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The increase of survival rate of shrimp *Penaeus monodon* after administrated with VP28

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Abstract
White spot Baculovirus (WSBV) infection of shrimp is characterized by high mortality of tiger shrimp *Penaeus monodon.* The gross signs of moribund shrimp are present of white spot, initially circular, inside cuticle, sometimes accompanied by overall red body coloration. The 5 major protein were isolated from WSBV are 15 kDa (VP15), 19 kDa (VP19), 24 kDa (VP24), 26 kDa (VP26) and 28 kDa (VP28). The isolated protein were then purified and characterized to be VP19, VP24, and VP28. Purified protein injected to shrimp to detect whether they have a protective immunity against WSBV infection. The result showed protection of infected shrimp against WSBV after administration of VP19, VP24 and VP28 are 20%, 0% and 60%, respectively. VP28 injected shrimp have a high production of phenoloxidase 24.1822 U/min/mg and after WSBV challenged 44.6067 U/min/mg. Based on the result suggest that VP28 of WSBV is potentially to increase immune response in penaeid shrimp.

Keywords: white spot baculovirus (WSBV), VP15, VP19, VP24), VP26, VP28, survival rate (SR)

Introduction
The White spot Baculovirus (WSBV) represent a devastated virus shrimp *Penaeus monodon* disease that infected East Asian region and rest of the world [5]. Shrimp infection was increasing in Indonesian farms since 1999-2001 [22] and mortalities reach 90-100% within 2-7 day [24]. WSBV infected shrimp age are 50-60 day or 5-15 g in weight, infection occurred especially at ectodermal, mesoderm, nerve, muscle, lymphoid, hematopoietic, stomach, hepatopancreas, antenna, gill and eye [4]. WSBV infection marked by the existence of white spot below/under exoskeleton that known by White Spot Syndrome. Infected shrimp become lethargic, show reduction in food consumption, and lose cuticle, often exhibited white spot under their exoskeletons. The virus has a wide host range among crustacean, infecting about 40 different species [6]. The virions are bacilliform, envelope nucleocapsid, and size 275 x 120 nm [3] and the viral genome contains double-stranded DNA (dsDNA) of 305 kb [1]. WSBV consist of five major and about 14 minor structural protein. Five major protein name according to their size namely viral protein (VP) 28, VP26, VP24, VP19 and VP15. The disease prevention is very difficult, due to lack of crustacean cell line to propagated the virus. Immunostimulan or subunit vaccine is a material that can be used for stimulating the immune responses in the shrimps [10]. Parts of WSBV potentially regarded for expanding immunostimulant or subunit vaccine is protein envelope. Vlak *et al.* [19], reported that the virion of WSBV has five main proteins weighing of 15-28 kDa. VP28 is the protein envelope and it's considered to have an important role in WSBV infection [9]. The present study were focuses on the VP28 to understand whether VP28 have the immune potent to shrimp to prevent infection of WSBV.

Materials and Methods
Infected WSBV shrimps
The WSBV infected shrimp weighing of 10 g were collected from East Java province farms during outbreak in 2015-2016. Shrimps were transported to laboratory by oxygenated air in the plastic bag and immediately store in -80°C until used for the experiments.

Virus isolation
Freshly prepared or frozen (-80°C) of whole shrimp were homogenized with 9-volumes of

Hank's balanced salt solution (HBSS, Gibco) and centrifuged at 2,000 x *g* for 15 min. The supernatant was filtered thought a 0.45µm-membrane filter. The filtrate was immediately used for experiments and the other were store at -80°C until used for the next experiments.

Histopathology and electron microscopy

The tissue of moribund shrimp were taken from the hepatopancreas, fixed in Bouin's solution for at least 24 h, embedded in paraffin wax, and cut at 3 µm. Sectioned specimens were stained with Haematoxyline and Eosin (H&E). For electron microscopy, infected tissue of shrimp was fixed in 2,5% glutaraldehyde in phosphate buffer (pH 7.2) at 4 °C for 4 h, and post-fixed with 1% osmium tetroxide in phosphate buffer (pH 7.2) at room temperature for 2 h. After post fixation, tissue were dehydrated in series of ethanol and embedded in Epon 812. The tin section were cut with ultra microtome, stained with uranyl acetate and lead solution, and finally observed with a JEOL 1200 EX-2 electron microscope.

