accumulation of Cu and Zn in bivalves (filter feeders) are more related to the total concentrations in water colomn.

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The Effect of CpG-ODN on The Immune System on The Indonesia Black Tiger Shrimp (*Penaeus monodon*)

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ABSTRACT

The objective of the study was to examine the immunostimulatory effect of the Synthetic oligoedoxynucleotides containing CpG (CpG ODN) on Tiger Shrimp (*Penaeus monodon*). Shrimp size used in this study ranging from 5-7 g, while the CpG – ODNs used were CpG-ODN 1668, CpG - ODN 2006 and CpG - ODN 2133 . Data were analyzed using analysis of variance (ANOVA). The Results showed that CpG-ODN 2006 and 1668 can increase the phagocytic index and lysozyme activity of the black tiger shrimp. CpG - ODN 2006 has the highest capacity to be as an immunostimulant in Tiger Shrimp, therefore The CpG-ODN 2006 is indicated as a specific sequence for stimulating the immune response on the black tiger shrimp.

Keywords : CpG-ODN, Immunostimulant, Tiger Shrimp

INTRODUCTION

In recent years, tiger shrimp (*Penaeus monodon*) is one of the commodities in the fisheries sub-sector which is expected to increase the country's foreign exchange and is favored by the public because of its delicious meat taste and high nutritional value .

The problem that arises in the cultivation of tiger shrimp *(P. monodon)* is the occurrence of disease. One of the diseases found in tiger prawns is the pathogenic bacterial disease *Vibrio harveyi* . The disease that often attacks shrimp both in hatchery and rearing is Vibriosis. Vibriosis can cause harm due to the death it causes. The disease is usually caused by the bacterium *Vibrio harveyi* . The disease caused by *V. harveyi* is very acute and malignant because it can kill the affected shrimp larvae population within 1 to 3 days (Rukyani *,* 1999).

Alternatives that can be done are the use of antibiotics and probiotics as well as the application of vaccination, but the use of vaccines besides being expensive is also specific to certain disease agents, besides that it cannot be applied to shrimp because shrimp do not have memory cells to recognize the type of vaccine given. Therefore, it is necessary to use other substances that can prevent diseases that help increase immunity against disease agents known as immunostimulants. The use of immunostimulants is the safest solution as an effort to protect against disease, because it can increase the natural immune system (*innate immunity*) and *adaptive immunity* in fish (Sakai, 1999).

One type of immunostimulant that is very potential and effective in increasing the immunity of mammals, fish and shrimp is in the form of a specific nucleotide sequence called *the unmethylated cystidine phosphate guanosine* motif (Tassakka, et al , 2004). The CpG motif was found in bacterial DNA with a very high frequency and was not methylated, while in mammalian DNA the CpG motif was found at a very low frequency and was methylated. Along with technological developments and the demand for CpG is increasing, then synthetic DNA immunostimulants are found whose sequences resemble bacterial DNA *CpG* called *CpG oligodeoxynucleotides* or CpG-ODN (Tokunag et al., 1994). This CpG-ODN also functions as a defense agent against disease in

vertebrates (Krieg *et al* ., 1995). In fish, in addition to an increase in the expression of immune genes in fish lymphoid organs, such as *cytokine* and *lysozyme* genes (Tassakka *et al* ., 2006).

Other studies have shown that administering CpG-ODN to Atlantic salmon (*Salmo salar*) can also increase resistance to diseases such as *amoebic gill disease* . CpG-ODN can function as an adjuvant (stimulates vaccination to work well) in *goldfish* (Kanellos *et al.,* 1999). In addition, CpG-ODN can also increase the *innate immune system* of tiger grouper and can increase the prophenoloxidase immune response in giant prawns (Kaun-Yu Lu *et al* ., 2005).

Nevertheless, research on the role of CpG-ODN in the defense system of fish and other aquatic animals is still very few compared to mammals, especially crustaceans. Therefore, research on the effect of CpG-ODN on economically important species such as tiger shrimp *(P Monodon*) is very important to prevent the spread of disease, as well as providing new information on CpG sequences -OND specific boost the immune response and the specific sequence as anti-bacterial in tiger prawns which later can contribute to the development of science and technology i.

The purpose of this study was to examine the potential of the three types of CpG-ODN (CpG-ODN 1668. 2133, and 2006) in enhancing the immune response of tiger prawns *(P. monodon)* as well as to obtain specific sequences of CpG-ODN that can enhance the immune response

MATERIALS AND METHODS

Research Location and Design

Research was conducted in the Laboratory of Fish Health and Aquaculture Wet Laboratory Department of State Agricultural Polytechnic Pangkep. This experimental design was designed using a completely randomized design (CRD) with 4 treatments and 3 replications each and static testing with ANOVA. Black tiger shrimp size used was 5-7 gr obtained from Pangkep Regency. Data from the percentage value of phagocytic index and lysozyme activity obtained in the study were analyzed by ANOVA. To find out the different treatments, the LSD (lenst Singificant Difference by Student's T) test was performed with a significance level of 95%.

