USE OF dsRNA WSSV VP19 WITH DIFFERENT DOSE FOR INFECTION CONTROL White Spote Syndrome Virus (WSSV) IN VANAME SHRIMP Litopenaeus vannamei

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Abstract- Increased production of lithopanaeus vannamei vannamei shrimp continues to be pursued, one of which is by increasing shrimp immune response to infection with WSSV disease. This study aims to evaluate the best dosage of dsRNA VP19 WSSV *in-vitro* production to increase the immune response of vannamei shrimp (*L. vannamei*). The isolation of the VP19 gene and the construction of the VP19 vaccines were carried out using the Megascript kit with VP 19 genomic DNA as a template. Vaccination was carried out by injection method on vannamei shrimp with an average weight of 10.5 ± 3.5 g and a length of 11.05 ± 1.95 cm. The vaccine dose tested was 0.02 µg; 0.2 µg; 2 µg; and as controls are shrimp that are not vaccinated. The study consisted of four vaccine dose treatments with each of two replication and maintained for five days. The challenge test was carried out for five days by injecting the WSSV virus in saline solution (1:3 v/v). The differential hemocyte count (DHC) calculation was carried out before the challenge test and after the challenge test on days 1, 2, and 3. Data analysis was performed statistically by analysis of variance (ANOVA). VP19 gene isolation, construction with T7 promoter, and making of WSSV VP19 dsRNA were successfully carried out, and application of dsRNA vaccine in vannamei shrimp with a dose of dsRNA vaccine of 2.0 µg had a significant effect (P <0.05) on granular hemocytes cells

Index Terms- VP19 dsRNA, differential hemocyte count (DHC), vannamei shrimp, dose.

I. INTRODUCTION

Shrimp is one of the fisheries commodities which have high economic value and are widely cultivated in Indonesia, especially vannamei shrimp (*Litopanaeus vannamei*) and Black tiger *Penaeus monodon* (Tassanakajon et al., 2013). Vannamei *L.vannamei* shrimp is widely cultivated today, because it is known to have superior resistance to virus attacks and environmental changes compared to tiger shrimp. But the current reality of vannamei shrimp cultivation also often fails due to virus attacks (Subyakto et al., 2009). One common virus that infects shrimp is white spot syndrome virus (WSSV). During the last 20 years, WSSV has been considered a viral pathogen that has the most negative impact on the shrimp culture industry. This is due to its high virulence, rapid spread, occurring in all shrimp producing countries, and mass mortality which can reach up to 100% within 3 to 7 days after the first clinical signs seem, reducing production with a large economic loss (Lucero at all, 2016)..

Prevention and treatment of viral infections is very challenging because the virus uses stem cell structures to replicate, inhibiting viral replication without damaging the structure of the host cell. Researchers have recently begun to use RNA interference (RNAi) technology to selectively inhibit viral genes that are important for virulence (Shuey et al, 2002). The RNAi technology has the role of inhibiting post-gene transcription through formation of dsRNA segments in the mRNA transcript, resulting in proper mRNA degradation. Vaccination technology uses viral dsRNA agents to develop very rapidly, especially in the fields of medical, agricultural, and aquaculture (Jin et al., 2010; Lichner et al., 2003; Rowley & Pope, 2012). In the field of aquaculture, this technology developed to overcome diseases caused by viruses in shrimp (Mejía-Ruíz et al., 2011; Ahanger et al., 2014). Some shrimp vaccination studies using WSSV agents as vaccine sources have shown progress and obtained encouraging results (Huang et al., 2014; Kumar et al., 2015; Puneeth et al., 2017).

