ANALYSIS OF IMMUNE RESPONSES ON TRANSGENIC TIGER SHRIMP (*Penaeus monodon*) AGAINST PATHOGENIC BACTERIUM *Vibrio harveyi*

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ABSTRACT

Vibriosis is one of main diseases of the black tiger shrimp *Penaeus monodon* infected by pathogenic bioluminous bacterium *Vibrio harveyi* that can cause mass mortalities in shrimp culture. The bacteria can also trigger the disease *white spot syndrome virus* (WSSV). An effort to produce shrimp disease-resistant strains has been done through transgenesis technology with antiviral gene transfection. By this technology, it is expected an increase in the immune response of shrimp in a variety of disease-causing pathogens. This study aimed to determine the immune responses (total haemocytes, haemocyte differentiation, and phenoloxydase activity) of transgenic tiger shrimp against pathogenic bacterium *V. harveyi*. Research using completely randomized design, which consists of two treatments and three replications. Test animals being used were transgenic and non-transgenic shrimp with size, weight 3.93±1.25 g and a total length of 7.59±0.87 cm. Treatments being tested were the injection of bacterium *V. harveyi* (density of $5x10^6$ cfu/mL) of 0.1 mL/individual on transgenic (A) and non-transgenic shrimp (B). Immune response parameters such as total haemocytes, haemocyte differentiation, and phenoloxydase activity were observed on day 1, 3, and 6 days after challenging. Data were analyzed using t-test by SPSS software. The results showed that the total haemocyte of transgenic shrimp was not significantly different (P>0.05) from non-transgenic shrimp, but haemocyte differentiation and phenoloxydase activity were significantly different (P<0.05) especially on sixth days after being exposed to the bioluminescent bacteria. The study results implied that transgenic shrimp has a better immune response compared than non-transgenic shrimp.

KEYWORDS: **immune response, transgenic, tiger shrimp, bacteria, *Vibrio harveyi***

INTRODUCTION

Vibriosis is the term used to refer to a multitude of infections caused by bacteria belonging to the genus of *Vibrio*. The direct economic implications of vibriosis can be devastating, particularly when acute outbreaks occur. The major species causing vibriosis in shrimp are *Vibrio parahemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. harveyi*. The bacterium *V. harveyi*, a luminescent species, is considered as the most devastating bacteria that causes extreme losses both in the hatcheries and shrimp-rearing farms. Vibrio bacteria is popularly known to make infection to the aquaculture sector such as shrimp culture (Baticados

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et al., 1990; Karunasagar *et al.*, 1994; Moriarty, 1998; Zhang & Austin, 2000), several species of fish and oyster (Austin & Zhang, 2006) as well as a coral (Ben-Haim *et al.*, 2003). Infection of *V. harveyi* in tiger prawn (*Penaeus monodon*) larvae in the hatchery can cause larval mortality up to 100% and in the area of juvenile shrimp farms can kill before the maintenance period of 80 days (Moriarty, 1998) as well as trigger the emergence of a secondary infection of white spot virus (white spot syndrome virus-WSSV) (Gunarto & Mansyur, 2010; Rantetondok, 2011).

The pathogenicity of bacteria *V. harveyi* is caused by the presence of haemolysin that can cause lysis of red blood cells by damaging their cell membrane (Conejero & Hedreyda, 2004). Haemolysin is activated by ToxR gene in bacteria to produce toxins such as haemolysin production (Pang *et al.*, 2006). Prevention and disease control efforts in tiger shrimp have been carried out, such as by application of β-glucan immunostimulatory (Effendy *et al.*, 2004), lipopolysaccharide (Rantetondok, 2002), *Vibrio* vaccine (Tompo & Susianingsih, 2008), seaweed extract (Jasminandar, 2009), Vitamin C and E (Kanagu *et al.*, 2010). An advance in molecular science has also provided some results that can be used to improve the immune system of shrimp. The discovery of the gene encoding the antimicrobial penaid is one of the opportunities in genetically improving immunity of whiteleg shrimp (*Litopenaeus vannamei*) against pathogen (Destoumieux *et al.*, 1999). Induction on shrimp immune through vaccination by using recombinant WSSV proteins in shrimp *Penaeus chinensis* has been reported by Kim *et al.* (2004) and double-stranded RNA vaccines (double-stranded RNA, dsRNA) in whiteleg shrimp (Robalino *et al.*, 2004). Transfer of antiviral gene in the whiteleg shrimp through the introduction of genes encoding coat protein of TSV (TSV-CP) has also been reported by Sun *et al.* (2005), where survival rate of transgenic whiteleg shrimp showed a significantly higher than normal shrimp (Lu & Sun, 2005).