PCR detection of WSBV and VP28

The infected shrimps, which were showed the white spot in the exoskeleton, were submitted for PCR detection. DNA were extracted with DNAzol regent (Gibco BRL, USA) according to the manufacturer instruction. The upstream primer are 5'-AGAGAATTCATGGATCTTTCTTTCAC-3' as sense primer and 5'-CACGTCGACTTACTCGGTCTCAGTGC-3' served as antisense primer. A 30 µl reaction mixture was prepared in a thin wall tube with 3 µl 10 x *Taq* polymerase buffer (Qiagen), 0.3 of each primer (25 pmol μl^{-1}), 2 µl dNTPs (10 mm μl^{-1}), 4 µl template DNA, 0.3 µl (5 U μl^{-1}) *Taq* polymerase and 20.4 µl sterile distilled water. Tube then were placed in a GeneAmp9600 (PE Apllied Byosistem) for amplification. After pre-denaturating step at 94 °C for 10 min, a 50 cycle reaction was employed for the amplification. Each cycle consisted of 94°C for 1 min, 55°C for 1 min and 72 °C for 1 min. Final step of 72°C 10 min was applied. A 10 µl of aliquot was subjected to electrophoresis on 1.2% agar gel.

SDS-PAGE and Western Blotting

SDS-PAGE was carried out according to the methods of Laemmli [25] and Towbin *et al.* [26], respectively. The PCR product were mixed with 0.17M Tris-HCl buffer (pH 6.8) containing SDS (5.3%) and 2-mercaptoethanol (13.2%). Electrophoresis using 10% gel and Tris (0.025M) glycine (0.192M) buffer containing 0.1M SDS was performed at a constant voltage of 140 for 1 h. The protein bands were stained with Coomasie Brilliant Blue. After SDS-PAGE, the protein in the gel were transferred electrophoretically to a nitrocellulose membrane filter. The membrane was blocked with TBS buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) containing 2% skim milk. After washing, the membrane was reacted with the anti-toxin rabbit serum diluted 1:500 with TBS buffer for 1 h, washed with the buffer, and the reacted with alkaline-phosphatase-conjugated anti-rabbit Ig swine Ig (Dako). Finally, the protein band were visualized by 5-bromo-4-chloro-3-indoryl phosphate (X phosphate) and 4-nitroblue tetrazolium chloride (NBT).

Two-dimensional electrophoresis

This analysis was performed according to Nishizawa (1991) briefly, first dimention, isoelectric focusing (IEF) tube gels were prepared with 4% acrylamide, 2% pharmalyte pH 3-10 (Pharmacia), 8 M urea and 2% NP-40. After adding overlay solution of 2% pharmalyte, 8 M urea, and 2% NP-40 the tube gels were placed between a cathode buffer (0.02 M NaOH) and anode buffer (0.01 M H3PO4) and prefocused at 300-400 V for 30 min.

Preparation samples for IEF by adding 2 volume of purified virus to 3 volumes of an SDS/urea solution (5% SDS, 9 M urea, 2% pharmalyte, 5% 2-mercaptoethanol). After incubation at room temperature for 15 min, 3 volume of NP-40/urea solution (4% NP-40, 9 M urea, 2% pharmalyte, 5% 2-mercaptoethanol) were added and the mixture incubated for another 15 min. Samples were placed in the top of IEF tub gels and protein separated by at 500 V for 16 by electrophoresis.

After IEF, a control gel were sliced into 1 cm section and transferred to 1ml portion of distilled water for 5 h incubation at room temperature when pH of each fraction were measured. Gel containing sample for 2 DGE were removed from the tube, incubated for 30 min in equilibrium buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol) and fixed to top of 2% polyacrilamide gel with melted 2% agarose in equilibrium buffer. Second dimendion electrophoresis condition and gels were the same as for SDS-PAGE.