WSSV is a double-stranded DNA (dsDNA) virus formed from complex macromolecules that are specifically attached and arranged for the protection and transfer of viral genomes. WSSV genes transcribed in the late phase include genes that encode WSSV main structural proteins, namely VP28, VP26, VP24, VP19, and VP-15. Where, this viral protein is likely to used as a recombinant vaccine (Sánchez-Paz, 2010). VP19 is a WSSV wrap protein that is important because of its involvement in systemic infections in shrimp (Nadala et al., 1998; van Hulten., 2001b; Zhang et al., 2002). The latest results of the study were reported by Nurhaeda (2017), who succeeded in making VP19 dsRNA vaccine which was able to increase the survival, immune response and histology of vannamei shrimp.

Isolation and characterization of the WSSV VP19 virulent gene has been successfully carried out (Nurhaeda, 2017), but as a dsRNA vaccine in increasing survival, immune response and histology of shrimp with various doses after challenge tests WSSV is still lacking, so this study needs developed with using various doses as an effort to control WSSV infection in vannamei shrimp. This study aims to evaluate the best dosage of WSSV VP19 DNARNA *in-vitro* production to increase the immune response of vannamei shrimp (*L. vannamei*).

II. MATERIALS AND METHODS

2.1 Research Materials

The shrimp genome material that was use was the vannamei that was positively infected with the WSSV virus. The genomic DNA collection from the Laboratory of Brackish Biotechnology Research Center for Brackish Water Aquaculture and Maros Fisheries Extension (BRPBAP3) in Maros.

2.2 Research Methods

Stages carried out in the research to produce VP19 dsRNA are: Isolation of VP19 gene, Construction of VP19 WSSV gene with *in-vitro* RNAi method, and production of WSSV VP19 dsRNA, then application to test animals to see Differential Haemocyte Count (DHC) after challenge test.

a. Isolation of WSSV VP19 Encoding Genes

Isolation of the VP19 gene carried out using the PCR technique. The DNA genome that has been isolated from vannamei shrimp used as a template for DNA (template) in the PCR process, using the forward primer VP19 F 5'-CGC GGA TCC GAT CAC CAC GAC TAA CAC-3 and reverse VP19 R 5'-CCG primer GAA TTC TTA CTG CCT CCT CTT GGG-3 with a target of 366 bp (Sarathi *et al*., 2010).

Amplification performed on the System 2700 PCR GeneAmp PCR engine (Applied Biosystem). The PCR reaction used is the PureTaq Ready-To-Go PCR Beads-GE Healtcare kit (2.5 units of PuReTaq DNA polymerase; 200 µM each dNTP; 10 mM Tris-HCl pH 9 at room temperature; 50 mM KCl; 1.5 mM MgCl2) and primer 1 µL each, 0.5 µL vannamei shrimp DNA genome, and nuclease free water 22.3 µL with a last volume of 25 µL.

Then sample the spin down for ± 10-15 seconds. Samples entered into PCR machines with PCR amplification reactions as follows: pre-denaturation 94°C for 5 minutes in 1 cycle; 35 cycles for denaturation of 94°C for 30 seconds, annealing 53°C for 30 seconds, extension 72°C for 30 seconds; and the last 72°C extension for 7 minutes then added 4°C at the end of the cycle.

Next to see the success of amplification of target DNA fragments, 1 µL sample was electrophoresis on 1% agarose gel with 20 mL TAE 1 buffer solvent, then 1µL gelred added documented with Gel Documentation System. The molecular weight of the DNA fragment determined using a 100 bp plus marker.

b. WSSV VP19 Gen Construction with T7 Promoter

Construction of WSSV VP19 gene with T7 promoter carried out by PCR technique. The VP19 WSSV gene is used as a template using the T7 VP19 primer: 5'-TAA TAC GAC TCA CTA GAC GGA GCG GAT CCG ATG GCC ACC CTA ACC-3 CTA 'and T7 VP19R primer: 5'-TAA TAC GAC TCA CTA TAG GGA CGG AAT TCT TAC TGC CTC CTC TTG GGG-3'(bottom line is T7 primary sequence).

