Comparison of the protective efficacy between monomeric and dimeric recombinant VP28 against White Spot Syndrome Virus (WSSV) infection in shrimp

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Abstract

White spot syndrome virus (WSSV) is one of the most serious biological threats that caused considerable economic losses in shrimp farming industry worldwide. Although vaccination of shrimp using recombinant subunit protein, such as VP28, was significantly improved shrimp survival, the commercialization of shrimp vaccine has been largely delayed due to the problems such as unsatisfactory efficacy. In this study, the efficacy of monomeric and dimeric recombinant VP28 (rVP28) proteins were compared in WSSV challenged *Penaeus monodon*. First, the construct of recombinant plasmids harboring a single copy or double copies of VP28 were transformed and expressed in *Escherichia coli* (BL21). The 6x-histidine tagged rVP28 were purified using Ni-NTA column prior to western blot analysis. Injection challenge tests were conducted in triplicate with 8 shrimp per replicate for each group and 9 shrimp per replicate for 1×rVP28 group. To examine the protective efficacy of the rVP28, the naïve shrimp were co-injected with 25 µg of either monomeric or dimeric rVP28 in combination with WSSV (10^{-8} dilution of the stock virus). While the positive control showed 100% cumulative mortality at the 9th day post-injection, the mortality of shrimp co-injected with monomeric and dimeric rVP28 were 41% and 21% mortalities, respectively. This result suggested that the dimeric rVP28 could be one of the promising strategies to increase the protective efficacy of the shrimp viral vaccine.

Keywords: *Penaeus monodon*, White spot syndrome virus (WSSV), monomeric recombinant VP28 (1×rVP28), dimeric recombinant VP28 (2×rVP28)

Introduction

White spot disease was first reported in Southern Asia in the 1990s (Wang et al., 1995) and East Asia during 1992-1993 (Yan et al., 2007) before rapidly spreading worldwide. The shrimp infected with White spot syndrome virus (WSSV) show cumulative mortality up to 100% within 3 to 10 days (Johnson et al., 2008). The economic impacts of WSSV is not limited to only shrimp but also other crustacean species such as crab, crayfish and lobster (Lo et al., 1996).

There are several different strategies including the administration of the viral envelope protein, e.g. VP28, have been used to reduce mortality caused by WSSV infection in the laboratory scale (Mavichak et al., 2011). Namikoshi et al. (2004) demonstrated that the intramuscular injection of rVP28 showed the highest protection against WSSV with 95% relative percent survival (RPS) when double injected with rVP28 (100 µg/g shrimp/time)

before challenging whereas a single vaccination of rVP28 only showed 17% RPS. This result indicated that the high protective efficacy was resulted from the injection of relatively high amount of rVP28. Hence, for practical use in farm-scale application, the lower amount of effective dose of rVP28 with improved protective efficacy is desirable.
To make VP28 suitable for a commercial production, there are several factors that need to be considered such as maximization of protective efficiency, long-term protection period, and suitable delivery method for large scale application, etc. (Mavichak et al., 2011). Recently, the idea of multimerized immunogen for improving immune response has been used in HIV vaccine research (Jardine et al., 2013). Therefore, the proposed study would like to examine whether the multimerized VP28 could enhance protective efficacy against WSSV infection in shrimp.

Methodology

Bacterial expression of VP28
The VP28-PCR product was amplified from the stock pCR4-TOPO-VP28 in our laboratory using the primers design to clone the product of single or double copies of VP28 into pET-HeLipop5 (Novagen). The recombinant pET-HeLipop5-VP28 plasmid was used to transform into *Escherichia coli* (BL21) strain. A single colony of bacteria containing the recombinant plasmid was grown in Luria-Bertani broth supplied with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL). Overnight cultured bacteria were inoculated into Luria-Bertani broth to increase the amount of bacteria until optical density (A_{600}) reached 0.6 prior to adding IPTG to obtain the final concentration of 0.1 mM to induce expression of recombinant proteins. At 4 h post-induction at 30°C, the cultures was centrifuged (5000 x g, 10 min, 4°C) and the pellet was kept frozen in -20°C. Then frozen bacterial cells were thawed on ice for 15 min and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1% Triton X-100, 5 mM 2-mercaptoethanol, 10 mM Imidazole, pH 8.0) + 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspended cells in lysis buffer were sonicated using Sonics (Vibra-cells) at 21% (pulse 2 sec, stop 2 sec for 10 min). The supernatant were collected for protein purification using Ni-NTA column, according to the manufacturer's instructions (Qiagen), prior to western blot analysis.