Administration of CpG-ODN

The CpG-ODN used were CpG-ODN 2133, CpG-ODN 2006 and CpG-ODN 1668. These CpG-ODNs obtained from Japan SAWADY. The administration of the CpG-ODN to the test animals was carried out by the injection method. Each CpG-ODN with a different motif was suspended in 0.01 M distilled water with a concentration (50 g/ml) and 0.1 ml was injected into tiger prawns. The control tiger prawns were injected with the same volume of pure distilled water. The injection was carried out in the ventral sinus in the second segment of the tiger prawn's abdomen using a 1 mL syringe . Observations of immune response *Pagocytosis Index (IP) and Lysozyme activity were* carried out before injection and on days 1, 3, 5, 7, after injection.

Phagocytosis Index

Shrimp hemolymph was added as much as 0.1 ml into the microplate and mixed evenly with 25 l of *Staphylococcus* sp bacteria and incubated for 20 minutes. Then as much as 5 l was dripped on a glass object and made preparations for review. It was then fixed with 100% methanol for 5 minutes and stained with Giemsa (10%) for 15 minutes. Phagocytic activity was measured based on the percentage of phagocytic cells showing the phagocytic process (Anderson and Siwicki, 1993). The phagocytic index is calculated by the formula:

$$\text{Phagocytic Index} = \frac{\textit{Number of Phagocytic Cells}}{\textit{Total Number of Phagocytic Cells}} \times 100\ \%$$

Lysozyme activity

To determine the activity of lysozime, the following procedure can be performed: *Micrococcus luteus was* cultured in a liquid medium, namely TSB (Tryptate Soy Broth) for 24 hours as much as 3 ml, made a mixture of *Micrococcus luteus* 1940 l, added PBS pH 7.4 as much as 60 l. Make a mixture of *Micrococcus luteus* 1940 mL, add shrimp blood (without treatment) by 60 µl.Membuat mixture *Micrococcus luteus* 1940 mL, add shrimp blood (control 1) 60 µl.Membuat mixture *Micrococcus luteus* 1940 mL, add shrimp blood (control 2) as much as 60 l. Control Dissolved Water as much as 2000 l. Take as much as 2000 l from each solution and then put it in the plate well. The calculation of the losozyme value using simple linear regression (Gaspersz, 1991). The reading results on the Bioo Microplete Reader were analyzed using a regression equation. Enzyme unit (EU) is the amount of enzyme that causes a decrease in absorbance of 0.001/min.

RESULTS

Phagosity Index

Figure 1 shows that CpG-ODN 2006 has the highest potential to increase the phagosity index with a value of 54.67%, followed by CpG-ODN 1668 with a value of 42.33% . The increase in the Phagosity Index reached its maximum point on the 3 days (H3) after treatment then began to increase on the day 5 to the day 7

