Effect of different doses of dsRNA VP15 vaccine for controlling white spot syndrome virus infection in tiger shrimp *Penaeus monodon*

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Abstract. White spot syndrome virus (WSSV) is the most virulent pathogen in cultured penaeid shrimp, including *Penaeus monodon*. RNA interference (RNAi) technology has been applied for silencing of viral gene expression during infection, through induced by double-stranded RNA (dsRNA) of WSSV viral protein (VP). This study aimed to evaluate the effect of different doses of dsRNA VP15 WSSV on the survival rate and immune response of tiger shrimp. Production of dsRNA was conducted by in-vitro using the MEGAscript RNAi kit. Different doses of dsRNA vaccination as treatments were: (A) 0.02 µg, (B) 0.2 µg, (C) 2 µg and (D) control (injected with 50 µL of saline solution) for each shrimp. Vaccination was carried out by intramuscular injection to tiger shrimp (15.88 ± 3.50 g) and the shrimp were challenged by injection with WSSV. Survival rate was counted daily, while total hemocyte count (THC), prophenoloxidase (proPO) activity, and antiviral gene expression were observed before and 1-, 3- and 5-days post-challenge (dpc). The results showed that the dsRNA VP15 vaccine was successfully produced by the MEGAscript RNAi kit. The different doses of dsRNA VP15 significantly influenced the tiger shrimp survival (P<0.05), in which a dose of 0.02 µg/shrimp showed the highest shrimp survival (15%) compared to the other doses and control. The THC and proPO activity of vaccinated shrimp showed a higher value compared to the control. A trend to increase the antiviral gene expression was obtained until 3 dpc and then decrease at 5 dpc. The results indicated the application of dsRNA VP15 as a potential way to control WSSV infection of tiger shrimp.

1. Introduction

White spot syndrome virus (WSSV) is a widespread viral pathogen and highly virulent in penaeid shrimp as well as many other crustaceans by distinctive clinical signs of white spots [1,2]. The protein components of WSSV virion have been well established by proteomic methods, and at least 39 structural proteins are currently known [3], including viral protein (VP) involved in shrimp disease infection. The WSSV consists of an enveloped rod-shaped nucleocapsid enclosing a large circular double-stranded DNA genome of 293 kbp. The virion envelope contains two major proteins of 28 (VP28) and 19 kDa (VP19) and the nucleocapsid consists of three major proteins of 26 (VP26), 24 (VP24), and 15 kDa (VP15) [4].

A strategy of prevention and treatment of viral infections is very challenging, due to the virus uses the host cell machinery to replicate. The development of antiviral vaccines is needed to be understood...
both of host immune system and viral gene function. Recently, the application of RNA interference (RNAi) technology has been reported to be a potential technique to control the shrimp white spot disease by using the virulence genes from WSSV as known as a dsRNA vaccine [5–9]. The RNAi technology plays an important role by activating a sequence-specific RNA degradation process (post-transcriptional gene silencing). The development of vaccination technology using dsRNA agents grows very rapidly, especially in the fields of medicine and agriculture [10–12]. In the field of aquaculture, this technology has been developed to overcome diseases caused by viruses in cultured shrimp [13,14]. Several studies on shrimp vaccination using a viral protein from WSSV have shown great progress and obtained encouraging results [15,16].

A gene encoding WSSV VP15 has been successfully characterized and cloned as well as transformed to the bacteria [17]. Moreover, a previous study was confirmed that the application of dsRNA VP15 by in-vitro using MEGA Sscript RNAi kit showed a potential strategy for controlling tiger shrimp white spot diseases [18]. However, the dsRNA VP15 vaccine is needed to be developed by assessment of various doses to combat WSSV infection in tiger shrimp. This study aimed to evaluate the optimal dosage of WSSV VP15 dsRNA by in-vitro production to increase tiger shrimp survival and immune response to control WSSV infection.

2. Materials and methods

2.1. Isolation of VP15-WSSV and construction of VP15 with T7 promoter

A gene encoding VP15 WSSV was isolated from infected tiger shrimp by genome DNA extraction [16], using a pair of primers i.e: forward primer VP-15 F: 5'-cgc gga tcc gat gac aaa ata ccc cga gaa c-3 and reverse primer VP-15 R: 5- ccg gaa ttc tta acg cct tga ctt gcg gg-3' [5]. DNA amplification was performed in GeneAmp PCR System 2700. PureTaq RTG PCR beads kit was used as the PCR reaction and mixed with primer for each 1 µL (50 pmol/mL). The PCR was programmed at pre-denaturation temperatures of 94°C for 3 minutes; for 35 cycles (denaturation at 94°C for 1 min, annealing at a temperature of 57°C for 45 seconds, extension at a temperature of 72°C for 1 min); and a final extension at a temperature of 72°C for 5 minutes. The VP15 was then cloned and transformed to the competent bacteria [17].