Identification and isolation of promoter antivirus (ProAV) and *P. monodon* antiviral (PmAV) gene of tiger shrimp has been done by Parenrengi (2010). Promoters are DNA sequences that play a role in the transcription of specific genes located in the upstream of structural genes. Promoter is functioned to regulate when, where and under what conditions, and the target genes that are activated. Construction of gene-PmAV ProAV have successfully transferred the shrimp through transfection techniques to the embryo which is a first step in the formation of disease-resistant strains of black tiger shrimp (Parenrengi *et al.*, 2009a; 2009b). Introduction of antiviral gene encoding the tiger shrimp may increase resistance to WSSV virus approximately 24.5% higher compared to normal shrimp (Parenrengi, 2010). The resulting performance of tiger shrimp needs to be studied more in depth, especially related to the resistance that involves non-specific immune response of shrimp PmAV transfection results. The nature of the non-specific immune response in shrimp antiviral gene transfection results that allowed to high resistance against bacterial pathogen such *V. harveyi*. Parenrengi *et al.* (2011) has proven that transgenic tiger shrimp had higher survival rate compared than normal shrimp with relative percentage survival (RPS) of 64.3% to 66.7%, when challenged with bioluminous bacterium *V. harveyi*. Therefore, analysis of transgenic shrimp immune response is needed to be done in order to determine the immune responses of transgenic shrimp to pathogenic bacterium *V. harveyi*.

MATERIALS AND METHODS

Animal Testing

Animal testing used in this study was transgenic shrimp (F_0) produced by antiviral gene transfection and non-transgenic shrimp spawned from the same parental broodstock. The shrimps in weight of 2.68-5.18 g (3.93±1.25 g) with a total length of 6.72-8.46 cm (7.59±0.87 cm) were obtained from the hatchery of Station of Research Institute for Coastal Aquaculture (RICA) in Barru, South Sulawesi. The shrimp were reared in aquarium with volume of 15 L and stocked at 10 shrimps/aquarium. Each aquarium was equipped with aeration for oxygen supply and filled with 10 L seawater having salinity of 30 ppt. Commercial feed was given as much as 2% of body weight twice a day. Before infected with bacterium *V. harveyi*, the shrimp was acclimatized in aquarium for a whole night.

Bacterial Isolate of *V. harveyi*

Isolate of *V. harveyi* was obtained from the Laboratory of Fish Health and Environment, RICA Maros. The isolate was molecularly iden-

tified based on 16S rRNA sequence by Kadriah (2012). *V. harveyi* was activated by culturing in nutrient broth (NB) and incubated for 24 hours in the incubator shaker. Bacterium was sub-cultured again in NB and then incubated in the shaker incubator for 4 hours. The density of stock was calculated and diluted by using physiological solution (0.85% NaCl) to get appropriate bacterial population to be injected to the shrimp for challenge test.

Challenge Test

Infection was done by injecting *V. harveyi* 0.1 mL for each shrimp. Concentration of the injected bacteria was 10^6 cfu/mL, based on the pathogenicity test conducted by Kadriah (2012). Bacterium was injected in the abdominal (intra-muscular) into the second segment using a 1 mL syringe volume (26" gauge). Test animals were then maintained and the moribund phases of shrimp were observed their immune responses.