Purify of protein

Shrimp infected with WSBV were collected during an outbreak of WSBV at a farm in East Java Province and stored at -80 °C. Whole bodies of infected shrimp were homogenized with 9 volumes of Dulbecco's PBS (D-PBS) and centrifuged (10000 g, 10 min, 4 °C). The supernatant was ®ltered through a membrane (0±45 lm; Advantec) and centrifuged (150000 g, 40 min, 4 °C). The pellet was resuspended in D-PBS and centrifuged (100000 g, 60 min, 4 °C) through a discontinuous gradient composed of 20, 35 and 50% (w}v) sucrose in D-PBS. The virus was collected from the interface between 35 and 50% (w}v) sucrose and pelleted by centrifugation (150000 g, 40 min, 4 °C). The virus pellet was resuspended in CsCl (q¯1±32) and centrifuged (100.000 *g*, 12 h, 4 °C). The virus was collected, diluted and then pelleted by centrifugation (150000 g, 40 min, 4 °C). The pelleted virus particles were resuspended in TE (10 mM Tris±HCl, pH 8±0, 1 mMEDTA).

Injection VP28 into shrimp

Shrimp weighing 10-15 g were used in this experiment. Ten shrimps in each treatment were injected intra-mucular (im) injection with 5 µg of VP19, VP24 and VP28 and submitted to challenge test by injection of 0.234 ng/ml of viral protein. Control shrimp injected with Phosphate Buffered Saline (10 nm PBS, pH 7.0) in equal dose.

Hemocyte collection

Haemolymph (about 300 µl) was obtained from the ventral sinus located at the base of the first abdominal segment. Samples were collected by using a 23-gauge needle and 1 ml syringe containing 300-µl pre-cold (4 °C) 10% sodium citrate solution in distilled water, used as anticoagulant. Haemolymph were counted with a haemocytometer (Erma).

Phenoloxidase activity assay

Phenoloxidase assay in hemocyte was estimated spectrophotometrically using L-3, 4-dihydroxyphenilalanine

(L-DOPA; Sigma) as substrate, and trypsin (Sigma) as elicitor according to method of Smith and Soderhall [28]. Two hundred microliter of hemocyte was incubated with 200 µl of 0.1% trypsinin ice cold cacodylate (CAC) buffer for 30 min in room temperature, following 200 µl of L-DOPA in 0.3% in CAC buffer. Finally diluted with 600 µl of CAC buffer and measured at 490 nm optical density (OD). One unit of enzyme activity was defined as increase of absorbance of 0.001/min/mg protein.

Results and Discussions

The management of viral disease in shrimp has been challenged because of lack of sensitive methods therefore until to date the WSBV infected sporadically in entire world. During epizootic in 2001-2002 both tiger shrimp *Penaeus monodon* and white shrimp *Penaeus merguiensis* were infected by WSBV in East Java farms. Electron microscopy of infected shrimp showed the present of ovoid virus particles with 235-275 nm in length and 80-100 in width.

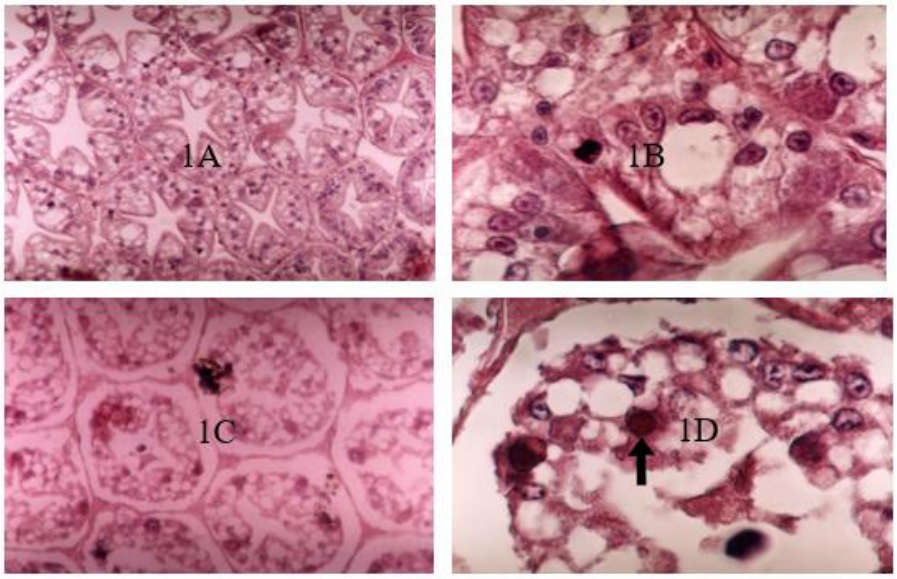

Fig 1a: Histology of hepatopancreas of healthy shrimp (100X); b. (400X); c. Hepato-pancreas of infected WSBV shrimp (100X); d. (400X). Arrow, *inclusion bodies*.