Gene construct was done by adding the sequence of a T7 promoter (pT7) at the 5'-region of each primer. DNA amplification was performed on a PCR machine using the RTG kit as a PCR reaction, 1 µL (50 pmol/mL) each primer and dH2O to reach the final volume of 25 µL. The PCR program was set according to the procedure of VP15 isolation that was explained earlier. PCR product was run on agarose gel 2.0% in 1X TBE and documented by Gel Documentation System.

2.2. Production of dsRNA VP-15 WSSV

The production of dsRNA VP-15 WSSV by in-vitro was performed according to the MEGAscript RNAi Kit procedure [6]. A total of 1-2 µg of purified PCR product of VP-15 was mixed with ATP, CTP, GTP, UTP, 10×T7 reaction buffer, T7 enzyme mix in 2 µL for each. The solution obtained was incubated at 37 °C for six hours, then followed by incubation at 75 °C for five minutes. The dsRNA was left on the bench to cool at room temperature for 3-4 hours and then stored at -20°C.

Nuclease Digestion was applied to remove DNA and dsRNA. A total of 20 µL of dsRNA product was reacted with 21 µL of nuclease-free water, 5 µL of 10 x digestion buffer, 2 µL of DNase, and 2 µL of RNase. The solution was incubated at 37°C for 1 hour and then purified by a mixture of 50 µL dsRNA with 50 µL of nuclease-free water, 150 µL of 10x binding buffer, and 250 µL of 100% ethanol. The reaction was gently mixed by pipetting up and down. A total of 500 µL dsRNA mixture was applied onto the filter in the filter cartridge and then centrifuged at maximum speed for 2 min. The filter cartridge was washed with 500 µL of wash solution twice and then left for 10-30 sec to remove the last traces of liquid. A hundred µL of elution solution (preheated to ≥95°C) was applied to the filter in the filter cartridge and then centrifuged for 2 min at maximum speed. This step was
conducted twice. The reaction product was conducted by measuring its absorbance at 260 nm to calculate the concentration of dsRNA. The dsRNA was stored at –20°C before applying for vaccination.

2.3. Vaccination of dsRNA VP15 and challenge test with WSSV

Vaccination was carried out by the intramuscular injection of tiger shrimp (15.88 ± 3.50 g in weight and 11.91 ± 1.11 cm in length). The vaccine doses as treatments of this present study referred to the previous study [6], namely: 0.02 µg/shrimp; 0.2 µg/shrimp; 2 µg/shrimp; and injected with saline solution (SS) without vaccine as a control. Shrimp that has been vaccinated are reared in a controlled tank in size of 80 cm x 80 cm x 60 cm equipped with aeration, with a density of 10 shrimp per tank for three replications (two replications for survival observation and one replication for response immune analysis).

The WSSV isolate was obtained from the hemolymph of tiger shrimp infected with WSSV. The hemolymph was then centrifuged at 3,000 g for 20 minutes at 4°C. The supernatant was removed and then centrifuged at 8,000 g for 30 minutes at 4°C. The supernatant was filtered using a cellulose nitrate membrane filter of 0.2 µm and then stored in a bio-freezer (–80°C) as a stock of the WSSV virus. The WSSV stock was diluted with saline solution in a ratio of 1:3 (v/v) and injected into shrimp with a volume of 100 µL/shrimp. After five days of vaccination, the shrimp were challenged with WSSV whose pathogenicity has been confirmed. Shrimp survival was observed every day, while the response immunes (THC and proPO) and gene antiviral expression were observed at the beginning before WSSV injection, and on 1-, 3-, and 5-days post-challenge (dpc) [19,20]. The present study was designed with a completely randomized design. To determine the effect of treatments, the data of survival rate, THC, and proPO were statistically analyzed by using an analysis of variance (ANOVA) program, while antiviral gene expression data were descriptively analyzed.

3. Result and discussion

3.1. Production of dsRNA VP15-WSSV

The gene construct of the VP15-WSSV gene with T7 promoter (pT7-VP15) was successfully created using MEGAscript RNAi kit, indicated by the increasement of DNA molecular weight, due to the addition of nucleotide sequence of a T7 promoter (Figure 1). The pT7-VP15 gene construct consisted of a T7 promoter, in approximately 27 bp, and a gene encoding VP15-WSSV in approximately 243 bp [16].