Injection challenge test
Penaeus monodon shrimp (8-10 g body weight) were used in the vaccination trial by injection challenge test. The monomeric and dimeric rVP28 were intramuscularly injected in combination with 10^{-8} dilution of purified stock WSSV (this viral concentration was confirmed to cause the 100% mortality in *P. monodon* within 7-10 days after injection) into naïve shrimp with 25 µg/shrimp, respectively. For control groups, the negative control was intramuscularly injected with PBS (100 µl/shrimp), whereas the positive control was co-injected with PBS (50 µl) and WSSV (10^{-8} dilution of the stock virus) in PBS (50 µl), as indicated in Table 1. After injection challenge, the shrimp mortality was observed every six hours until the shrimp in the positive control group reached 100% mortality. Moribund shrimp were collected for confirmation of WSSV infection by using semi-nested PCR and histopathology.

Table1: Experimental design for injection challenge test

Group Type	rVP28/shrimp in PBS (50 µl)	WSSV in PBS (50 µl)	No. of shrimp
Negative control	0	No	8×3
Positive control	0	Yes	8×3
1×rVP28	25 µg	Yes	9×3
2×rVP28	25 µg	Yes	8×3

*Total volume of co-injection is 100 µl

Semi-nested PCR for confirmation of WSSV infection

Genomic DNA were extracted from the gill of moribund shrimp by standard method of phenol-chloroform extraction (Sambrook et al., 1989). The PCR protocol for the first step of semi-nested PCR was performed as 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s with final extension at 72°C for 7 min. The reaction PCR of 12.5 µl reaction mixture consist of 10×PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 10 µM 447F1 primer and 10 µM 447R1 primer, 0.125 µl of Taq-DNA polymerase and 2 µl of DNA template (50 ng/µl).

For the second step of semi-nested PCR, the 12.5 reaction mixture was the same to the first step, but DNA template obtained from PCR product of first step and amplified by 10 µM 229F2 and 10 µM 447R1 primers. The PCR protocol for the second step consist of 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s followed by 72°C for 7 min. The size of PCR product from first step of semi-nested PCR was about 447 bp, whereas the second product was about 229 bp. All primer sequences are shown in Table 2.

Table 2: Primer sequence for semi-nested PCR

Primer name	Primer sequence
447F1	5'ATGAGAATGAACTCCAACTTTAA3'
447R1	5'CAGAGCCTAGTCTATCAATCAT3'
229F2	5'GATGGAAACGGTAACGAATCTGAA3'

Histopathology for confirmation of WSSV infection

After gill collection for PCR, the sample were immediately fixed and preserved in Davison's fixative for 24 h, then, changed to 75% ethanol for the long-term preservation. The samples were stored in 75% ethanol tube waiting for tissue sectioning processing (dehydration, embedding, sectioning, and stained with haematoxylin and eosin) to detect the presence of WSSV infected tissue under the microscope. The histopathology processing in this study were follow the method described by Bell & Lightner, 1998.

Data Analysis

The data of mortality were compared using chi-squared test. The protection against WSSV after vaccination were calculated as the relative percent survival (RPS) [(1 - vaccinated group mortality/control group mortality) × 100] (Amend, 1981).

Results

Mortality of injection challenge test

The shrimp mortality was observed until the 9th day post-challenge (dpc) because the mortality of positive control group reached 100%. The cumulative mortality of negative control, 1×rVP28, and 2×rVP28 group was 8%, 21%, and 41%, respectively, as indicated in Figure 1. Depending on statistical analysis by Chi-square test, there was no significant difference among 1×rVP28, 2×rVP28 and negative control group, however p-value suggest that 2×rVP28 group showed the least difference to negative control (p = 0.479), as indicated in Table 3.

