Expression of Antiviral Genes from Several Organs of Tiger Shrimp (*Penaeus monodon*)

Andi Aliah Hidayani[1], Asmi Citra Malina[1] and Andi Parenrengi[2]

[1] *Faculty of Marine Sciences and Fishery, Hasanuddin University, Makassar*
[2] *Research Institute for Coastal Fisheries, Maros*
E-mail: lhia_achsan@yahoo.com

Abstract

One of shrimp disease prevention is by improving the genetic quality of the shrimp through transgenesis methods. Genetically modified organisms that are resistant to pathogens can be produced by introducing genes that play a role in the immune system and genes encoding immunogenic proteins of pathogens. Antiviral gene encoding the tiger shrimp, named *PmAV* known as genes that play a role in the immune system in black tiger shrimp. This study was performed to look at the expression of antiviral genes in multiple organs such as the shrimp hepatopancreas, carapace haemolimph and an attempt to understand the mechanism of resistance of shrimp against WSSV. The method used consists of two phases, the first covering the extraction of RNA from natural shrimp and cDNA synthesis by RT-PCR that aimed to get *PmAV* antiviral genes. The second stage was the isolation and observation *PmAV* antiviral gene expression. The results showed that the highest percentage for both females and males of shrimp antiviral gene expressions was found in the hepatopancreas organs was 100% and 66.7% of the total female and male organ of shrimp, respectively. This proves that the hepatopancreas has an important role in the body's defense mechanism against pathogens of shrimp.

Keywords: Antiviral gen, expression, WSSV, Tiger shrimp

Introduction

Tiger shrimp (Penaeus monodon) is one of the local species of crustaceans that are cultured in brackish water ponds in Indonesia and has generated significant foreign exchange. Nevertheless, since the 1990s, experiencing a variety of tiger shrimp deaths, due to both the aquatic environment and the lack of support to bacterial and viral diseases. At least 20 types of viruses that cause diseases in shrimp farming have been reported (Zhang et al. 2004 in Parenrengi, 2010).

Disease which is the cause for the loss of shrimp farming in Indonesia, one of which is called White Spot Disease. Until now the disease is caused by White Spot Syndrome Virus (WSSV) is one of the leading causes of death in the black tiger shrimp ponds in Indonesia. WSSV assault case first emerged in East Asia in 1992-1993 and

quickly spread by infected seed and stock holding, across the continent of Asia to Southeast Asia and India is a major cause of the pandemic, and then causing significant losses in some areas (Lightner, 2003).

Various efforts have been done to prevent the disease, such as vaccine delivery (Tizard, 1988; Anderson, 1974; Ellis, 1988, Witteveldt et al., 2004), the use of immunostimulants and probiotics (Robertson et al., 1990; Anderson, 1992, Effendy et al., 2004, Zar et al., 2006). In addition, other ways that can be done is by improving the genetic quality of the shrimp through selection and genetic engineering. Development of molecular techniques are more and more nowadays, provides great opportunities to learn the defense mechanisms of shrimp against WSSV attacks. Antiviral gene encoding the shrimp, named PmAV (Penaeus monodon anti-viral) has been identified by Luo et al. (2003). Antiviral genes (PmAV) isolated from black tiger shrimp were collected from aquaculture farms in China, where the gene has been identified as a gene that plays a role in the immune system in black tiger shrimp. Parenrengi (2010) have successfully performed antiviral gene transfer (PmAV) and showed an increase in resistance of shrimp against WSSV.

Luo et al. (2007) have reported the expression of antiviral genes PmAV naturally on non-transgenic tiger shrimp through the test challenged with WSSV. However, until now there has been no study on the distribution of antiviral gene expression in hepatopancreas, haemolimph and shrimp carapace. Therefore, this study has the purpose to see the expression of antiviral genes in hepatopancreas, carapace haemolimph and an attempt to understand the mechanism of resistance of shrimp against WSSV.

This study aimed to examine the expression of antiviral genes in hepatopancreas, carapace, haemolimph and an attempt to understand the mechanism of resistance of shrimp against WSSV. By knowing the distribution of antiviral genes PmAV on the organ, can be used as the location of genes PmAV information that will be isolated for

purposes of gene transfer in an effort to get a shrimp species that has a high resistance against WSSV.