![Figure 1. Electrophoresis result of dsRNA VP15-WSSV production using the MEGAscript RNAi Kit. M=DNA marker; 1 and 3 = gene construct of pT7-VP15; and 2 = control gene of VP15-WSSV (without pT7).](image)

3.2. Survival rate

Observation of tiger shrimp survival after the challenge test with WSSV showed that shrimp mortality has begun to appear on 1 dpc, and the shrimp mortality was getting higher on 3-6 dpc. However, a dose of 0.02 µg showed higher a cumulative survival rate than the control and other doses. Moreover,
on 7 dpc the tiger prawns for all treatments had 100% death, while at a dose of 0.02 µg was still 15% remaining until the end of the study (Figure 2). The statistical analysis indicated that the different doses of application dsRNA VP15 have significantly influenced (P<0.05) the survival rate of tiger shrimp. This result indicated that the application of the 0.02 µg dsRNA VP-15 vaccine could increase the survival rate of tiger shrimp up to 15%. This value was relatively lower when compared to the 65% survival rate increase in the application of 0.2 µg dsRNA VP24 in tiger shrimp and relatively close with the application of antiviral PmAV overexpression on tiger shrimp (24.5%) [21,22].

Some studies reported that recombinant DNA vaccine construct was able to protect tiger prawns up to 62% against WSSV infection [23]. By injection of in-vivo VP24 vaccine, the cumulative mortality of P. monodon increased up to 37% [5] and the survival rate of tiger shrimp 50% at a dose of 2.5 µg/g shrimp weight [9].

The interesting point of this result showed that the increased doses of dsRNA did not influence to increase the survival rate after the challenge test. Application of higher doses of 0.2 and 2.0 µg showed the shrimp survival lower than a dose of 0.02 µg. It was assumed that the higher doses of dsRNA may be acted as immune suppression to the shrimp, such as the use of immunostimulant. The use of immunostimulants exceeding the optimal dose could have a negative response to cause immune suppression in the shrimp, even they could protect shrimp against disease [24].

The study suggested that the application of the dsRNA vaccine plays an important result in the enhancement of 15% tiger shrimp survival. RNAi plays a key role in innate immune responses to viral infections in animals, plants, and insects. In crustaceans, RNAi mechanisms occur in several stages, in which initiation of RNAi involves the generation of small interfering RNAs (siRNAs) of 21-23 nucleotides in length by a protein known as dicer. Then, the siRNA degrades the mRNA, followed by gene expression becomes specifically in-active at the post-transcription stage [25,26].

### 3.3. Total haemocyte count

The total hemocytes count (THC) was observed at the beginning (before the challenge test) and then on the 1-dpc, 3-dpc and 5-dpc. A trend of decline in the number of THC after the challenge test was shown in Figure 3. At the 3-dpc there was a slightly increasing for the higher doses and then decreased to the 5-dpc, indicating the effort to combat virus infection. The result indicated that the higher doses showed the higher average number of THC, where the end of observation (5-dpc) showed that the highest THC cell was obtained in the dose of 0.02 µg/shrimp (390×10^4 cell/mL), followed by 0.2 µg/shrimp (270×10^4 cell/mL), 2.0 µg/shrimp (240×10^4 cell/mL) and the lowest was
control (120×10⁴ cell/mL). Statistical analysis showed that the different doses of dsRNA VP15 did not significantly influence (P>0.05) the THC of tiger shrimp. However, the vaccinated shrimp showed higher THC than the control shrimp. The previous study reported that application of dsRNA VP24 vaccine on tiger shrimp at the dose of 0.2 µg/shrimp was optimal for injection method to reach the highest number of THC (1,550 × 10⁴ cell/mL), compared to the control (without dsRNA), namely 490 × 10⁴ cell/mL [21].

![Figure 3](image)

**Figure 3.** The number of THC (in log value) of tiger shrimp vaccinated with different doses of dsRNA VP15 before and post-challenge test

In general, the result of this study showed that the number of THC tended to decrease after the WSSV challenge for both in the dsRNA application and the control (without vaccination). The decrease in THC after the challenge test was caused by the migration of hemocytes from the body's circulatory system to the tissues, with a large number of infected cells [27]. The critical phase of WSSV infection causes the reaction of hemocyte cells to carry out various mechanisms of body defence, consisting of the process of pathogen recognition, phagocytosis, melanization, and cytotoxicity.

The main effector of immune system cells in crustaceans is hemocytes, while the hepatopancreas is responsible for the biosynthesis of several humoral factors. The increase in hemocyte cells in the shrimp body plays an important role in inhibiting or destroying pathogens that enter the shrimp body. In shrimp, as in other crustaceans, there are three main types of hemocytes (blood cells) namely hyaline cells, semi-granular cells, and granular cells (granulocytes), all of which play a role in immunity and defense against infectious diseases. Immunostimulants that are carried out continuously can regulate and maintain the immune system in optimal conditions until the administration stops [12,28].