Observed Immune Responses Parameters

To determine the effect of shrimp immune response against bacterial challenge test *V. harveyi*, haemolymph was taken from the shrimp before injection and on day-1, day-3, and day-6 after challenge test. Parameters of immune responses were observed:

Total haemocyte

Observations of total haemocyte count (THC) was conducted by taking haemolymph 0.1 mL of the second abdominal segment using a 1 mL syringe and needle size of 26 gauge (Braak, 2000) contained 0.3 mL anticoagulant Na-citrate 3.8%. The solution was homogenized by shaking it with hand to form a figure of eight. The first droplet was discharged, and then the next droplet was dripped into the haemocytometer (improved neubauer type). Haemocyte cell number was counted under a binocular light microscope with 100 times magnification (Blaxhall & Daishley, 1973). Total haemocyte cell 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)

Haemocyte differentiation

Observations of differential haemocyte type in term of differentiation haemosit count (DHC) was performed by the method of Martin & Graves (1985). Haemolymph was dripped on glass object and created a slide-review, then dried in the room temperature. After drying, the preparation was fixed with methanol for 5-10 minutes and then dried again in the room temperature. This preparation was soaked in 10% giemsa dye solution for 15-20 minutes. Then, the preparations were washed with water and allowed to dry. The slide preparation was examined under a microscope with 400 times magnification and identified cell types. Haemocyte number was counted up to 100 cells in order to determine the percentage of each type, by formula:

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

where:

H = Percentage of haemocyte cell types (%)

N = Number of each type of haemocyte cells (Cell)

Activity of phenoloxidase

Phenoloxidase (PO) activity was measured according to the formation of dopachrome produced by di-hydroxyphenil L-alanine (L-dopa) using a spectrophotometer. Activity of PO activity measurement was based on the procedure developed by Liu & Chen (2004). A total of 0.1 mL haemolymph and 0.9 mL anticoagulant solution was homogenized in microtube. The mixture was then centrifuged at 700 × g at 4°C for 20 minutes. Supernatant was discarded and pellet was rinsed with 1 mL cacodylate-citrate buffer (0.01 M sodium cacodylate; 0.45 M sodium chloride; 0.10 M trisodium citrate; pH 7) and recentrifuged at the same speed and conditions. Supernatant was discarded and the pellet was dissolved with cacodylate buffer (0.01 M sodium cacodylate; 0.45 M sodium chloride; 0.01 M calcium chloride; 0.26 M magnesium chloride; pH7). The solution was then divided into two parts in 100 mL for each. The first solution was incubated with 50 mL trypsin (1 mg/mL cacodylate buffer) as an activator, while the second solution was added with 50 mL cacodylate buffer (trypsin replacement). Both solutions were incubated for 10 minutes at temperature of 25°C-26°C. Then, each solution was added wit 50 mL L-dopa (3 mg/mL cacodylate buffer) and the solution was left for

5 minutes before being added with 800 mL cacodylate buffer. PO activity was then measured using a spectrophotometer with optical density of 490 nm absorbance. Optical density (OD) of PO activity in all test conditions was noted as dopachrome formation in 50 mL haemolymph.

The Presence of *Vibrio* sp. in Shrimp Haemolymph

To determine the success of the bacterial infection into the shrimp, the presence of *Vibrio* bacteria was observed in the haemolymph shrimp test on 1 hour after injection and followed by observation on day-1, day-3, and day-6. Haemolymph was taken from the abdomen of the second segments of shrimp. The bacteria were isolated using TCBSA (thiosulfat citrat bile salt sukrose agar) medium and then incubated for 24 hours at room temperature. The successful infection was indicated by adjusting the characteristic bacterial colonies that were injected and fluorescent characteristic colony was used as the main criterion of bacterial identification. Observation of luminous *Vibrio* colonies was carried for six days.