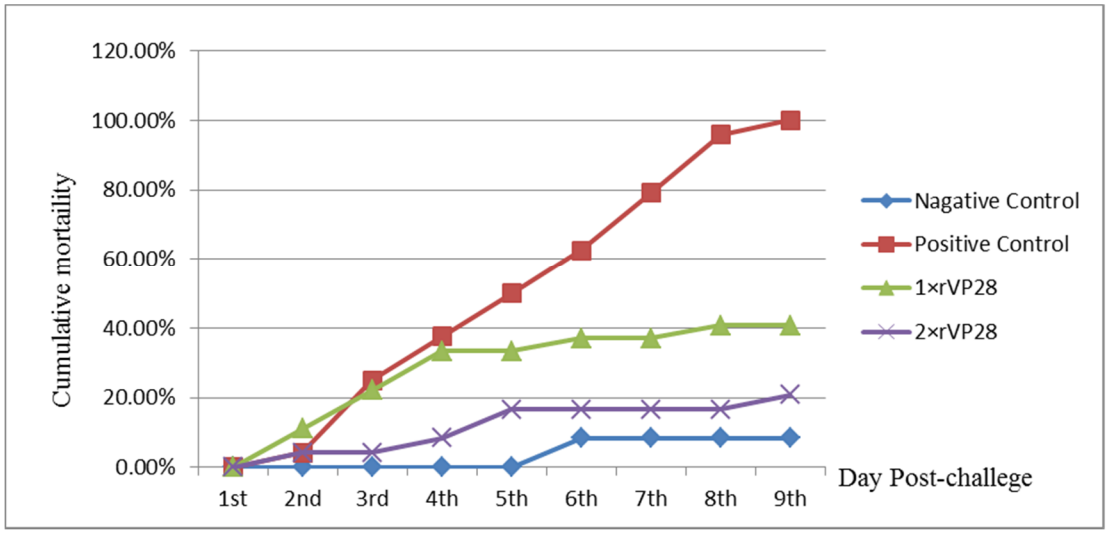

Figure 1: Comparison of the protective efficacy between monomeric and dimeric rVP28 against WSSV infection in *P. monodon*.

Table 3: Statistical analysis on cumulative mortality by Chi-square test

Chi-square test (significant level of 0.05)		
Control Group	Treatment group	p-value
Positive control	1×rVP28	0.005
Positive control	2×rVP28	0.001
Negative control	1×rVP28	0.174
Negative control	2×rVP28	0.479

Confirmation of WSSV by semi-nested PCR

All samples of the treatment groups including positive control group were positive of WSSV infection because the 447 bp and 229 bp PCR amplicons were detected in semi-nested PCR reaction. Unexpectedly, negative control samples were also positive of WSSV infection (Figure 2).

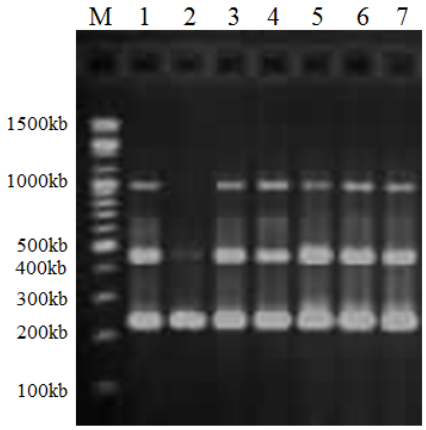

Figure 2: Semi-nested PCR result: Lane M: 100 bp marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3 and 4: moribund sample of 2×rVP28 group; Lane 5, 6, and 7: terminated sample of 2×rVP28. WSSV positive sample shows 447 bp and 229 bp PCR products.

Confirmation of WSSV by histopathology

As expected, the histopathology of positive control samples showed the presence of WSSV infection (n = 8), while the sign of WSSV infection was absent in negative control group (n = 8). It is noteworthy that only 50% (n = 4) of the moribund shrimp samples in the treatment groups showed WSSV infection (Figure 3). All terminated samples were absent of WSSV infection, suggesting the protective effect of the recombinant VP28 protein.