In the histology studies of naturally infected shrimp, it was showed by the present of inclusion bodies in the hepatopancreas (Fig 1d). Inclusion bodies were not found in the artificially infected shrimp, may the reason shrimp dead in the short time due to high injection of viral particle. The establisment of countermeasure for WSBV in Indonesia shrimp culture is urgently needed to prevent heavy looses. One way to achieved the goal is to developed vaccine against the disease. The transmisión of the disease in farms also very rapid because the present of crabs which was served as the WSBV reservoir.

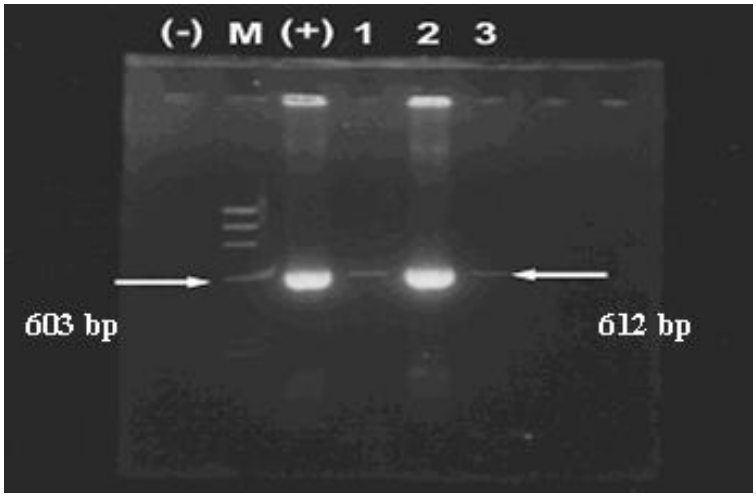

Fig 2: PCR products of electrophoresis on 2% gel have around 615 base pairs. Lane -: negative control, M : marker, + : infected shrimp, 1 : sample of infected shrimp, but not clear visulaized due to low viral particle 2 : highly infected shrimp, 3 : sample of infected shrimp, but not clear visulaized due to low virual particle

Base on VP28 region the result of PCR showed that shrimps positively infected by WSBV with 615 base pairs nucleotide (Fig.3). White spot as usually present in the naturally infection can not produced in the laboratory trial, the reason could be shrimp injected with the high dose of viral protein caused shrimp dead imediately without present white spot. Protein was used for the sake of immune responses such as phagocytosis, forming capsule, melanization, coagulation, prophenoloxide enzyme activity, opsonization, blood freezing, anti microbial activity and the process of humoral

and other cellular activity [12]; Soderhall *et al*, 1996 [13, 14, 16]. Lo *et al.* (1997) [7] has reported, based on the sequence analysis from DNA that codes WSBV protein capsid, it was not difference among China, India, Thailand and USA isolates. Analysis of protein profile [20] and restriction fragment length polymorphism [9] are not identical. The result of this analysis showed that there is a posible genetic heterogeneity (van Hulten *et al.*) [18].