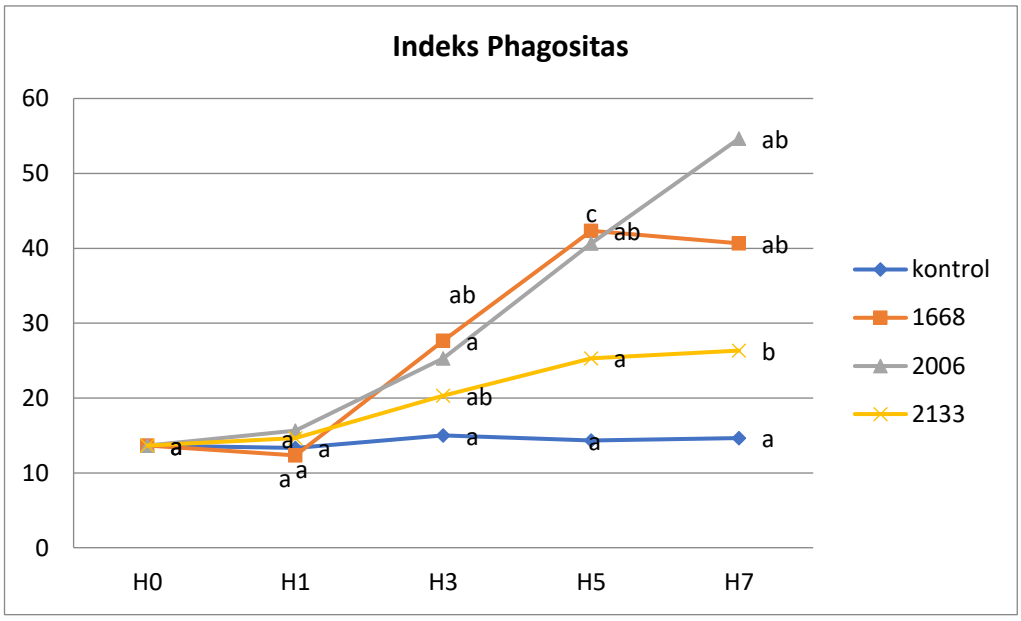

Figure 1. The Percentage of the Phagocytic Index in the Tiger Shrimp (Penaeus monodon) After Treated by CpG ODN

Lysozyme Activity

Figure 2 shows that before the administration of CpG-ODN the average value of lysozyme activity was 0.016 units and the observation on day 1 increased with the highest value found in the 2006 CpG-ODN treatment with a value of 62 units following CpG-ODN 1668 with a value of 55 units, 2133 with a value of 24 units and controls. The graph of lysozyme activity continued to increase from day 1 to day 7 can be seen in (Figure 2) .

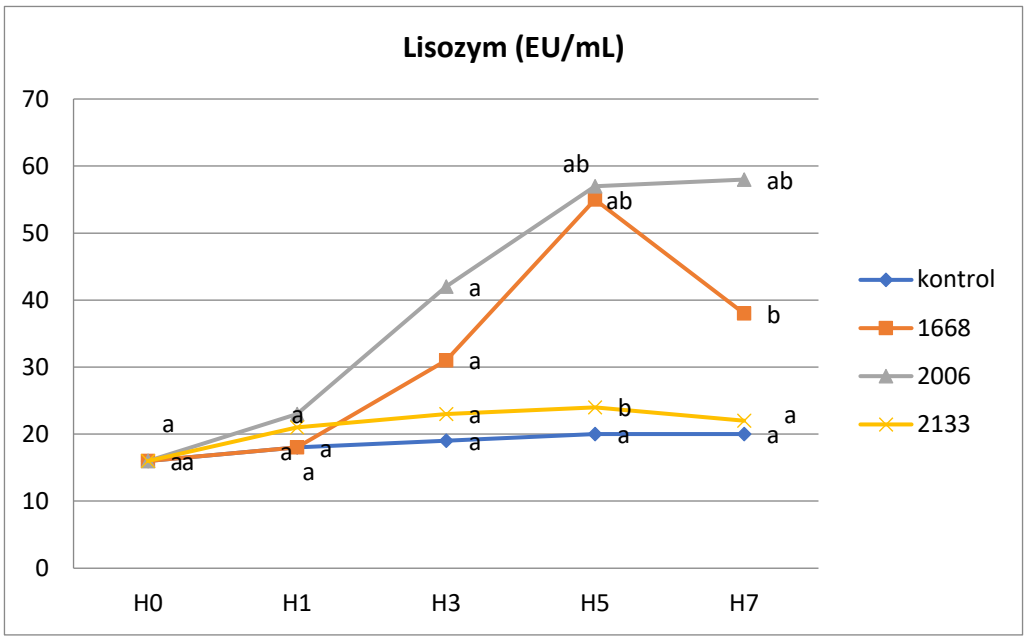

Figure 2. The Percentage of the Lysozyme Activity in the Tiger Shrimp (Penaeus monodon) After Treated by CpG-ODN.

DISCUSSION

Research was carried out on the shrimp using three types of CpG-ODNs namely CpG 1668, 2006 and 2133. These CpG-ODNS were injected to Tiger shrimp (*Penaeus monodon)* and could improve the Phagocytic index. The results of the analysis of variance on day 3 (H3), showed that CpG-ODN had an effect on the phagosity index . The results of the Tukey CpG-ODN test 2006 and 1668 were significantly different from CpG-ODN 2133 and controls, this shows that, the 2006 and 1668 CpG-ODN types on the day 3 had good ability to increase the phagosity index.

On the day 5 (H5), the results of the analysis of variance showed that the type of CpG greatly influenced the phagosity index ($p<0.001$), and continued with the Tukey test with the result that CpG-ODN 2006,1668 and 2133 were significantly different from the control, indicating that the type CpG-ODN on day 5 was very influential in increasing the phagositic index compared to other CpGs .

