthening their immune systems and preventing the development of the WSSV virulence genes, resulting in a non-pathogenic gene. Our previous investigation showed that the application of dsRNA VP15 increased the survival rate of tiger shrimp by up to 36.7% compared to that without the use of vaccines or controls (3.3%) or the equivalent of an RPS (relative percentage survival) value of 37.9% (41). A study on tiger shrimp survival during a dsRNA VP-24 vaccine challenge test at a dose of 0.2 µg revealed a significant increase in survival by 65% compared to the tiger shrimp control, in only 10% (19). Although the challenge test analysis was conducted only up to the 5th dpc, the dsRNA treatment demonstrated significant efficacy, with survival rates stabilizing by that time (29). The data indicated that the test duration was sufficient to assess the vaccine's effectiveness. Consequently, the highest survival rate was observed in the group administered with 10^8 cells, suggesting this as the optimal dose for the dsRNA vaccine. However, the long-term effect of the dsRNA application still needs to be assessed in future studies.

Based on the proPO activity profile analysis on tiger shrimp, the concentration of 10^8 cells on the feed indicated the optimal dose of dsRNA. The proPO value of this treatment was significantly different from other treatment including the control and even the higher dsRNA concentration (10_9 cells). This indicated that the dsRNA has a point of optimal concentration; however, the higher dsRNA concentration stiil need to be comprehensively assessed. Crustacean immune response of proPO activity, commonly referred to as the humoral and cellular nonspecific immune systems, is a crucial immune protein of the shrimp's innate immunity (42). According to Robalino et al. (43), the specific RNAi response, a phenomenon triggered by dsRNA, serves antiviral functions in invertebrates, and innate antiviral immune reactions induced by dsRNA, including the proPO activity. When dsRNA enters the shrimp, it acts as a pathogen-associated molecular pattern to activate the proPO by converting into active PO, which catalyzes melanin formation (44). The cellular melanotic encapsulation, also known as melanization, is one of the most effective invertebrate immune strategies against foreign particles among the several types of humoral immune responses, whereby triggering the proPO system, which involves PO enzymes, melanin production is carried out to increase proPO activity (45), Conversely, a reduction in proPO system activity may result in tissue injury and phagocytosis failure (46). Parenrengi et al. (20) reported that the dsRNA vaccine treatment significantly improved the proPO activity of tiger shrimp compared to the control groups (without dsRNA). The highest proPO value using the shrimp intramuscular injection was obtained from the dsRNA by in vivo (0.138), followed by in vitro (0.093), and the lowest from control or without dsRNA (0.061). Applying VP24 dsRNA to tiger shrimp by injection showed that proPO activity increased from 24 to 72 hours post-WSSV challenge test (19). Different dosages of dsRNA injection significantly influenced the proPO activity, with the optimal dose of 0.2 µg per shrimp.

Blood parameters, such as THC, can be used to detect tiger shrimp's resistance level (47). The decrease of THC in the hemolymph after the challenge test is commonly known as the suppression system of the tiger shrimp's immune response due to infection (29). According to Yeh et al. (48), the migration of hemocyte cells from the body's circulatory system to the tissues as a result of numerous infected cells is typically what causes the decrease in THC. Unlike vertebrates, haemocytes rely entirely on innate immunity, and haemocytes are central to that system (49). A higher THC is strongly correlated with better protection because haemocytes are the frontline defenders in crustacean immune systems, responsible for phagocytosis, production of

antimicrobial molecules, and activation of immune pathways. They engulf and destroy invading pathogens such as bacteria, fungi, and viruses (50). The trend of THC response was relatively the same as survival and proPO pattern, where the highest THC was obtained in the concentration of 10^8 cells compared to other treatments. It was suspected that a density of 10^8 cells provided better protection than the control treatment (without dsRNA). Our previous study on the application of the VP24 dsRNA by the injection method suggested a similar response to the THC on tiger shrimp hemolymph post-challenge test. Based on the study of Mulyaningrum (19), the VP24 dsRNA application showed a significant influence on the amount of THC in tiger shrimp hemolymph, where 0.2 µg/shrimp was the optimal dose with a THC amount of 1.55×10^7 cells/mL. Increasing the amount of THC is one of the shrimp's defense efforts in enhancing its resistance to pathogenic infections. On the other hand, the survival and THC at a higher concentration (10^9 cells) of dsRNA were lower compared to 10^8 cells. The dramatic reduction in survival and THC on the 3rd dpc at the highest dsRNA concentration suggested the potential toxicity or adverse effects. However, the results of this study still need to be thoroughly investigated in terms of the mechanism of using high dsRNA concentrations and whether it will have a negative impact on the tiger shrimp.

The observed DHC in tiger shrimp treated with the dsRNA vaccine provides valuable insights into the immune response mechanisms activated upon pathogen exposure (5). The data indicate that the highest percentage of hyaline cells was consistently observed across all treatments, while semi-granular cells exhibited the lowest percentages. Notably, a trend emerged where the percentage of hyaline cells decreased from the 1st to the 3rd dpc in all treatments, except for the group administered with 10^8 cells, which showed an increase in hyaline cell percentage during the same period. The observed increase in hyaline cell percentage in the 10^8 cell treatment group implied that this concentration may optimally stimulate the shrimp's innate immune system, leading to a more robust cellular defense response. Hyaline cells, semi-granular cells, and granular cells are the three primary types of blood cells found in crustacean species, including tiger shrimp, which are involved in immunity and protection against infectious diseases (32).