Study Design and Data Analysis

The study was arranged in a completely randomized design with two treatments and three replications, where each treatment has one replicate for only used for sampling haemolymph of shrimp. For comparison, the negative control was also performed by injected physiological solution sterile to the shrimp. The treatments of this present study was the challenge test to the transgenic (A) and non-transgenic (B) shrimp, by injecting the *V. harveyi* for both in concentration of 10^6 cfu/mL.

Data obtained in this present study were presented in average value±SD. The evaluation of differences of immune responses (total haemocyte, haemocyte differentiation, and phenoloxidase activity) between transgenic and non-transgenic shrimp after challenge test with *V. harveyi* was performed by t-test analysis by SPSS software. The presence of bacteria *Vibrio* sp. in haemolymph was analyzed descriptively.

RESULT AND DISCUSSION

Total Haemocyte

Total haemocyte of transgenic shrimp (1.57 x 10^7 cells/mL) were not significantly different (P>0.05) compared to non-transgenic shrimp (1.13 x 10^7 cells/mL). Similar pattern of total haemocyte was obtained between transgenic and non-transgenic shrimp (Figure 1). This may reflect a pattern of shrimp defense against bacterial infections of *V. harveyi*. The Figure 1 showed that the total haemocyte prior to the challenge test (initial) was 0.37-0.62 x 10^7 cells/mL. Total haemocyte was likely to be lower compared to that reported by Braak (2000), namely that the total haemocyte of tiger shrimp *P. monodon* was 5.09±1.77 x 10^7 cells/mL using a coulter counter ZM. While according to Tampangallo & Susianingsih (2011), total haemocyte of shrimp exposed by

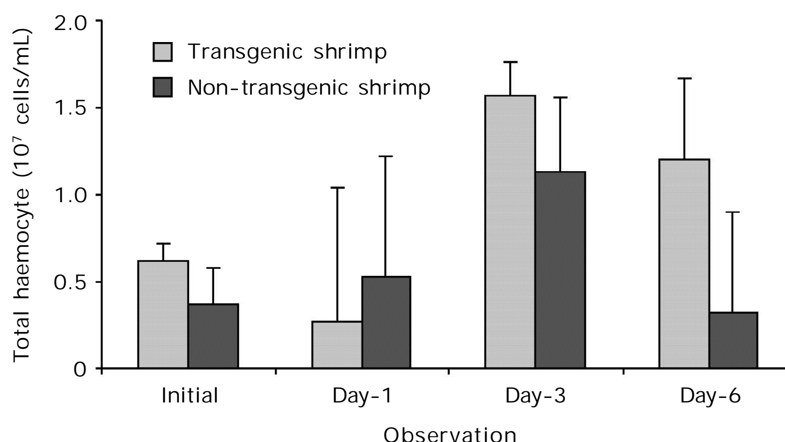

Figure 1. Total haemocyte on the haemolymph of transgenic and non transgenic tiger shrimp (*P. monodon*) during the challenge test

bacteria *V. harveyi* in concentration of 10^6, 10^4, and 10^2 cfu/mL was 2.0 x 10^7 cells/mL, 9.74 x 10^6 cells/mL, and 7.23 x 10^6 cells/mL, respectively.

A day after a challenge test, haemocyte of non-transgenic shrimp (0.53 x 10^7 cells/mL) was higher compared to the haemocyte transgenic shrimp (0.27 x 10^7 cells/mL). But further observation in day-3, haemocyte of transgenic shrimp (1.57 x 10^7 cells/mL) was higher compared to the non-transgenic shrimp. On the observations in the last day (day-6), total haemocyte of transgenic shrimp was still higher (1.2 x 10^7 cells/mL) compared to non-transgenic shrimp (0.32 x 10^7 cells/mL). However, total haemocyte of the three observations were not significantly different. The results of this study, indicating that transgenic shrimp tended to have ability to multiply the haemocyte in order to get more resistance to attack pathogenic bacterium *V. harveyi*.