Amplification performed on the System 2700 PCR GeneAmp PCR engine (Applied Biosystem). The PCR reaction used was the PureTaq Ready-To-Go PCR Beads-GE Healtcare kit by adding primers of 2.5 µL, 0.5 µL of the mold genome, and nuclease-free water 19. 5 µL with a final volume of 25 µL.

Samples were inserted into the PCR machine with PCR conditions as follows: 94°C for 5 minutes, followed by 25 cycles of 94°C for 30 seconds, annealing temperature of 72°C for 30 seconds and extension 72°C for 30 seconds, followed by last extension of 72ºC for 7 minutes.

Next to see the success of amplification of target DNA fragments, 1µl sample was electrophoresed on 1% agarose gel with 20 ml TAE 1 buffer solvent, then added 1 µl of gel documented with Gel Documentation System. The molecular weight of the DNA fragment determined using a 100 bp plus marker.

Nexted PCR with the premier T7 VP19, on the PC 27 GeneAmp PCR (Applied Biosystem) PCR engine. The PCR reaction used was the PureTaq Ready-To-Go PCR Beads-GE Healtcare kit by adding primers of 2.5 µL each, 0.5 µL of the mold genome and nuclease-free water 19.5 µL with a final volume of 25 Ml.

Samples were inserted into the PCR machine with PCR conditions as follows: 94°C for 5 minutes, followed by 25 cycles of 94°C for 30 seconds, annealing temperature of 72°C for 30 seconds and extension 72°C for 30 seconds, followed by last extension of 72°C for 7 minutes.

Next to see the success of amplification of target DNA fragments, 1µl sample was electrophoresis on 1% agarose gel with 20 ml TAE 1 buffer solvent, then added 1 µl of gel documented with Gel Documentation System. The molecular weight of the DNA fragment determined using a 100 bp plus marker.

c. Manufactures WSSV dsRNA-VP19

The PCR results used for the synthesis of dsRNA using the MEGAscript RNAi (Ambion) kit. The sample used to produce dsRNA is a sample that was successfully constructed with the T7 promoter.

The concentration of PCR products measured to find the number of PCR products to be used in making the RNase transcript reaction. Subsequently added to the new tube: Adenine triphosphate (ATP), Cystidine triphosphate (CTP), Guanosine triphosphate (GTP), Uridine triphosphate (UTP) (2 µL); 10x T7 Buffer (2 µL); T7 enzyme mix (2 µL); PCR products (1.5 µL); Nuclease-free water 6.5 µL; Total tube 1 (20 µL) Appendix 1.

Then in the spin down at medium speed and incubated at the incubator at 37ºC for 6 hours (every 30 minutes the sample shaken). For the Annealing process, it was re-incubated at 75ºC for 5 minutes then cooled to room temperature for about 3 hours then the dsRNA stored at -20ºC and -80ºC until it reused.

Then the process of Digestion DNA and ssRNA uses MEGAscript RNAi kit (Ambion) with the following composition: dsRNA VP19 (tube 1) 20 µL; Nuclease-free water (21 µL); Digestion buffer (5 µL); DNase I (2 µL); RNase A (2 µL) Total tube 1 (50 µL).

After all the ingredients mixed then spindown and incubated at 37ºC for 1 hour (not more than 2 hours). Then leave it at room temperature for 5 minutes. The next process is purification which aims to remove proteins, free nucleotides, and nucleic acid degradation products from dsRNA, namely dsRNA (tube 1) (50 µL); 10x binding buffer 50 µL; Nucleace-free water (150 µL); 100x ethanol (250µL); Total tube 1 (500 µL).