Materials and Methods

Samples of Tiger Shrimp

Tiger shrimp *P. monodon* measuring between 20-30 grams as 6 individuals (3 males and 3 females), obtained from shrimp aquaculture in South Sulawesi. Shrimp samples collected in life and then taken to the Laboratory of Biotechnology, Research Institute Brackish Water Aquaculture, Maros for taking the hepatopancreas, carapace haemolimph and for extraction of total RNA.

RNA Extraction

Total RNA was extracted using *isogen* kit (Nippon Gene) in the manner as described by Parenrengi (2010). A total of 20-25 mg samples of shrimp put in 1.5 mL micro tube, then diluted with 200 µL *isogen* in a container with ice. Sample, was crushed with a grinder micro tube isogen added back up to 800 µL, then incubated at room temperature for 5 minutes so that the sample can be lysis perfect. Sample was added to 200 µL of chloroform then divorteks and allowed to return to room temperature for 2-3 minutes. Sample centrifuged at 12,000 rpm for 10 min and then stored at room temperature for 5 min and the supernatant was transferred into a tube formed a new micro has been filled with 400 µL iso-propanol. Homogenized sample with micro tube slowly and then stored at room temperature for 5-10 minutes. Samples were centrifuged again at a speed of 12,000 rpm at 4°C for 15 minutes. Supernatant was discarded, while the pellet was dissolved in 1 mL of cold ethanol 70% and then centrifuged at 12,000 rpm at 4° C for 15 minutes. Supernatant was discarded, and subsequently dried pellets in vitro micro-

aired. RNA pellet was dissolved in DEPC 0.1% by 50 µL and followed by cDN synthesis.

cDNA synthesis by RT-PCR

Synthesis of complementary DNA (complementary DNA, cDNA) was performe using a kit Ready-To-Go You-Prime First Strand Beads (GE Healthcare) by RT-PCR RNA concentrations 3 µg in 30 µ L DEPC 0.1% homogenized by vortex in the micr tube, then incubated at 65°C for 10 minutes. Furthermore micro tubes put in ice for minutes, then put into the tube of RNA first strand reaction mix beads which alread contains 2 grains of white balls. Prime oligo (dT) 5' - gta ata cga ata act ata ggg cac gc tgg tcg acg gcc cgg gct ggt ttt ttt ttt ttt ttt t-3' with a concentration of 1 µg/ 3 µL added into the reaction, then left to stand for 1 minute. Micro tubes were incubated at 37° C fo 1 hour. Then cDNA formed added 50 µL SDW.

Isolation and Antiviral Gene Expression

Isolation of antiviral genes *PmAV* performed using cDNA as template DNA PmAV gene was isolated by using the *forward* primer specific *PmAV*-F 5'- tag tgc atg ca atg ggt cat aca atc cta -3 'and *reverse PmAV*-R 5'-ctg tctcgagct atg tgt cct gct ttc aca -3' with a target fragment 513 bp long. The primer has been equipped with the restrictio sites (underlined nucleotides), respectively SPHL and XhoI to assist in the process o cloning. One milligram of cDNA used as a template for PCR kit Pure Taq Ready-To-Go PCR Beads (GE Healthcare), and then mixed with each 1 µL (50 pmol / µ L) *Forward* and *Reverse* primer is then added SDW up to 25 µL. The kit contains 2.5 units of Taq polymerase; 10 mM Tris-HCl pH 9; 50 mM KCl; 1.5 mM MgCl2; and 200 µM each dNTP-mix.

Antiviral gene expression can be observed through antiviral gene amplificatio using PCR machine GenAmp 2700 (Applied Biosystems). **PCR process is run on pre-**

denaturation temperature of 94°C for 2 min for 35 cycles consisting of 30 seconds denaturation at a temperature of 94°C, 30 s annealing at a temperature of 61°C, at a temperature of 72°C extension for 45 seconds), and **final extension for 7 min at 72° C.** Used as an internal control β-actin gene expression shrimp (Parenrengi, 2010). β-actin gene amplification was performed using PCR machine (Applied Biosciences 2700) with the following program: pre-denaturation 94°C for 2 min, 35 cycles consisting of 30 seconds denaturation at 94°C, 30 s annealing at 55°C, extension at a temperature of 62° C (for 1 min), and final extension for 5 min at 68 °C. DNA fragment of β-actin gene expression targeted at position 400 bp. To see the success of amplification of the target DNA fragments, electrophoresis results of PCR using 2.0% agarose gel and documented with a Gel Documentation System (Biometra). To determine the molecular weight marker DNA fragments used VC 100 bp Plus DNA Ladder (Vivantis).