Total haemocyte is one indicator of the health status of shrimp that can be enhanced by providing immunostimulatory. Nucleotides can serve as an immunostimulant to increase the total of white shrimp haemocytes up to 87% higher than the control shrimp. These nucleotides can optimize the growth and multiplication of cells including cells haemocytes (Manopo, 2011). The application of vitamins C, E, and β-1, 3 glucan was performed by Kanagu *et al.* (2010) in order to increase the total shrimp haemocytes to 17 x 10^6, 15 x 10^6, and 18 x 10^6 cells/mL, respectively. The increase in total haemocyte of transgenic shrimp may be caused by the role of the function of antivirus gene that induce the production of haemolymph cell for the body's defenses.

Haemocyte Differentiation

Haemocyte differentiation is a comparison between the three types of shrimp haemocyte cell, such hyaline, granular, and semi-granular cell (Kakoolaki *et al.*, 2011). Hyaline cell in this study was characterized by its small size and did not have granules. Haemocyte differentiation of transgenic and non-transgenic shrimp during this study was shown in Figure 2.

Hyaline cell of non-transgenic tiger shrimp (69.67%) before the challenge test was higher than the transgenic shrimp (30.00%). Hyaline cells on non-transgenic shrimp in the early observations were higher than transgenic shrimp, but it decreased gradually until day-6. The percentage of hyaline cell of non-transgenic shrimp on the initial and day-1 was a quite different value from the transgenic shrimp, while on day-6, the transgenic shrimp (16.67%) had similar percentage with non-transgenic shrimp (19.00%). T-test analysis results indicated that the transgenic shrimp showed the ability to maintain the composition of haemocyte cell types to day-6, that may happen due to the over expression of antiviral gene on transgenic shrimp (Parenrengi, 2010). According to Kakoolaki *et al.* (2011), hyaline cell is responsible for immunity system as phagocytosis.

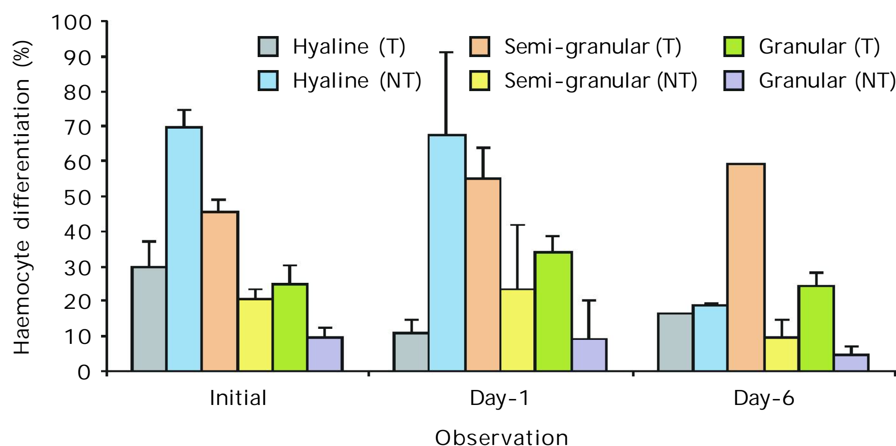

Figure 2. Haemocyte differentiation of transgenic and non-transgenic tiger shrimp (*P. monodon*) during the study (T = Transgenic and NT = Non-transgenic shrimp)

Granular cell in this present study was characterized by their large size and has a granule, while semi-granular also has a granule but the size is smaller than granular cells. Semi-granular and granular cells on the transgenic shrimp showed the tendency to be higher than non-transgenic shrimp. On day-6, the granular and semi-granular was significantly different (P<0.05) between transgenic and non-transgenic shrimp.

Both of granular and semi-granular cells have a responsible role for cytotoxic activity on production and release of prophenoloxidase system (proPO) (Kakoolaki *et al.*, 2011). The higher percentage of granular cell and semi-granular in particularly on the day-6 observation suggested that transgenic shrimp carrying PmAV gene would be able to enhance the immune system to tiger shrimp. A study of the use of nucleotide consisting of adenosine monophosphate (AMP), guanosine monophosphate (GMP), cytidine monophosphate (CMP), uridine monophosphate (UMP), and inosine monophosphate (IMP) on the food of white shrimp could increase the total haemocytes and activity of phenoloksidase (Manopo, 2011). The application of peptidoglycan isolated from bacterium *Bifidobacterium thermophilum* also improved granular cell of kuruma shrimp (*Penaeus japonicas*), and stimulated the activity of its phagocytosis (Itami *et al.*, 1998).

