A preliminary experiment on production of dsRNA by in-vivo and its application to tiger shrimp *Penaeus monodon* larvae for survival enhancement

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Abstract. RNA interference (RNAi) is one of the most recent tools against shrimp viral infection by gene constructs of dsRNA induction. This study aimed to produce dsRNA by in-vivo method and to analyse the effect of its application to tiger shrimp larvae. The dsRNA production was conducted by cloning of genes encoding VP15 and VP24 of WSSV into L4440 vector containing T7 promoter, then transformed to *Escherichia coli* and grew in LB media for mass production. The bacteria were in-activated using heat-killed bacteria method by immersion at 80°C for 5, 10, and 15 min. Before WSSV-challenge test, tiger shrimp PL-12 were vaccinated by immersion using in-activated bacteria in concentration of $1.3 \times 10^8$ cell/mL for 30 min as a treatment and without vaccination was as a control. The results showed the successful production of VP-dsRNA by in-vivo. In-activated bacteria was effective at 5 min, since dsRNA did not damage. Survival of larvae at 5 days post challenge (dpc) in VP15-dsRNA was relatively higher (28.3%) compared to control (24.4%), and application of VP24-dsRNA also showed higher survival (86.9%) compared to control (83.1%) at 10 dpc. The result implied that the application of dsRNA exhibited a sign of potential way to produce resistant larvae.

1. Introduction

Penaeid shrimp culture contributes significantly to the economic development of the Indonesian fisheries sector. However, especially tiger shrimp *Penaeus monodon* industry has faced an outbreak of disease infected by viral agents, including white spot syndrome virus (WSSV). WSSV caused a mass mortality of tiger shrimp and more than 50% of shrimp pond became idle [1]. At the moment, it is difficult to find a qualified strategy approach to combat the viral diseases of tiger shrimp.

DNA recombinant approach, especially DNA vaccines has started to be applied in aquaculture [2]. Application of biotechnology in aquaculture to increase an immune resistance to fish or shrimp to fight disease or pathogen is one alternative solutions to disease problems. A potential mechanism for improvement disease resistance is aquatic animal production through the application of RNA interference (RNAi). A gene silencing mechanism called RNAi is one of high interest methods for protecting shrimp from viral infection on the post-transcriptional that is induced by gene-specific constructs of double stranded RNA (dsRNA) [3].
Application of biotechnology in order to solve disease problems especially in crustaceans with was conducted at different approaches. Gene discovery penaeidin antimicrobial coding leads to open opportunities in an increase in shrimp immunity against pathogenic attack on *Litopenaeus vannamei* shrimp [4]. Immune response induction in shrimp through vaccination has been reported with use recombinant WSSV protein in *P. chinensis* shrimp [5] and antivirus use dsRNA in *L. vannamei* shrimp [6]. The other papers reported that the application dsRNA of gonadal-inhibiting hormone (GIH) could stimulate a gonad maturation in the shrimp and as an alternative method to eyestalk ablation in captive shrimps has been reported [7, 8].

The use of RNAi to control shrimp viral diseases has been intensively studied, which these have revealed the existence of two pathways of antiviral immunity in shrimp, such as: an innate immunity (sequence-independent) and an RNAi-mediated (sequence-specific) [9]. A mechanism of RNAi in aquatic animals, in particular of crustacean species consists of several stages, where dsRNA is induced into the target cell, siRNA formation occurs to degrade mRNA, so that the specific gene expression become inactive at the post-transcription stage [10, 11]. Application of dsRNA vaccine produced by in-vivo specifically the viral protein (VP) coding virulence gene from WSSV on tiger shrimp is still limited. Previous studies on production of dsRNA by in-vitro and its application to the tiger shrimp have been reported [12, 13, 14, 15]. Production of in-vitro dsRNA using commercial kits still remains some constrains in terms of high production costs and relatively small production yield [16]. Therefore, it is necessary an in-vivo dsRNA production to make easily to high number of quantities in production through bacteria mass culture. The aim of this study was to produce the dsRNA by in-vivo method and to analyze the effect of its application to tiger shrimp larvae to survival enhancement.

2. Material and methods

2.1. Production of dsRNA by in-vivo

Genes encoding VP15 and VP24 were isolated from WSSV infecting tiger shrimp and cloned into the L4440 vector containing the T7 promoter. The gene construct of T7-VP15 and T7-VP24 was transformed to *Escherichia coli* DH5α. Preparation of dsRNA vaccine material was carried out by culture of recombinant bacteria (*E. coli* strain DH5α). Recombinant bacterial colonies were grown on liquid LB media in a 15 mL test tube, then incubated overnight in temperature of 37°C at a speed of 120 rpm. Bacterial harvest was conducted by centrifugation at a speed of 5,000 rpm for 5 minutes and the formed pellet was used as a vaccine material and PCR template to verify the construction of gene constructs in bacterial plasmids. To determine the time of in-activation, the recombinant bacteria of VP-dsRNA were in-activated by heat-killed bacteria method immersion at 80°C for 5, 10 and 15 minutes and without heating as a control treatment. The in-activated bacteria were then stored at -20°C until the vaccine was applied. An application of dsRNA vaccine was carried out by immersion based on the method in the KHV DNA vaccine in carp [2] and recombinant GH in white shrimp [17].