These ingredients added to tube 1 and spin down, after which th added to the fliter cartidg. Then centrifuge at a speed of 14,000 rpm at 4ºC for 2 minutes. The supernatant is remove, add 500 µLwash solution and centrifuge at a speed of 14,000 rpm at 4oC for 2 minutes. The supernatant is discard, add 500 Ml wash solution and centrifuge with a speed of 14,000 rpm at 4ºC for 2 minutes. The supernatant removed and repeat the dry centrifuge, homogenized it with 50 µL elution solution (95ºC), centrifuge at a speed of 14,000 rpm at 4ºC for 2 minutes. homogenized again with 50 µL elution solution (95ºC), centrifuge at 14,000 rpm at 4ºC for 2 minutes. Homogenate is remove and the supernatant is remove. Furthermore, measuring WSSV VP 19 dsRNA concentration and to see the success of the target RNA in electrophoresis by using agarose 1% with TAE 1x buffer solvents as much as 20 mL and added as much as 1 µL and documented with Gel Documentation System (Appendix 2). The molecular weight of the DNA fragment determined using a 100 bp plus marker.

d. Application of WSSV dsRNA-VP19 to Vannamei Shrimp

Experiments conducted to test whether dsRNA VP19 with different doses can improve the survival and immune response of vannamei shrimp against WSSV virus infection and to see the level of hepatopancreas tissue damage in test animals. The limits to observed are DHC.

The study will be designed using a Completely Randomized Design (CRD) with 4 treatments and each treatment each has 3 replications. Thus this study consisted of 12 experimental units. The vaccine dose tested refers to the study of Loy et al. (2012) namely: a. Shrimp injected with saline solution (SS) without vaccine (control), 0.02 µg, 0.2 µg and 2.0 µg

1. experiment animals

The vannamei shrimp used came from Barru Regency ponds with an average weight of 10.5 ± 3.5 g and an average length of 11.05 ± 1.95 cm. The shrimp have been vaccinated maintained in a fiber-controlled tub measuring 80 cm x 80 cm x 60 cm which equipped with aeration, with density of 10 fish / tub, using sea water media and fed with commercial feed 2% of body weight and given 2 times a day before the challenge test and 4 times a day after the challenge test.

2. dsRNA vaccine

On the first day, the shrimp in each treatment injected with VP19 dsRNA with the dose tested in the second segment of the abdominal section using a syringe volume of 1 mL and 0.1 mL in each test shrimp. The test animals were then maintained until the third day, then tested challenged with the WSSV virus.

3. Test challenges using the WSSV virus

The challenge test was to infect the WSSV virus through the injection method into vannamei shrimp. WSSV isolates came from the Maros BRPBAP3 Fish and Environmental Health Laboratory which been tested molecular. On the 3rd day after vaccination, challenge tests carried out by injecting 0.1 mL WSSV virus in each test shrimp. Before the challenge tes carried out, first take a hemolim sample from each treatment randomly (4 individuals per treatment group) to observe the immune response. During the study, observation carried out.

4. Differential Haemocyte Count (DHC)

Differential hemocytes are then calculated by grouping hemocytes into 3 cell types (granular, semi granular, and hyaline), the percentage of each cell type calculated using the formula:

$$\text{DHC (\%)} = \frac{\text{number of each type of hemocyte cell}}{\text{hemocyte count}} x\ 100\%$$

2.3 Data Analysis

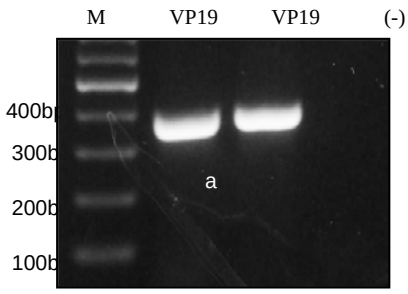
The parameters of the DHC immune response were analyzed using the ANOVA test at the level of 0.05.