Data Analysis

The resulting data were analyzed by descriptive pattern (DNA fragment thickness) antiviral gene expression in hepatopancreas, haemolimph and shrimp carapace. The success of isolation and antiviral gene expression is determined by the expression of β-actin gene as an internal control.

Results and Discussion

Antiviral gene expression *PmAV* sized shrimp average weight of 10.3 g and an average length of 10.8 cm, in some organs (hepatopancreas, haemolimph and carapace) shows that the results of six samples, five samples antiviral gene expression the hepatopancreas organ. But on the other organs do not express the gene antivirus *PmAV* (Table 2). Indicator of antiviral genes are expressed or not used in this study is evidenced by the presence of fragments of β-actin gene expression shrimp both male and female, because β-actin gene was always expressed at all times and in all organs so that

expression can be used as an internal control in the expression of other genes (Parenrengi, 2010). In Figure 1 and 2 show that the gene expression pattern of β-actin have relatively the same thickness and fragments found in all organs and tissues of male and female. This implies that the antiviral gene expression amplification by PCR has been going well.

Table 1. PmAV gene expressions from several organs of shrimp

No	Sample code	Size Weight (g)/ length (cm)	Sex	Gene Expression/organ *			Gene Expression percentage **
				Hp	H	C	
1	1 ♀	9,3/10,5		+	-	-	
2	2 ♀	9,4/10,7	Female	+	-	-	Hepatopancreas 100%
3	3 ♀	8,9/10,4		+	-	-	
4	1 ♂	12,8/11,5		+	-	-	
5	2 ♂	11,7/11,3	Male	+	-	-	Hepatopancreas 66,7%
6	3 ♂	9,8/10,2		-	-	-	

Caption: * Hp = Hepatopancreas; H = Haemolimph; C = Carapace.
+ = Organs that express antiviral genes *PmAV*
- = Organs that do not express the gene antivirus PmAV
** PmAV antiviral genes that are expressed in the hepatopancreas organ

As data in the table showed above, the percentage of males sampled shrimp antiviral gene expression only in the hepatopancreas organ is 66.7% of the total male organ of shrimp, whereas other organs do not express the gene PmAV antivirus. The same is the female tiger shrimp showing PmAV antiviral gene expression only in the hepatopancreas organ of 100%. From these data it can be seen that there is no difference between males and females in PmAV antiviral gene expression, where the expression of antiviral genes commonly found in the hepatopancreas. This is presumably because there is no difference in the immune system of shrimp labor between males and females.

Observations *PmAV* gene expression patterns in this study showed almost all organ samples positive hepatopancreas *PmAV* antiviral genes expressed in the amount of 83.3% of the total sample *PmAV* antiviral gene expression, whereas other organs do not express antiviral genes. Visualization of DNA fragment *PmAV* antiviral genes in DNA fragment gel electrophoresis showed the position approximately 513 bp (Fig. 3 and 4).

Research Parenrengi (2010) succeeded in isolating genes from cDNA antivirus PmAV hepatopancreas of shrimp (P. monodon) by RT-PCR. Among the 22 samples from several locations shrimp aquaculture, 6 samples (27.3%) showed antiviral gene expression.

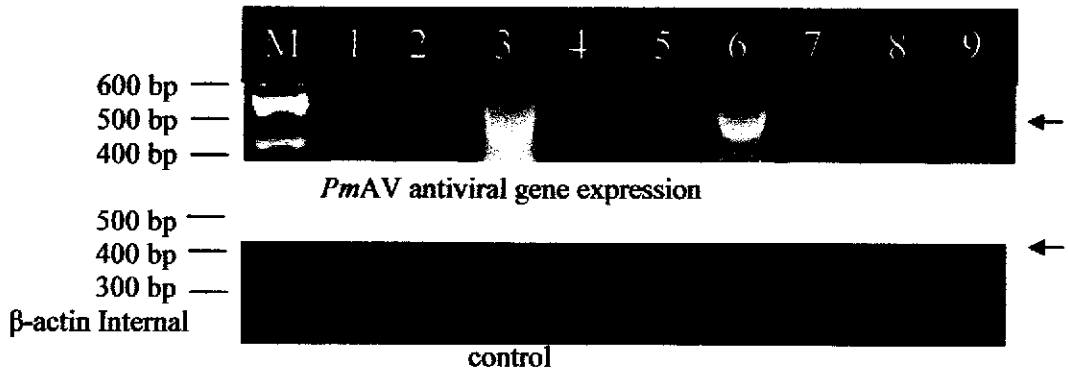

Figure 1. Electrophoresis results *PmAV* antiviral gene expression and internal control β-actin in the male tiger shrimp, M = marker 100 bp Ladder, Haemolimph = 1,4,7, 2,5,8 = 3,6,9 = carapace and hepatopancreas (3 and 6 = *PmAV* antiviral gene expression, 1,2,4,5,7,8, and 9 = no antiviral gene expression *PmAV*). The arrows indicate *PmAV* antiviral gene fragment in a position approximately 513 bp and β-actin control fragment at a position approximately 400 bp.

