Brevibacillus choshinensisより作製したN末端欠損組換えVP28タンパク質を投与したホワイトシュリンプのホワイトポットウイルス感染に対する抵抗性

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Protection of Pacific White Shrimp, *Litopenaeus vannamei* against White Spot Virus Following Administration of N-terminus Truncated Recombinant VP28 Protein Expressed in Gram-positive Bacteria, *Brevibacillus choshinensis*

Rapeepat MAVICHAK¹, Hidehiro KONDO¹, Ikuo HIRONO¹, Takashi AOKI¹,* Hiroshi KYONO² and Yoshikazu YUKI²

**Abstract:** To increase survival of different commercially important shrimp species against white spot virus disease (WSVD), a number of combinations using different recombinant VP28 protein forms expressed in Gram-negative or -positive bacteria have been tested. Here, we explored yet another combination to maximize utilization of VP28 specific to Pacific white shrimp, *Litopenaeus vannamei* to protect it against WSV. Large amounts of N- and an N+C-terminus-deleted recombinant VP28 (rN-VP28 and rNC-VP28) were produced in large amounts in a Gram-positive bacterium, *Brevibacillus choshinensis* and then administered to the shrimp by either oral feeding or intramuscular injection. Feeding rN-VP28 (50 µg day⁻¹ for 7 days) but not rNC-VP28 provided each shrimp significant protection against WSV. Furthermore, we showed that both oral delivery and intramuscular injection of rN-VP28 were effective for Pacific white shrimp.

**Key words:** White spot virus (WSV); Immunostimulant; Recombinant protein VP28; *Litopenaeus vannamei*; *Brevibacillus choshinensis*

Pacific white shrimp, *Litopenaeus vannamei*, is now considered the most important among the cultured shrimp species in the world, representing 75% of the total global shrimp farm production in 2006 (mainly from Central and South America, and Asia) where the species alone accounted for 2.1 million metric t valued at 7.8 billion US dollars culture (www.fao.org).

Like other commercially cultured shrimp species, however, Pacific white shrimp is also greatly impacted by white spot virus disease (WSVD) (Yanga et al. 2007), which can cause 100% mortality in the species within a few days of infection. The disease is caused by an enveloped double-strand DNA virus called white spot virus (WSV), the only member of the genus *Whisspovirus*, family *Nimaviridae*, having an ovoid or ellipsoid to bacilliform shape with a flagella-like appendage at one end (Jehle et al. 2006; Lo et al. 2004; van Hulten et al. 2001a; Yang et al. 2001).

WSV possesses five major WSV proteins (Tsai et al. 2006), one of which, VP28, has been extensively studied and shown to control WSVD in various cultured shrimp species. For example, full recombinant VP28 protein (rVP28) produced in Gram-negative bacteria *Escherichia coli* protected kuruma shrimp, *Marsupenaeus japonicas* against WSV (Namikoshi et al. 2004). Recently, in a similar study that used Gram-positive bacteria *Brevibacillus brevis* instead to eliminate the possibility of endotoxin contamination, the same full rVP28 also clearly protected kuruma shrimp from WSV infection.
(Caipang et al. 2008). On the other hand, partial rVP28, whose N-terminal hydrophobic region was truncated, was shown to be highly expressed in Gram-negative E. coli cells and significantly increased survival of black tiger shrimp, Penaeus monodon against WSSV infection when mixed with commercial feed pellets (Witteveldt et al. 2004). Later, E. coli expressing this same partial VP28 was shown to enhance the tolerance of Pacific white shrimp, L. vannamei against WSSV when it was fed to shrimp mixed with food pellets (Witteveldt et al. 2006).

Because of the importance of the shrimp industry, in particular of Pacific white shrimp, there is a need to explore more options to maximize the utilization of VP28 protein, one of only very few WSV viral proteins shown so far to effectively protect shrimps against the virus. One way to do this would be to combine and analyze the methods of previous studies that showed positive and promising results. In this study, therefore, we expressed in large amounts, an N- and N+C-terminus truncated VP28 (rN-VP28 and rNC-VP28) in a Gram-positive bacteria B. choshinensis and tested the proteins’ ability to protect Pacific white shrimp, L. vannamei against WSV using oral administration, and then testing whether oral administration or intramuscular injection of rN-VP28 is suitable for Pacific white shrimp.

**Materials and Methods**

**Shrimp culture**

L. vannamei (approximately 8 – 10 g body weight) were obtained from a commercial shrimp culture farm in Chachoengsao, Thailand and were checked with WSV using PCR method. For each treatment, shrimps were stocked in 1 ton plastic tanks, each fitted with an individual filter system with continued aeration containing 20 – 22 psu sea water at 23 – 24°C.