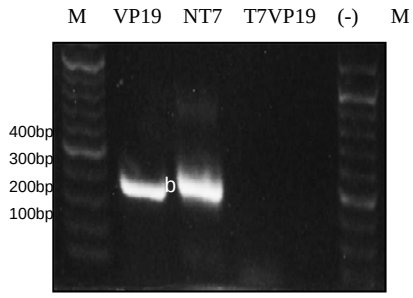
III. RESULTS AND DISCUSSION

1. Characteristics of the WSSV VP19 gene

a. Isolation of WSSV VP19 encoding gene

The VP19 encoding gene was successfully isolated from WSSV on the vannamei shrimp genome DNA which was positively infected with white spot disease using the PCR technique using VP19F and VP19R specific primer. Electrophoresis results showed that one DNA fragment at position of around 366 bp (Figure 1a) and VP19 gene construction was also successfully carried out on the nexted promoter T7 (T7 VP19). Can be seen in (Figure 1b) which shows an increase in DNA fragments from VP19 position between 400-500bp.



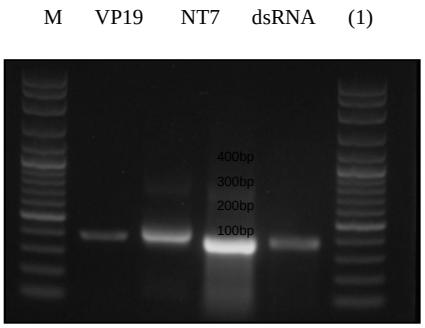


Gambar 1. Electrophoresis results of PCS VP19 WSSV (a) and nexted PCR T7 VP19 WSSV (b) isolated from the vannamei shrimp genome that were positively infected by WSSV. M = marker 100+, VP19 = gene VP19, NT7 = nexted T7 PCR, T7VP19 = promoter T7 VP19, (-) = negative control.

The results of PCR amplification and authentication with VP19 WSSV encoding gene electrophoresis, DNA fragment bands at position of around 366 bp which showed that the VP19 encoding gene was successfully isolated from the vannamei shrimp genome that was positively infected with the WSSV virus. This has also been successfully carried out in isolating other VP19 and VP-WSSV encoding genes in both vannamei shrimp and tiger shrimp. The results of the study carried out by Alim *et al*. (2011), reported that isolation of the WSSV VP19 gene encoding in tiger shrimp Situbondo isolates was 366 bp; Tenriulo *et al*. (2015), showed that the VP24 WSSV encoding gene was successfully isolated from tiger shrimp with a sequence length of about 641-648 bp; Parenrengi *et al*. (2017), reported that VP15 was successfully isolated from tiger shrimp with ORF DNA fragment length (open reading frame) of 243 bp; Hidayani et al. (2016), showed the success of isolating the coding gene for WSSV VP19 protein in vannamei juvenile of Indonesian isolates with a DNA fragment length of 387 bp; Hidayani *et al*. (2013), reported that gene encoding of the VP19 WSSV protein in tiger shrimp had a size of 386 bp; Malina *et al*. (2013), reported that isolation of VP28 surface protein encoding genes from tiger shrimp was successfully carried out with a fragment length of 672 bp. The results of the VP-WSSV encoding genes isolated from the shrimp genome infected with WSSV showed that the primary pair was specifically able to amplify the VP-WSSV gene from the shrimp genome infected with WSSV. Furthermore, it said that differences in genome size show chance of mutations caused either by deletion or presence of certain sizes of insertion in the WSSV genome (Yang *et al*., 2001).