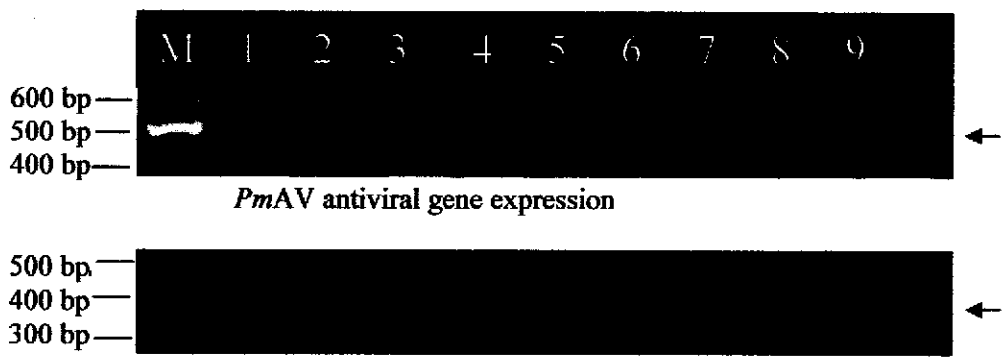

Figure 2. Electrophoresis results *PmAV* antiviral gene expression and β-actin internal control in shrimp females, M = marker 100 bp Ladder, Haemolimph = 1,4,7, 2,5,8 = 3,6,9 = carapace and hepatopancreas (3, 6 and 9 = *PmAV* antiviral gene expression, 1,2,4,5,7, and 8 = no antiviral gene expression *PmAV*). The arrows indicate PmAV antiviral gene fragment in a position approximately 513 bp and β-actin control fragment at a position approximately 400 bp.

Based on the above results indicate that the cells of different organs also respond differently to the same virus. The differences are thought to be related to the structural differences between the structure of the hepatopancreas tissue, haemolimph and carapace. It is also associated with the condition of the body's defense shrimp itself. Based on the

study Peng *et al.* (1998) *in* Mufidah and Koesharyani (2010) stated that the highly pathogenic WSSV infection in shrimp given conditions of pressure, this is because the body's defense mechanisms can not prevent or shrimp resist WSSV propagation under conditions of stress. WSSV can spread quickly to various organs such as the heart, epidermis, muscles and digestive system even in small amounts. Viruses also can be found in the shrimp haemolimph showing clinical symptoms, it is thought to WSSV spread through the circulatory system (Momoyama *et al.*, 1995 *in* Mufidah and Koesharyani, 2010).

Figure 1 and 2 shows that the distribution of gene expression is the highest in the hepatopancreas organ. PmAV high gene expression in hepatopancreas organ because hepatopancreas also one of the organs involved in digestion and metabolism play a role in the digestion of food and absorption of nutrients for shrimp (Vogt, 1992 *in* Littik, SAM., 2009). Hepatopancreas cephalotorax which is located on one of the organs that can produce digestive enzymes, store and dispose of the metabolism of the metabolic waste. The enzymes were successfully extracted from this organ are protease, amylase, and xylanase sellulase (Pavasovic, 2004). According to Karin (2002), the shrimp hepatopancreas organ composed of tubules were closed and the production of enzymes that flowed through the ductus hepatopancreas. The organ is very vital because it has the same functionality as the liver and pancreas in mammals.

In general, one can ideally reflect the health parameters of the immune function (immune) associated with the relevant health conditions and is easy to be measured and observed. Hepatopancreas is assumed to be the major site of production of molecules that play a role in the immune system of crustaceans (Hastuty, 2006). Gross *et al.*, (2001) *in* Hastuty (2006) stated hepatopancreas is an organ where digestion haemolimph filtration plays an important role in the immune system.