Phenoloxidase Activity

The pattern of phenoloxidase activity (PO) in transgenic and non-transgenic shrimp is similar from initial to the observation on day-6. During challenge test, the activity of phenoloksidase on transgenic shrimp showed to be higher than non-transgenic shrimp (Figure 3). The highest PO activity of transgenic shrimp obtained on day 3 (0.034) while the non-transgenic shrimp was only 0.018, followed by day-6 in value of 0.009 and 0.002, respectively. This present study indicated that the transgenic shrimp showed significant effect (P< 0.05) on increasing of phenoloxidase activity as well as improving total haemocyte and haemocyte differentiation in terms of granular and semi-granular cells. Kakoolaki *et al.* (2011) noted that the granular and semi-granular cells have responsibility for the storage and release of prophenoloxidase.

Phenoloxidase is one of the humoral immune systems of crustacean species (Flegel & Sritunyalucksana, 2011), which phenoloxidase enzyme functioned as a responsible agent for melanization process in response to the presence of foreign particles or pathogens attacked to the host. This enzyme also stimulated the act of biological reactions such as phagocytosys, encapsulation, and nodulation (Rodriquez & LeMoullac, 2000; Sritunyalucksana & Söderhäll, 2000; Vargas-Albores & Yepiz-Plascencia, 2000). Activity of phenoloksidase on black tiger shrimp could be enhanced through the use of β-glucan (Chang *et al.*, 2003) and lipopolysaccharide (Vargas-Albores *et al.*, 1998). The application of nucleotides 300 mg/kg in whiteleg shrimp diet significantly increased the phenoloxidase activity compared to the diet without nucleotides (Manopo, 2011).

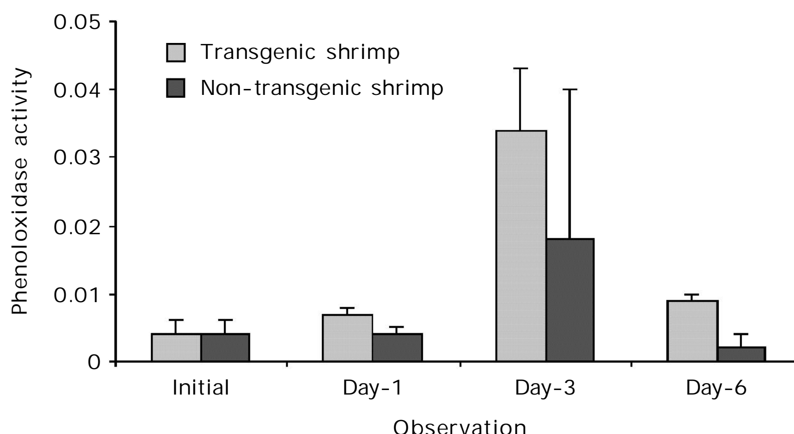

Figure 3. Phenoloxidase activity on transgenic and non-transgenic tiger shrimp (*P. monodon*) during the challenge test

Some studies regarding to the molecular technique have successfully isolated the responsible genes in order to increase the activity of phenoloxidase, such prophenoloxidase. Prophenoloxidase served to activate the enzymes as catalysts in initial step of melanin synthesis. The phenol was subsequently transformed into the quinones by phenoloxidase and then it was converted into melanin. PmMasSPH (*Penaeus monodon* masquerade-like serine proteinase homologue) has been successfully cloned by Amparyup *et al.* (2007) from shrimp cDNA of tiger shrimp. The increase PmMasSPH in haemolymph shrimp was observed 24 hours post-injected with *V. harveyi*. This suggested that PmMasSPH played an important responsibility in shrimp defense mechanism.