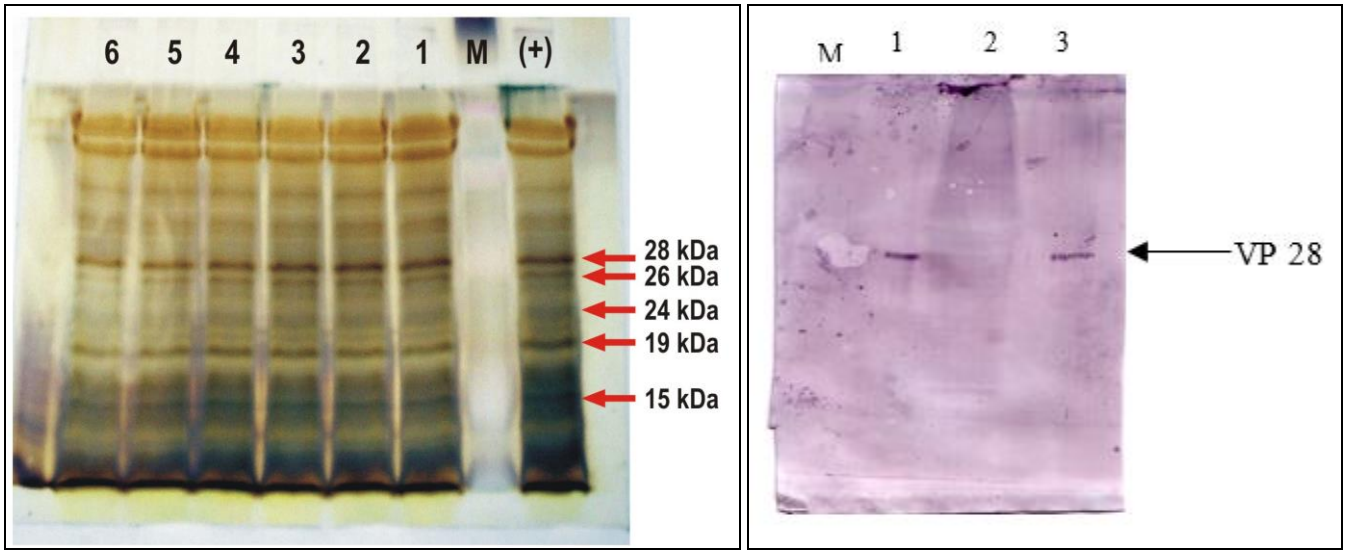

Fig 3: Protein profile of WSBV on SDS-PAGE 15% gel (A) and Western blotting (B). SDS-PAGE (A), Lane + : infected sample, M : marker, 1,2,3,4,5,6 : infected simple, Western blotting (B), M : marker, 1,3 : Protein *envelope* of VP28, 2 : *Whole protein* WSBV.

Protein analysis profiles (Fig 3A) showed especially in WSBV Indonesia isolates have five main proteins namely 15 kDa, 19 kDa, 24 kDa, 26 kDa, and 28 kDa, same character of envelope protein from China, Taiwán, and Korean isolate [17, 9]. Purified VP28 by elution was showed by Western blotting (Fig 3B) lane 1 and 3, in the center is whole protein of WSBV but not visualizad perfectly. The same characters protein envelope VP28 of WSBV Indonesian isolates and encoding gene have 100% to 99.8% homology among the WSBV isolates from Korea, China, Vietnam, USA, Japan, and India [21, 8, 7] (Fig. 4).