Hepatopancreas have antimicrobial activity against bacteria, fungi and viruses. Antimicrobial activity is due to the gene encoding C-lectins contained in the

hepatopancreas. Based on research conducted by Parenrengi (2010) in shrimp hepatopancreas cDNA indicates that the site has similarities to *C-type lectin-like domain (CTDL)* are considered the same as a group of genes C-type lectin on the species of crustaceans. CTDL existence has also been reported by Luo *et al.* (2003) on antiviral genes isolated from black tiger shrimp and Kong *et al.* (2008) on the crab *Portunus trituberculatus*.

Lectins are known to have an important role in non-specific defense system in invertebrates based on biochemical analysis and molecular studies on some species of crustaceans (Zelenksky and gready, 2005). Furthermore Luo *et al.* (2006), reported that lectins from shrimp cDNA P. monodon (*PmLec*) plays a role in non-specific immunity in particular the identification of proteins and their function as opsonin. Zhao *et al.* (2009) reported that *L. vanname* C-type lectin-1 (*LvCTL1*) expressed very high in healthy vannamei shrimp hepatopancreas. The same was reported by Soonthornchai *et al.* (2010) in hepatopancreas of shrimp (*P. monodon*) healthy.

All organs can express antiviral genes against WSSV. Luo *et al.*, (2007) reported that the antiviral gene expression in hepatopancreas PmAV 700 times higher than in the muscle, but the gene may also be expressed in haemosit (2.84 times), colon (7.4 times), stomach (6 , 81 times) and gills (12.9 times) by using real-time PCR method. However, gene expression resulting from organ healthy shrimp are usually lower or no expression compared with organs that have been tested shrimp challenged with WSSV. From the results of our study indicate that there is no distribution of gene expression in haemolimph and carapace. Gene expression is likely to emerge after the shrimp organs tested WSSV challenge. Research conducted by Zhao *et al.* (2009) reported that *L. vanname* C-type lectin-1 (*LvCTL1*) was not detected in healthy shrimp haemolimph, but the present and the concentration increased in haemolimph shrimp injected with WSSV. This suggests that *LvCTL1* expression induced by WSSV infection. Involvement of antiviral genes in shrimp immune system has also been studied by Tenriulo *et al* (2010).

The study revealed that the antiviral gene expression in hepatopancreas of shrimp *PmAV* increased when exposed to WSSV virus. It shows that the antiviral gene expression increased (up-regulation) due to viral infection.

The presence of DNA fragments at a position approximately 513 bp DNA marker can be used as resistant shrimp in the selection program. Expression of these genes is very high in the hepatopancreas that antiviral gene detection PmAV very difficult to do if the sample to be kept alive shrimp. Observation of antiviral gene expression in tiger shrimp, especially hepatopancreas provide an overview of the involvement of antiviral genes in the immune system of shrimp. Parenrengi (2010) adds that the results of the study showed that the shrimp has a high resistance to disease virus (shrimp that escaped the attack WSSV) has the ability to express antiviral genes PmAV better than the other shrimp.

Conclusions

The results showed that the highest percentage for both females and males of shrimp antiviral gene expressions is found in the hepatopancreas organs was 100% and 66.7% of the total female and male organ of shrimp, respectively. The antiviral gene expression is mostly distributed in the hepatopancreas organ. This proves that the hepatopancreas has an important role in the body's defense mechanism against pathogens of shrimp.

References

Anderson DP. 1974. Fish Immunology. TFH Publication.Ltd. Hongkong.

Anderson DP. 1992. Immunostimulants, Adjuvants and Vaccines Carrier in Fish. Aplications to Aquaculture. Annual Review of Fish Diseases 21 : 281- 307.

Effendy S, Rantetondok A, Tahir A. 2004. Peningkatan Haemosit Benur Udang Windu (*Penaeus monodon* Fabricius) Pasca Perendaman Ekstrak Ragi Roti *(Saccharomyces cerevisiae) Pada Konsentrasi Yang Berbeda.* J. Sains & Teknologi, Vol. 4 No.2: 46-53.

Ellis AE. 1988. Fish Vaccination. 1. General Principles of Fish Vaccination. Academic Press.London.

Hastuti SD. 2006. Pengaruh Penyuntikan Poly I:C Terhadap Ekspresi Protein Yang Bertanggung Jawab Terhadap Sistem Kekebalan Lobster Air Tawar *(Cherax quadricarinatus* Von Martens*)*. Scientific Journal, Vol. 14 No.2: 155-162.

Karin VDB. 2002. Haemocyte defense in black tiger shrimp (*Penaeus monodon*), Proefscrift, PhD thesis, Wageningen University, 158 pp.