The Presence of *Vibrio* sp.

Total *Vibrio* bacteria in haemolymph of shrimp during the challenge study was shown in Figure 4. One hour after injection, the total bacteria in transgenic shrimp did not increase, while the non-transgenic shrimp showed the population of 2.56 log cfu/mL. The highest *Vibrio* spp. was obtained in observations of day-3 after injection of the non-transgenic shrimp (5.12 x 10^3 cfu/mL). On day-6 observation, both transgenic and non-transgenic shrimp showed the decreasing of the bacteria population of 30 cfu/mL and 40 cfu/mL, respectively. The presence of *Vibrio* sp. in haemolymph after injection also reported by Braak (2000), in which population of bacteria in haemolymph started to decline after the first 5 minutes injection up to one week of observation, although its population reached to zero. The high population of *Vibrio* in haemolymph caused shrimp mortality. The pathogenycity of *Vibrio* sp. was caused by the presence of hemolysin gene such as *Vibrio harveyi* (Zhang *et al.*, 2001; Kadriah, 2012), *V. parahaemolitycus* (Bej *et al.*, 1999), which can cause blood cell lysis of the host.

Observation on day-6 showed that the bacterial population started to decline in shrimp. The decrease of population was caused by the ability of the host to destroy pathogens inside the body through the multiplication of haemocyte cells and increase phenoloxidase activity. A pattern of decline in total *Vibrio* sp. as shown in Figure 4 may be due to gene of C-type lectin group in crustaceans and in penaeid species have a common specificity on the N-acetylated amino sugar especially sialic acid (Marques & Barracco, 2000). Cation of antimicrobial penaidin (AMPs) was expressed in circulating haemocytes and moved toward infection to destroy invading pathogens (Tassanakajon *et al.*, 2011). Destoumieux *et al.* (2000) has also conducted a series of studies on gene as an antimicrobial peptide in crustaceans such the penaidin P3-A consisting of 82 amino acids. The function of penaidin gene was to reduce pathogenic bacteria especially gram-positive bacteria in shrimp (Aoki *et al.*, 2011).

CONCLUSION

The immune response of total haemocyte on transgenic shrimp revealed to be higher than non-transgenic shrimp, but not signifi-

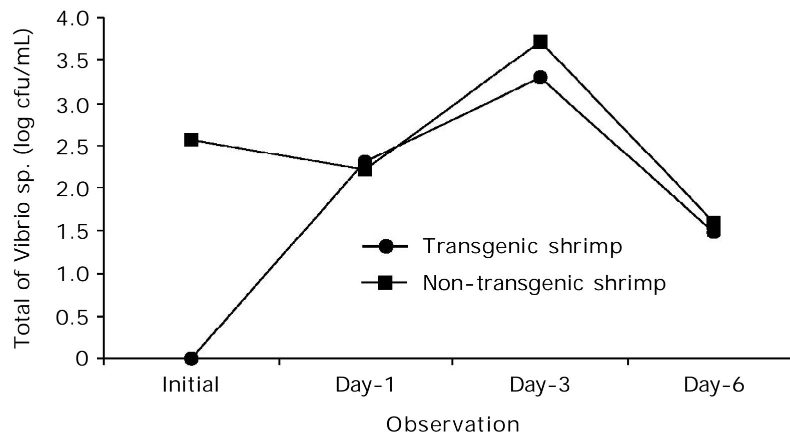

Figure 4. Total *Vibrio* sp. in haemolymph of tiger shrimp (*P. monodon*) during the challenge test

cantly different in statistical analysis. Haemocyte differentiation and phenoloxidase activity of transgenic shrimp was significantly different particularly on day-6 observation after being exposed to *V. harveyi* compared to non-transgenic shrimp. The implication of this present study was that the transgenic shrimp has ability to express more powerful immune responses as defense mechanism towards the invading pathogen bacteria.

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