2.2. Vaccination of tiger shrimp post-larvae

The tiger shrimp post larval stadia 2 (PL-2) were collected from production of Nucleus Canter, Tiger Prawn Hatchery Station of Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) in Barru regency, South Sulawesi. The larvae were already checked by PCR for a negative WSSV infection. As much 1,000 larvae were vaccinated by in-vivo dsRNA through immersion for 30 minutes in 1 L of seawater using plastic bag filled with oxygen:seawater at a ratio of 1:5. The treatments of this experiment was vaccination with dsRNA in concentration of $1.3 \times 10^8$ CFU mL$^{-1}$ (A) and immersion without dsRNA vaccination as a control (B). The vaccinated larvae were then keep in a 250 L fibre-tank for each treatment until the larvae reached to the post-larva PL-12 for challenge test.

2.3. WSSV challenge test

The PL-12 larvae were transferred to an aquarium filled with 18 L seawater with a density of 25 larvae L$^{-1}$ (450 larvae per aquarium), which each aquarium was equipped with aerator system. The
experiment was set in a completely random design with 2 treatments and 4 replicates. The experiment consisted of two trial groups, such as VP15-dsRNA and VP24-dsRNA. The WSSV challenge test was conducted by immersion method using the WSSV isolates from infected tiger shrimp. Survival rate of larvae was observed every day.

2.4. Data analysis
The results of the determination of in-vivo dsRNA production, growth of recombinant bacteria, and plasmid isolation were descriptively discussed. To determine the effect of the dsRNA vaccine treatment, a t-student analysis was performed at a confidence level of 0.05. The results of data analysis were descriptively presented in the form of tables and figures.

3. Results and discussions
3.1. Production of dsRNA by in-vivo
The single DNA fragment obtained in the present study indicated a successful in gene cloning of each VP into the vector and transformed to the bacteria. Three representative colonies of cloned bacteria for both of VP15 and VP24 were observed to determine the DNA construct of VP15-dsRNA and VP24-dsRNA in their plasmid (Figure 1). For more evidence, the position of appropriate DNA fragment was also observed, for instance for VP15 and VP24 in length of approximately 245 bp and 620 bp, respectively.

![Figure 1](image1.png)

Figure 1. The DNA fragment of VP-WSSV isolated from plasmid of recombinant bacteria. M=DNA Marker; 1-3=colony bacteria carrying VP15; 4-6=colony bacteria carrying VP24

Figure 1 showed that the VP15-dsRNA and VP24-dsRNA were successful produced by in-vivo with a very high level of purity in range of 1.8-1.9, which indicated by a very clean DNA band. It is recommended that DNA purity for molecular purposes should be in the range of 1.8-2.0 [18]. Before application of dsRNA, in-activated bacteria have to be produced to eliminate the negative effect to the larva, since the DNA constructs of dsRNA were not damage. The growth verification of bacteria on agar medium indicated that the bacteria did not grow after heating 5, 10, and 15 minutes, however the bacteria grew well in without heating treatment (Figure 2-a), while the VP-dsRNA constructs in plasmid were not broken (Figure 2-b). This result suggested that the production of in-activated bacteria was enough for 5 minutes of heating for efficiency purpose. A similar finding that in-activation of bacteria carrying KHV DNA vaccine was successfully performed by heat-killed bacteria for only 5 minutes [2].

The successful production of dsRNA by in-vivo through bacteria gives a potential perspective in the production of dsRNA on a larger scale, not only for immersion purpose, but also for feed enrichment for shrimp. Large-scale production of RNAi through bacterial cells is also a cheap and attractive method. The massive delivery of RNAi molecules through feed either using macromolecules encapsulating/binding RNAi or inactivated bacterial cells expressing RNAi, represented an easy and effective method to distribute RNAi in shrimp facilities (hatcheries or grow-out ponds) [19].
Figure 2. The growth verification of recombinant bacteria on agar medium (a) and plasmid DNA-dsRNA (b) after applied heat-killed bacteria method by using temperature 80°C. (a): A=without heating as a control; (B) heating for 5 minutes; (C) heating for 10 minutes; and (D) heating for 15 minutes and (b): M=DNA marker; T0=without heating as a control; T5=heating for 5 minutes; T10=heating for 10 minutes; T15=heating for 15 minutes; and (+)=positive control.