Kong HJ, Park EM, Nam BH, Kim YO, Kim WJ, Park HJ, Lee CH, Lee SJ. 2008. A C-type lectin like-domain (CTDL)-containing protein (PtLP) from swimming crab Protunus triberculatus. Fish Shellfish Immunol 25:311-314.

Lightner DV. 2003. The penaeid shrimp viral pandemics due to IHHNV, WSSV, TSV and YHV; History in the Ameicas and Current Status. In: Aquaculture and Pathobiology of Crustacean and Other Species. Proceedings of 32nd UJNR Aquaculture Panel Symposium, November 17-18 and 20th 2003, Davis and Santa Barbara, California, USA.

Littik SAM. 2009. Prevalensi dan Keparahan Infeksi Monodon-Baculovirus (MBV) Pada Organ Pencernaan Udang Windu (*Penaeus monodon*). Jurnal Ilmiah Satya Negara Indonesia Vol.2 No.1 :1-7.

Luo T, Zhang X, Shao Z, Xu X. 2003. *PmAV*, a novel gene involved in virus resistance of shrimp *Penaeus monodon*. FEBS Lett 551 : 53-57.

Luo T, Yang H, Li F, Zhang X, Xu X. 2006. Purification, characterization, and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon* [abstract]. *Dev Comp Immunol* 44:1516-1523.

Luo T, Fang L, Kaiyu L, Xu X. 2007. Genomic organization, promoter characterization and expression profiles of an antiviral gene *PmAV* from shrimp *Penaeus monodon. Mol. Immunol* 44 : 1516-1523.

Mufidah T dan I. Koesharyani. 2010. Histopatologi kasus multi infeksi alami *White Spot Syndrome Virus* (WSSV) dan *Infectious Hypodermal Haematopoetic Necrosis Virus* (IHHNV) pada *Penaeus monodon*. Prosiding Forum Inovasi Teknologi Akuakultur 2010. Hlm. 921-926.

Parenrengi A. 2010. Peningkatan resistensi udang windu *Penaeus monodon* terhadap penyakit White Spot Syndrome Virus (WSSV) melalui transfer gen *Penaeus monodon* antiviral. [Desertasi]. Bogor : Sekolah Pascasarjana, Institute Pertanian Bogor.

Pavasovic M. 2004. Digestive profile and capacity of the mud crab (*Scylla serrata*). [Thesis]. Brisbane : The Queenslend University of Technology.

Robertson B, G. Rostarad, R. Enggstad, and J. Raa. 1990. Enhancement of non specific diseases resistance in Atlantic salmon (*Salmo salar*) by a glucan from *Saccharomyces cerevisiae* cell walls. J. of Fish Diseases, 13 : 391 – 400.

Soonthornchai W, Rungrassamee W, Karoonuthaisiri N, Jarayabhand P, Klinbunga S, Söderhäll K and Jiravanichpaisal P. 2010. Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Developmental and Comparative Immunology* 34 : 19-28.

Tenriulo A, Tonnek B, Tampangallo BR, Widodo AF dan Parenrengi A. 2010. Analisis ekspresi gen Antivirus *PmAV* pada udang windu, *Penaeus monodon* yang ditantang dengan WSSV. Jurnal di Balai Riset Perikanan Budidaya Air Payau. Makassar, Hal. 541-546.

Tizard I. 1988. An Intoduction to Veterinary Immunology. Penterjemah P. Masduki dan S. Hardjosworo. Pengantar Immunologi Veteriner. Universitas Airlangga, Surabaya.

Witteveldt, J., Cifuentes, C.C., Vlak, J.M., van Hulten M.C.W., 2004. Protection of *Penaeus monodon* against White Spot Syndrome Virus by Oral Vaccination. *J. of virology*., 2057-2061.

Zar JLB, Blas ID, Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Mu´zquiz, J. L., 2006. The Role of Probiotics in Aquaculture. *Vet. Microbiol*., 114: 173–186.

Zelensky AN, Gready JE. 2005. The C-type lectin-like domain superfamiliy. *J. FEBS* 272:6179-6217.

Zhao ZY, Yin ZXm Xu XP, Weng SP, Rao XY, Dai ZX, Luo YW, Li Zs, Guan HJ, Li SD, Chan SM, Yu XQ, He JG. 2009. A novel C-type lectin from the shrimp *Litopenaeus vannamei* possesses anti-white spot syndrome virus activity. Journal of Virology 83 (1):347-356.