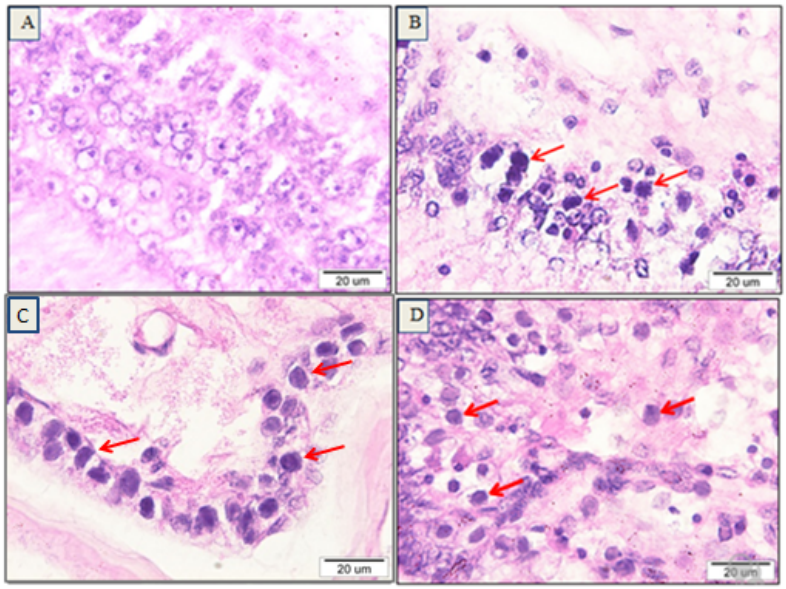

Figure 3: Histopathological analysis indicated in stomach epithelium: (A) Sample of negative control was absent of WSSV infection. (B) Sample of positive control, (C) sample of 1×rVP28 group, and (D) sample of 2×rVP28 group show the presence of WSSV infection indicated by red arrow.

Discussion

The idea of multimerized immunogen for improving immune response has been successfully used in HIV vaccine research. This research team demonstrated that the multimerized immunogen on nanoparticles led to the activation of germline and mature VRC01-class B cells that produce antibodies against HIV (Jardine et al., 2013). Although the mechanism that stimulated the immune response in mammals is completely different from crustaceans (Loker et., 2004), the similar concept for boosting protective response against WSSV by multimerization of antiviral peptides such as VP28 can be applied.

In our study, we expressed two constructs of rVP28, and then the efficiency of protection against WSSV was compared between them by injection challenge test in *P. monodon*. At the 9th dpc, the mortality observation showed that, 1×rVP28 group showed lower ability of protection with 59% RPS, while those injected with 2×rVP28 showed higher protection with 79% RPS. Chi-square test shows the result of non-significant difference among 1×rVP28, 2×rVP28 and negative control group, however p-value suggest that 2×rVP28 group showed the least difference to negative control ($p = 0.479$), suggesting that 2×rVP28 have more ability of protection against WSSV infection in *P. monodon*. According to the analysis of WSSV envelope protein complexome, the structure of envelop protein VP28 was revealed as homotetrameric form (Li et al., 2011). At the same concentration of rVP28, we hypothesized that, the dimeric rVP28 could accelerate mimicking of the signal that generated by homotetrameric VP28 when compared to monomeric rVP28.

Confirmation of WSSV infection by semi-nested PCR showed the technical problem during PCR processing that caused to the contamination of genomic DNA. We conclude that the contamination was occurred during PCR processing because the histopathology result showed that it was no contamination occurred during experiment. Although the 477 bp and 229 bp amplicons were proved to be the non-structural gene of WSSV, the unexpected band of 1000 bp will be determined by sequencing.

The confirmation of WSSV infection by histopathology revealed that only 50% (n=4) of moribund samples were found to have the presence of WSSV infection. It suggested that not every single dead shrimp was killed by WSSV. The cause of mortality might be due to environmental stress or side effect of rVP28 protein. Hence, we plan to repeat experiment by designing with the other treatment control of each recombinant protein which will be injected only with rVP28 protein alone to determine the side effect of treatment.

Conclusion

The vaccine against WSSV in the commercial scale for shrimp aquaculture industry has been largely delayed due to the problems such as low vaccine efficacy. The vaccination trial in this study suggested that dimeric rVP28 should be considered as the candidate vaccine against WSSV.

Acknowledgements

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