### 3.4. ProPO Activity

As with the amount of THC, the observation of prophenoloxidase (proPO) activity in tiger prawn hemolymph was carried out before and after the challenge test (1, 3, and 5 dpc). In general, proPO activity increased in the 3 dpc observation but after that, there was a decrease in activity until the end of the study (Figure 4). The average of proPO activity was relatively similar among doses, which the highest value was obtained in a dose of 0.2 µg followed by control, 2.0 µg and 0.02 µg, namely: 0.0448, 0.0321, 0.0280, and 0.0234 respectively. However, statistical analysis indicated that the different doses of dsRNA VP15 did not influence (P>0.05) the activity of proPO in tiger shrimp. A previous study reported that the highest proPO activity (0.042) on tiger shrimp was obtained on the application of dsRNA VP24 at a dose of 0.2 µg [21]. For this result of the present study, it was
supposed that the dsRNA VP15 was more referred to be functioned as a degradation process or post-transcriptional gene silencing, before induced the response immunes, including proPO activity.

![Figure 4](image.png)

**Figure 4.** The value of proPO activity in tiger shrimp vaccinated with different doses of dsRNA VP15 before and post-challenge test

ProPO is one of the major components of the innate immune system in shrimp. Humoral and cellular systems are characterized as two main components of which work together and are activated to combat invading pathogens in the event of an immune challenge, such as the coagulation of hemolymph and melanization by the ProPO system [29]. Both immune systems act synergistically to protect shrimp and remove foreign particles and pathogens. A cellular melanotic encapsulation, a type of humoral immune response, is known as the most effective invertebrate immune technique against foreign particles/pathogens. The melanization pathway is a principal innate immune response in shrimp, which is activated by the proPO system by involving proPO-activating enzymes [30,31].

3.5. Antiviral gene expression

One of the tiger shrimp resistance parameters to viruses is an expression of the PmAV antiviral gene by increasing the tiger resistance to the pathogen [32]. Expression of tiger prawn antiviral genes can be done through RNA extraction and cDNA production and gene amplification through PCR technology. To determine the success of cDNA synthesis, beta-actin gene expression was used as an internal control. The present study showed that the tiger prawn antiviral gene was expressed in all treatments, both dsRNA vaccinated shrimp at various doses and a control treatment or without application of dsRNA vaccine (Figure 5). There was a trend to increase the expression post-challenge test until 3 dpc and then decrease at 5 dpc. This gene expression indicated that tiger shrimp had a molecular effort to fight the virus when challenged with WSSV. However, the expression of these antiviral genes was relatively similar for all treatments.

Compared to transgenic tiger shrimp, after being challenged with WSSV, PmAV antiviral gene expression was observed for both transgenic and non-transgenic tiger shrimp and it showed up-regulated response by increased induction of antiviral gene expression [22]. This study reported antiviral gene expression in transgenic shrimp began to be induced 6 h after the challenge test and continued to increase sharply until day-4 and slightly decreased on day-5. While, non-transgenic tiger shrimp, even also increased induction until day-1 and decreased on day-2, and then the expression showed relatively lower until the end of the experiment.
Figure 5. Expression of antiviral genes in vaccinated tiger shrimp and challenged with WSSV. Antiviral gene expression (top) and β-actin tiger shrimp expression (bottom) as internal controls, 100+ = DNA marker 100 bp plus; (-) = negative control; (+) = positive control; A=0 µg; B=0.02 µg; C=0.2 µg; and D= 2.0 µg; and 0-5=days of post challenge test.

Base on the results of the present study, the enhancement of survival rate and immune response suggested that the application of the dsRNA vaccine had to be a potential strategy in the future development of tiger shrimp culture. For a more optimal application, it is still necessary to develop an indeed research to improve the performance of the dsRNA. Production by in-vitro and the application of the injection method have some advantages in preventing WSSV infection, but they have also limitations regarding the small scale of mass production by in-vitro and difficulty on apply injection for a huge individual in the population. Therefore in-vivo production of dsRNA is necessary and the application of immersion methods or application through feed will also be considered in future research implementation.

4. Conclusion
The gene construct of pT7-VP15 and dsRNA VP15 vaccine by in-vitro were successfully produced using the MEGAscript RNAi Kit. The application of dsRNA VP15 at the doses of 0.02 µg/shrimp by muscular injection increased the tiger shrimp survival up to 15%. The THC and proPO activity of vaccinated tiger shrimp showed a higher value compared to the un-vaccinated shrimp (control treatment). This finding suggested the use of dsRNA V15 has a potential technique for controlling the WSSV infection of tiger shrimp.

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