An increased immune gene expression response on the 1st dpc was suspected because the tiger shrimp tried to fight the virus. After that, due to the very strong infection attack, suppression of the immune response occurred, which resulted in decreased gene expression. RNAi technology can directly damage WSSV, but can also induce immune response genes (5,39,40). Therefore, the proPO gene's expression trended similarly between dsRNA treatments and the control, presumably due to dsRNA responses that occurred more in the process of directly damaging pathogens than in inducing tiger shrimp antiviral genes. Even so, to support this finding, detailed research still requires more in-depth quantitative research using quantitative real-time qPCR. The present study revealed that the use of dsRNA upregulated the proPO expression when exposed to the pathogen. A similar finding has been reported by Paria et al. (51) that introducing dsRNA to tiger shrimp increased the expression of the proPO gene in a sequence-independent way. This upregulation expression occurred more acutely and early in nature and was constant until 72 hours. Therefore, the present study suggested that the induction of dsRNA increased proPO expression as a promoting factor in protecting tiger shrimp against WSSV in both specific and nonspecific dsRNA-injected shrimp. Taken together, these findings support the hypothesis that dsRNA-mediated induction of proPO contributes significantly to antiviral defense in tiger shrimp. The study therefore highlights the potential of dsRNA for both as a sequence-specific RNAi therapeutic agent and as a broader immunomodulatory tool to enhance resistance against WSSV infection in aquaculture systems. So, the dsRNA can be harnessed not only for gene-specific silencing but also for stimulating innate immune responses in crustaceans (52).

The symptoms of clinical infection indicators of this present study are in line with those documented in previous studies, where WSSV infection in shrimp led to a rapid onset of clinical signs and high mortality rates within a short period post-infection (53). The tiger shrimp death was characterized by clinical symptoms in the form of white spots on the carapace, and they were molecularly confirmed by the PCR technique. These symptoms are among those mentioned in previous studies (54–56). The decrease in feed response was caused by the disruption of the function of organs such as the antennae and antenula, caused by the infection of the test shrimp, which caused changes in the body's metabolism (57). All infected shrimp exhibited less activity and more passive swimming behaviour as a result of their inability to detect food efficiently, which lowers the quantity of energy taken by the body. While the body of the test shrimp itself requires a lot of energy to boost the body's immune system in fighting WSSV infection attacks, as a result, the shrimp are susceptible to disease (58). Apun-Molina et al (59) also reported that reduced hemocyanin levels and impaired glucose stress responses in WSSV-infected shrimp indicated that the immune activation consumes energy reserves and weakens resilience.

Conclusions

The different concentrations of VP24 dsRNA showed a significant effect on the survival rate, proPO activity, and THC of tiger shrimp. The inactive bacteria concentration of 10^8 cells per 0.02 g of feed was an optimal dose of the dsRNA vaccine. The highest average proportion of DHC was hyaline cells, and the lowest was semi-granular cells. The

highest expression of the proPO gene was also shown in the dsRNA concentration of 10^8 cells. The present study suggested that the concentration of 10^8 cells for the VP24 dsRNA vaccine was recommended to be applied to enrich with 0.02 g of shrimp feed.

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Conflict of interest

The authors declare no conflict of interest.

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جرعة فموية مثالية من VP24 dsRNA تعزز البقاء على قيد الحياة والمناعة في *Penaeus monodon* خلال تحدي WSSV

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الخلاصة

وقد أجريت تطبيق dsRNA عادة عن طريق الحقن والإعطاء عن طريق الفم. بسبب القيود المفروضة على حقن العضلات، وقد ثبت أن اعطاء dsRNA عن طريق الفم ليكون نهجا فعالا للاستخدام في الروبيان. أحد العوامل الرئيسية في تحسين فعالية dsRNA هو تركيز dsRNA في غذاء الجمبري. أجريت هذه الدراسة لتقييم تأثير تركيزات مختلفة من dsRNA المخصبة في علف الجمبري عن طريق التطعيم الفموي على جمبري النمر. تم إنتاج لقاح dsRNA VP24 عن طريق تعطيل البكتيريا المؤتلفة الإشريكية القولونية DH5α باستخدام البكتيريا المقتولة بالحرارة بواسطة تقنية الغمر. كانت كثافة البكتيريا المعطلة (الخلايا) إشارة إلى تركيز الحمض النووي الريبي في ٠,٠٢ غرام من علف الروبيان، مثل العلاجات، مثل (أ) السيطرة من دون dsRNA. (ب) ١٠^٧ خلايا، (جـ) ١٠^٨ خلايا، و (د) ١٠^٩ خلايا. تم اجراء التحدى مع WSSV للروبيان (العدد =١٦٠) عن طريق الحقن العضلي مع ٥٠ مايكروليتر/ روبيان بعد ١٥ يوما من التطعيم. وأظهرت نتائج الدراسة الحالية أن تركيزات مختلفة من dsRNA كان معدل البقاء على قيد الحياة كبيرة، ونشاط proPO ، THC ($P<0.05$) في الروبيان النمر. تم الحصول على أعلى معدل البقاء على قيد الحياة (٣٣,٣٪)، ونشاط proPO (٠,٠٥٤)، و THC (٥,٣٩ x ١٠^٦ خلايا/مل) في تركيز ١٠^٨ خلايا. أظهر جين proPO أيضا تعبيرا أعلى في ١٠^٨ خلايا مقارنة بالعلاجات الأخرى. تحتوي الخلايا الهيالينية على أعلى متوسط نسبة مئوية من هيدروكلورايد، بينما تحتوي الخلايا شبه الحبيبية على أدنى نسبة. أشارت هذه النتيجة إلى أن التركيز الأمثل لـ ١٠^٨ خلايا بكتيرية معطلة لكل ٠,٠٢ جرام من علف الجمبري للتطعيم ضد VP24 dsRNA يوصى بتطبيقه بتردد تغذية ثلاث مرات في اليوم بنسبة ٣,٥ ٪ من وزن الجسم.