b. Production of WSSV dsRNA-VP19 *in-vitro*

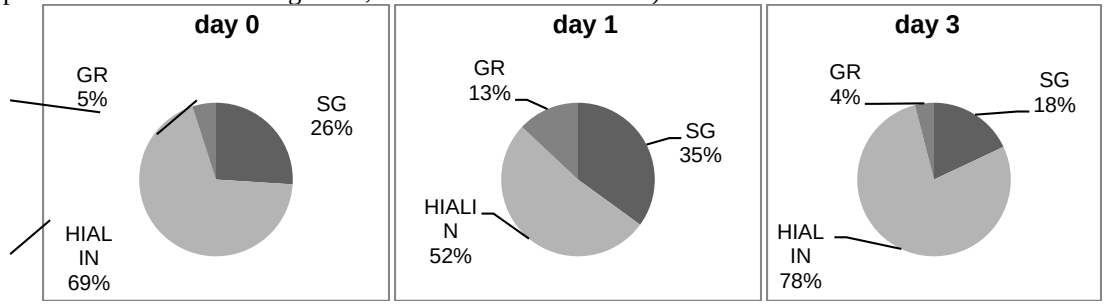
Production of WSSV VP19 dsRNA was successfully carried out in-vitro using the MEGAscript RNAi kit with purity of 1.96 and a concentration 850 µg / mL, where a purity level above 1.8 was indicated as a product for production of dsRNA. Linacero et al. (1998), recommend the ratio value for pure double-stranded DNA between 1.8–2.0. The same method carried out for production of in-vitro gonad inhibiting hormone (GIH) dsRNA using the MEGAscript RNAi kit (Wulandari, 2010). The success of in-vitro dsRNA production using the MEGAscript kit is characteristic by high levels of purity, and an increase in fragments or parallel to the control fragments, and the WSSV VP19 dsRNA fragment band seen as verified by 1% agarose electrophoresis. This result can be seen in (Figure 2) there is a VP19 DNA fragment that constructed with the T7 promoter.

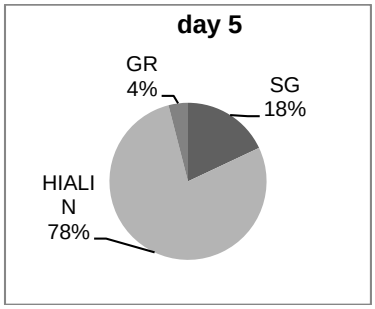


Gambar 2. Electrophoresis produced by WSSV dsRNA-VP19. M = marker100 +, VP19 = VP19 gene, NT7 = nexted promoter T7, dsRNA = WSSV dsRNA-VP19 production.

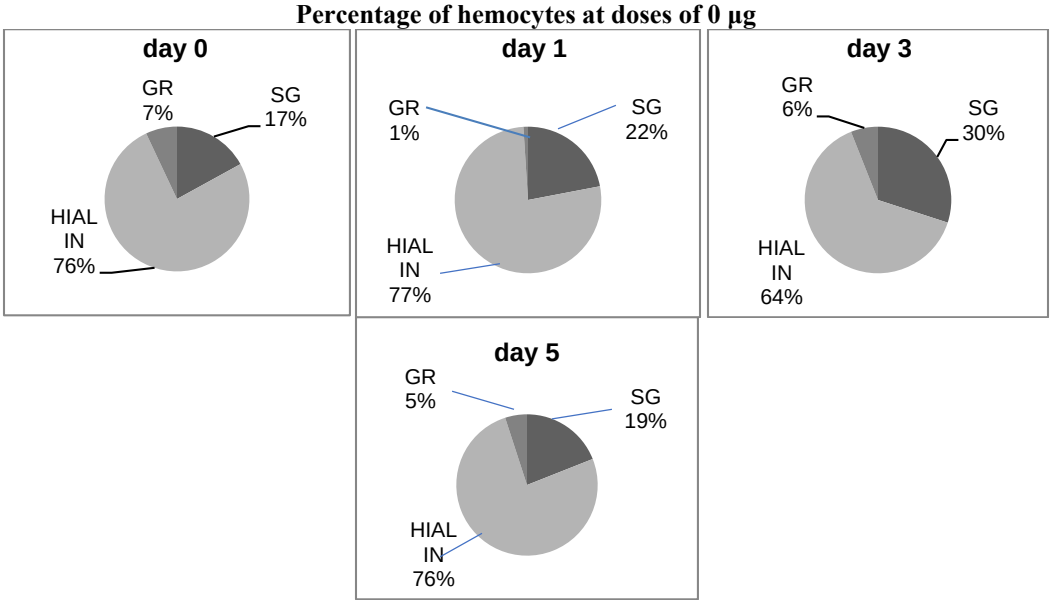
2. Differential Haemocyte Count (DHC)