The results of the analysis of variance on day 7 (H7), showed that CpG-ODN had an effect on the phagosity index . The results of the Tukey CpG-ODN test 2006 and 1668 were significantly different from CpG-ODN 2133 and controls, showing that, the 2006 and 1668 CpG-ODN types on day 3 had good ability to increase the phagocytic index. Statistical test results from all observation times showed that CpG-ODN 2006, 1668 and 2133 could increase the phagosity index. The paghosity index value based on the time of observation showed that it was quadratic ($p<0.05$), where the 2006 CpG-ODN had the highest response value occurred on the 7th day.

The increase in the phagosity index value of tiger prawns after obtaining CpG-ODN 2006 was basically an implication of the increase in hemocytes. Shrimp haemocytes have the same biological properties and functions as macrophage cells, granulocytes, and natural killer vertebrates (Van de Braak et al., 2002). These cells play a role in phagocytosis, encapsulation, nodule formation, wound healing, clotting, and proPO activation. These cells also aid in the production of adhesive, agglutinating, and antimicrobial peptide (AMP) molecules (Bachere et al., 2004). Phagocytosis involves the internalization of foreign material. Phagocytosis is the main cellular defense mechanism in invertebrates, and is carried out by semi-granulocytes and granulocytes,

these cells consist of chemotaxis, attachment, engulfment, pathogen destruction and exocytosis (Kondo et al., 1998).

The results of analysis of variance showed that all CpG-ODN treatments had no significant effect on increasing lysozyme activity from day 1 to day 3 but on the 5th and 7th day observations only 2006 CpG-ODN had a significant effect on total lysozyme ($p<0.05$). Tukey's further test results showed that 2006 CpG-ODN species had the highest lysozyme activity and was significantly different from other CpG-ODN types on day 7 .

On the day 5 (H5), the results of the analysis of variance showed that the type of CpG had an effect on lysozyme ($p<0.005$), and continued with the Tukey test with the result that CpG-ODN 2006 and 1668 were significantly different from CpG-ODN 2133 and control, in the sense that CpG-ODN 2006 and 1668 on day 5 were very influential in increasing lysozyme compared to other CpGs .

On the day 7 (H7), the results of the analysis of variance showed that the type of CpG had an effect on lysozyme ($p>0.05$), and continued with Tukey's test with the result that 2006 CpG-ODN was significantly different from other CpGs, showing that 2006 CpG-ODN on day 7 was very influential in increasing lysozyme compared to other CpGs .

The effect of 2006 CpG-ODN which was significantly different on the day 7 was related to the cell maturation process. The cells will experience peak maturity on the seventh day and that's when the lysozyme enzyme will be active and work according to its function as one of the non-specific body defenses.

The ability of CpG-ODN 2006 and 1668 in enhancing the immune response , phagosity index and lysozyme activity had an impact on the high survival rate of the test animals . CpG-ODN 2 006 had a significant effect on the lysozyme activity of *C. quadricarinatus in* line with Chen (2007)'s opinion that administration of a recombinant plasmid containing a copy of the CpG-ODN 2006 motif in giant prawns had an effect on increasing lysozyme activity. CpG-ODN 1668 had no effect on lysozyme activity because it was specific for fish .

Lysozyme is an indicator to see the level of immunity or immune response in shrimp that are given immunostimulants. Lysozyme is found in serum and mucus (*mucus*) in fish, especially those associated with leukocyte-rich tissues such as the kidneys, stomach and spleen. Lysozyme is a major source of monocytes, macrophages and neutrophils (Tasakka, *et al., 200 4*). In shrimp, lysozyme can be derived from hyaline (in vertebrate macrophages), which is known in shrimp haemocytes divided into hyaline (agranular), semigranular and granular (Braak , *et al* ., 2002).

Lysozyme enzyme is a non-specific immune response that plays a very important role in shrimp body defense. Lysozime are enzymes that sever the bond of β-1,4-glycoside between acid-N-acetyl glucosamine with acid-N-acetyl muramat on peptidoglycan , which can damage the cell walls of bacteria. Water can then enter the cell and cause the cell to swell and eventually burst, the process is called Van de lysis (Braak , *et al* ., 2002) .

The highest survival results were CpG-ODN 2006 of 96.67% and CpG 1668 of 90.00% in increasing the immune response, phagocytic index and lysozyme activity which had an impact on the high survival rate of the test animals. Phagocytic activity is the main defense possessed by shrimp, so that with the increasing phagocytic activity, the humoral and cellular body defense systems will also increase .

CONCLUSIONS

CpG-ODN 2006 and 1668 can increase the phagocytic index and lysozyme activity of the black tiger shrimp. CpG - ODN 2006 has the highest capacity to be as an immunostimulant in Tiger Shrimp, therefore

The CpG-ODN 2006 is indicated as a specific sequence for stimulating the immune response on the black tiger shrimp.

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