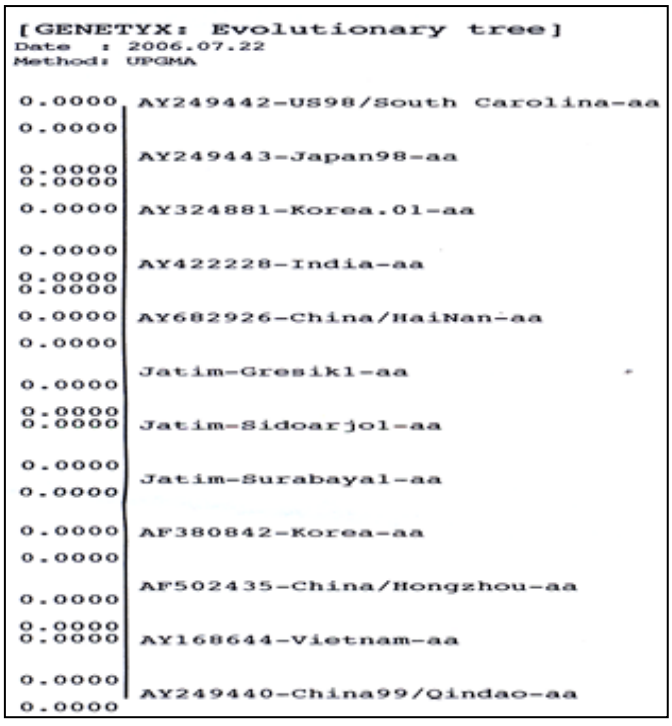

Fig 4: Phylogenetic analysis of WSBV

The analysis of VP28 through two dimensions was VP28 has specific characters and four kinds of protein with different isoelectric-points (Figure 5). The different value of isoelectric-point caused by the differences in contents, kinds of arranging amino acids, weight and the structure of the molecule. Based on immunization test to forty shrimps showed that the shrimps, which were given VP28 can, produced 60 percent survival rate (Table.1) until the end of experiments for 14 days. The control shrimp, which were, received only PBS (pH 7.0) died for 2-4 days post infection. To confirmed that the dead shrimp caused by WSBV the PCR test was done both for dead shrimp and control. The control shrimp of course no viral particle was detected in tissue. There is also the question how the WSBV sporadically infected shrimp in Indonesian until today, may the role of other crustacean which is living near shrimp farms have the significant role as virus reservoir. Crustacean such as crabs easily enter the shrimp farms without any preventing measure by farmer.

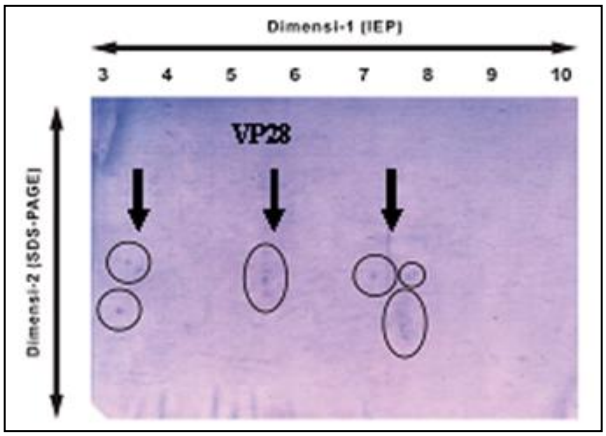

Fig 5: Protein profile of WSBV by Two Dimension, isoelectric point of VP28 between 3-4, 5-6 and 7-8.

Table 1 showed that the protection of *P. monodon* after immunization with VP19, VP24 and VP28 isolated from WSBV. The salinity during the research is 20 ppm and water temperature is 25 °C. Control shrimps were dead after one-two days post infections without any white spots on carapace, in entire laboratories trial we failed to produced white spot in the carapace as normally present in natural sign of infection [15].

Table 1: The protection of *P. monodon* shrimps after immunization with VP19, VP24 and VP28 isolated from local isolated.

Groups	Treatment	Survival Rate (%)
I	Control (0/10)	0
II	VP19 (2/10)	20
III	VP24 (0/10)	0
IV	VP28 (8/10)	60

Even though survival rate of shrimp were high, we do not know whether it's caused by neutralization of VP28 against WSBV infection or increasing the immune response of shrimps. The increasing immune response becomes very important to prevent infection in shrimp. Wu *et al* [23]; Namikoshi [2] reported when quasi-immune responses of shrimps increases there is followed by increasing immune response of shrimp, and this is not caused by natural resistant selection or innate resistant of shrimps.

Table 2: The production of *phenoloxidase* and total hemocyte count of shrimp after administration of envelope protein VP28.

No.	Treatment	Enzyme *Phenoloxidase* (U/min/mg)	Total *Haemocyte* (X10^7 sel/ml)
1.	Control shrimp	3,1167^d ± 0,06325	140,78^c ± 3,667
2.	WSBV infected shrimp	30,4000^b ± 0,06305	117,44^d ± 1,324
3.	Injected VP28	24,1822^c ± 0,06685	160,44^b ± 0,959
4.	Injected VP28 WSBV challenged	44,6067^a ± 0,03162	193,89^a ± 4,314

^{a,b,c,d} Superscript indicate the significantly

The protection system of invertebrates is often regarded as primitive compare with mammalian. The mechanism of self-defense could be acquainted with self and non-self for crustacean [11], efficiently rapid defense process with cellular response (clotting, phagocytes, and encapsulation) [14] and humoral responses (agglutination, lectin, killing factors, neutralization) [12]. It has been known that defense mechanism in many invertebrate accompanied by melanization. The enzyme involved in the melanization formation is phenoloxidase, this phenomenon of defense mechanism was exhibited by phenoloxidase in shrimp (Table 2). Phenoloxidase was low in control shrimp and increase significantly in VP28 injected shrimp, WSBV infection and VP28 injected shrimp after WSBV challenged.

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