Based on the results of the study before and after the challenge test with the WSSV virus at each dose showed an average DHC value, the number of hyaline hemocytes cells dose 0 µg, 0.02 µg, 0.2 µg, and 2 µg before the challenge test which is 69%, 76 %, 74% and 72%. After that, semi granular 26%, 17%, 20%, and 22%. Furthermore, granular is 5%, 7%, 7% and 6%. The results after the challenge test were obtained for the average DHC values of hyaline, semi granular, and granular hemocytes with doses of 0 µg, 0.02 µg, 0.2 µg, and 2 µg on days 1, 3 and 5th is hyaline (52%, 77%, 64%, and 68%), (78%, 64%, 64%, and 68%), (69%, 76%, 69%, and 69%), Variety analysis showed no significant difference (P> 0.05) in each treatment. Semigranular (35%, 21%, 27% and 25%), (18%, 30%, 24%, and 20%), (7%, 19%, 20%, and 17%), variance analysis showed no significant influence (P> 0.05) on each treatment. Granular (13%, 1%, 9%, and 7%), (4%, 6%, 13% and 12%), (4%, 5%, 11% and 14%) the results of the tukey advanced test show differences that real (P <0.05) in each treatment. This result can seen in Figure 3.

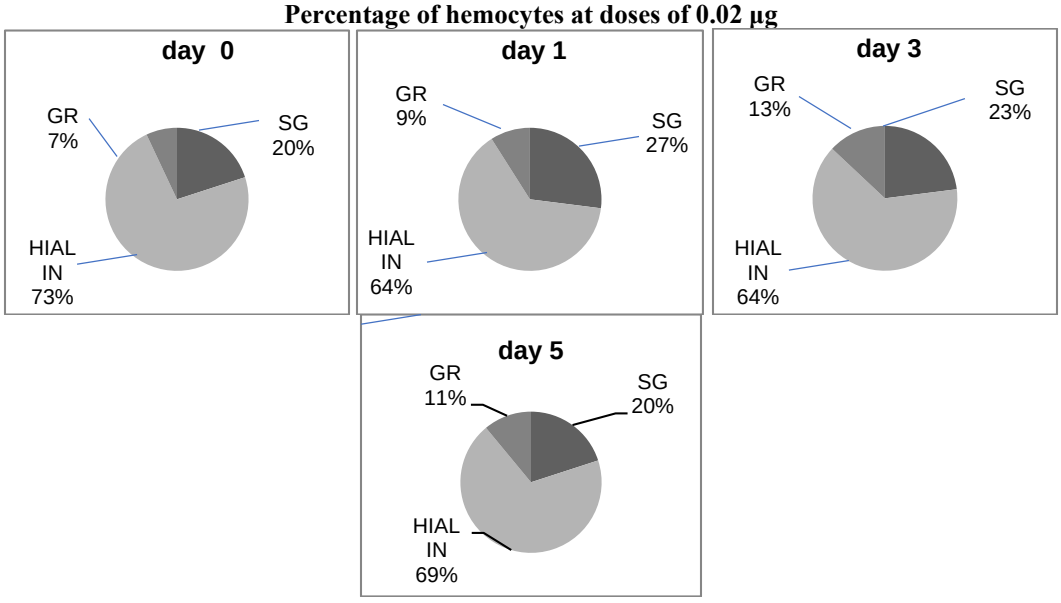




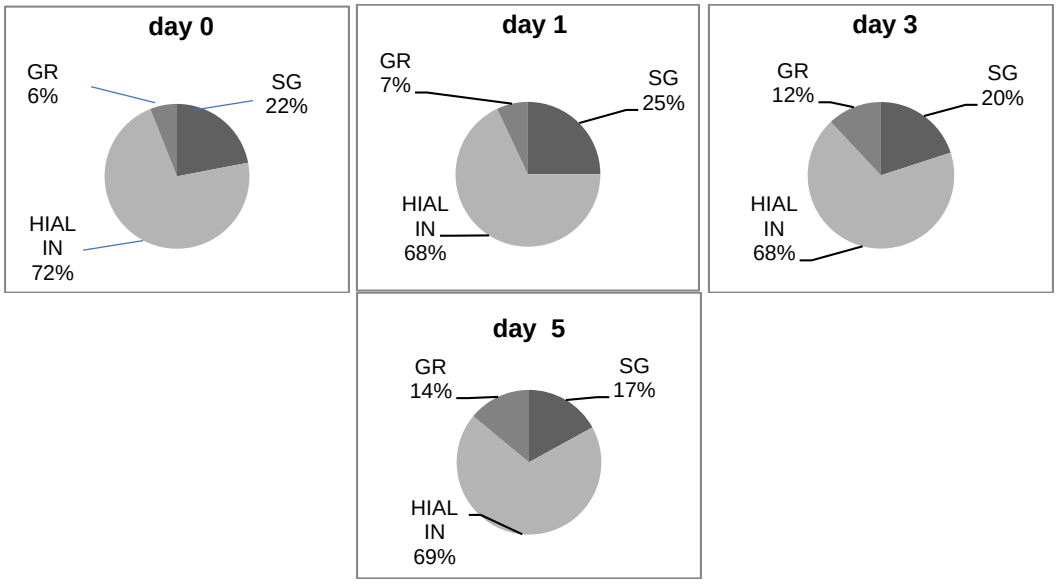
Percentage of hemocytes at doses of 0 µg



Percentage of hemocytes at doses of 0.02 µg



Percentage of hemocytes at doses of 0.2 µg



Percentage of hemocytes at doses of 2,0 µg

Gambar 3. Percentage of differential haemocyte count (DHC) of vannamei P. vannamei shrimp before and after testing challenged with WSSV virus. ANOVA analysis showed no significant difference (P> 0.05) hemocytes cells in each treatment.