3.2. WSSV challenge test
A trend of larvae mortality during WSSV challenge test showed the similar pattern between both treatments, even the dsRNA application exhibited a relatively higher survival rate (Figure 3), while the average survival of larvae after stable larva mortality on day-5 for VP15 and day-10 for VP24 was presented in figure 4. The enhancement of survival larvae in the present study was 4.4% and 13.8% on the application of VP24-dsRNA and VP15-dsRNA compared to the control, respectively.

Figure 3. Cumulative survival rate of tiger shrimp after WSSV challenge test on larvae immersed with in-vivo dsRNA of VP15 (A) and VP24 (B).
Figure 4. The average of survival rate of tiger shrimp larva in VP15-dsRNA at 5-days (A) and in VP24-dsRNA at 10-days (B) after WSSV challenge test.

Unlike an intra-muscular injection method of dsRNA application, dsRNA application by an immersion method in this present study showed a relatively lower survival response to the larvae, resulting survival rate was not statistically different (P>0.05) between treatments for both trials of VP15-dsRNA and VP24-dsRNA. The injection of VP24-dsRNA vaccine to tiger shrimp in dosage of 0.2 μg increased the survival rate 55% higher than control treatment [15], while injection of VP15-dsRNA in tiger shrimp also showed the higher survival, 40.0% in vaccinated shrimp and 3.3% in control shrimp [14]. The experimental efficacy of RNAi has been exhibited a great result through intra-muscular injection on the shrimps. However, this method seems to be not suitable for a huge delivery and continuous production of RNAi molecules to large populations of animals in shrimp hatcheries or grow-out ponds [19].

The survival enhancement of 3.9% for VP15-dsRNA and 3.8% for VP24-dsRNA (equivalent with 4.6% and 15.9% in percentage of increase) in this preliminary study indicated a potentiality for utilization of dsRNA to improve tiger shrimp larvae resistance to combat WSSV. However, it needed some improvements on method, dosage, immersion time, larval stadia, etc. to obtain the proper RNAi technology for tiger shrimp larvae. The high survival rate of dsRNA vaccinated tiger prawns compared with those not vaccinated indicated that VP-WSSV was predicted to provide protection against WSSV infection. This opinion is in line with the argument of the VP15 gene had a high homology with histone proteins; therefore, this protein is known to have a role in binding of WSSV DNA, forming a nucleoprotein core, playing a systemic infection, as well as stimulating the immune system in tiger shrimp [20].

Application of VP28 and VP28+VP29 mixture vaccine to tiger prawn showed the higher relative percentage survival (RPS) value compare to the control in value of 44% and 33% 2 days post-challenge test, respectively [21]. The other study reported that VP26 and VP28 vaccine application in shrimp P. japonicus increased the resistance to WSSV with RPS values of 80% and 95%, respectively [22], while the RPS of VP28-dsRNA application in Macrobrachium rosenbergii was 44.5% [23]. The use of dsRNA vaccines at a dose of 2.0 μg in white shrimp L. vannamei increased survival rate of 100% against IHNV [24], while injected with plasmids containing VP28, VP39, and WSV-477 showed survival rate of 50%, 60%, and 90% compared to controls (0%) on the day 9th after being challenged with WSSV [25]. Oral vaccination through a feed on black tiger shrimp using VP-19 increased survival rate with a RPS value of 77% on the 7th day and 29% on the 21st day after the WSSV challenge test [26].

To validate the shrimp larvae mortality during the challenge test caused by WSSV infection, the death of tiger prawn larvae was extracted to get the DNA genome for PCR detection. The result
showed that the DNA genome of larvae was positively infected with WSSV with an indicator of the presence of a single band at a position of approximately 200 bp in the electrophoresis gel (Figure 5). This indicated that the death of tiger shrimp larvae was caused by the WSSV infection or not a cause of other disease agent.

![Figure 5. Validation of death tiger shrimp larvae during the challenge test at 0.8% agarose gel. 1-9= death shrimp samples; (+) = positive control; (-) = negative control; M= DNA marker; and arrow sign indicates position of DNA WSSV fragment.](image)

### 4. Conclusion

The VP15-dsRNA and VP24-dsRNA were successfully produced by in-vivo with a very high level of purity and the in-activated bacteria was conducted by heating period of 5-15 minutes without causing damage of VP-dsRNA constructs, however, for efficiency purpose, the use of 5 minutes was enough to in-activate the bacteria. Application of dsRNA showed higher the survival compared to the control. The result of the experiment suggested a potential way to improve the technique to obtain the suitable RNAi technology for produce the resistant tiger shrimp and also more researches are needed to address issues related to food safety and environmental safety of RNAi-treated shrimp.

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