The results of the study of hemocyte cell differentiation consisting of hyaline, semigranular, and granular cells of vannamei shrimp before and after the challenge test showed differences in the effect that varied in (Figure 3). Hyaline cell results showed no significant difference (P> 0.05) in each treatment. Vannamei shrimp hyaline cells were vaccinated and then injected with WSSV decreased on day 1 and day 3, while on day 5 there was an increase compared to controls. Hyaline cells depicted by the absence granular function as cells that do phagocytosis (Smith at al. 2003). Increasing hyaline cells can increase phagocytes activity against pathogens that enter the crustacean body (Le Moullac et al. 1998; Smith et al. 2003).

Semigranular cells described as having small granules, which function in recognizing and responding to pathogens that enter the crustacean body (Soderhall and Cerenius 1992). While granular cells described as cells that have a large number of granules which function in storing and releasing the proPO system and as toxicity together with semigranular cells (Smith et al, 2003). Although there was a decrease in the percentage of semigranular in this study, granular cells showed a significant effect (P <0.05) which was able to increase phenoloxudase activity which plays a role in the melanization process. Each active cell type in the immune reaction, such as hyaline cells involved in phagocytosis, semigranular cells are active in encapsulation, and granular cells are active in the storage and release of proPO and cytotoxic systems.

IV. Conclusion

The results showed that VP19 gene isolation, construction with T7 promoter, and making of WSSV VP19 dsRNA were successfully carried out, and the application of dsRNA vaccine in vannamei shrimp with a dose of dsRNA vaccine of 2.0 µg had a significant effect on granular hemocytes

V. Suggestion

Further research needs to developed on vaccine production in-vivo and evaluates on the day that vaccines increase and survive.

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