**WSV stock and in vivo titration**

The virus suspension used in this study was obtained from WSV-infected M. japonicus. Briefly, under aseptic condition, the muscle tissues were removed from the cuticle and homogenized using sterile Dounce homogenizer with an equal amount of phosphate-buffered saline (PBS) on ice and centrifuged at 1,500 x g at 4°C for 10 min. The supernatant was stored at −80°C until use. The WSV stock was diluted ten-fold with PBS and titrated by in vivo experiment as described by van Hulten et al. (2001b) with some modifications. In brief, 25 μl of the different WSV stock dilutions were IM injected into kuruma shrimp (approximately 2 – 3 g in body weight). The injected shrimp were monitored for mortality twice a day. Dead shrimp were tested for the presence of WSV using PCR as described by Kiathathomchai et al. (2001) or a commercial detection kit (Shrimple, EnBioTec Lab. Co., Ltd., Tokyo). The mortality rate was used to desire the challenged pressure in succeeding infection experiments.

**Expression of recombinant VP28**

The VP28 gene (accession no. AF173993) lacking strong hydrophobic region 29 amino
acids (a.a.) at the N-terminal region (30-204 a.a.) or lacking 29 a.a. at the N-terminal and 36 a.a. the C-terminal regions (30-168 a.a.) were ligated to expression vector pNY326 (Takara, Japan) as VP28N/pNY326 and VP28NC/pNY326, respectively (Fig. 1). The promoter and signal peptide-encoding region of cell-wall protein gene of B. choshinensis were prepared as described by Yuki et al. (2005). The vectors were introduced into B. choshinensis HPD31-SP3 by electroporation (Okamoto et al. 1997). The culture supernatant (2 l) of recombinant N-VP28 (rN-VP28) or NC-VP28 (rNC-VP28) was concentrated 10 fold with an ultrafiltration device (Millipore, Massachusetts, U.S.A.) with a 10,000 molecular weight cut-off filter, and then dialyzed against 10 mM phosphate buffer (pH 7.4) followed by sterilization by 0.22 µm filter.

**SDS-PAGE and Western blot**

rN-VP28 and rNC-VP28 in B. choshinensis were separated in 5–29% precast e-PAGE (ATTO Co., Tokyo, Japan) under reduced conditions and stained with Coomassie brilliant blue R-250. The separated proteins in a second gel were electro-blotted an AE-6667 (ATTO Co., Tokyo, Japan) membrane, and then the membrane was incubated with an anti-his tag monoclonal antibody (Amersham Biosciences, U.S.A.) or a rabbit polyclonal antibody (1:2,500) against rN-VP28 and rNC-VP28 of WSV (Genesis Biotech INC., Taiwan). The second antibody was rabbit anti-mouse Ig G or goat anti-rabbit Ig-G conjugated with alkaline phosphatase (Promega, WI, U.S.A.). The last interaction was revealed with 5-Bromo-4-Chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate (Sigma-Aldrich, St. Louise, MO, U.S.A.).

**Preparation of feed pellets**

Shrimp commercial pellet was ground and individually mixed with equal parts (W/V) of the purified rN-VP28 and rNC-VP28 dissolved in PBS. To form feed pellets, mixtures were placed in 50 ml syringe and slowly extruded, allowed to dry the moist pellets at 4°C for 3–4 days before mixed with 1% fish oil (W/V) to prevent diffusion of feed particles in the water. The same protocol was used to prepare the negative control treatment. Finely ground shrimp pellets were also mixed with supernatant of B. choshinensis sonicated culture media (BSCM) dissolved in PBS and PBS only.

**Feeding experiment**

The study consisted of two experimental runs (Table 1). The first run was undertaken to compare rN-VP28 and rNC-VP28. It consisted of four treatments where in each treatment, 50 shrimps were fed with approximately 4% shrimp body weight/day. In one day, shrimps in 1st and 2nd treatments were fed with pellets containing 50 µg of rN-VP28 and rNC-VP28. Shrimp in the 3rd treatment were fed pellets mixed with BSCM and shrimp in the 4th treatment were fed with pellets mixed with PBS. In each treatment, shrimps were fed for 7 days followed by virus challenge at 3 days post feeding. The second experiment run was conducted in order to compare the efficacy of oral administration and intramuscular (IM) injection of rN-VP28. There were also four treatments

<table>
<thead>
<tr>
<th>Experimental Run</th>
<th>Treatment No.</th>
<th>Experimental scheme and virus challenge</th>
<th>No. of shrimp</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2wks feeding, 1 feeding contain rNC-VP28 50 µg/shrimp, challenge 3 days post last feeding</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2wks feeding, 1 feeding contain rN-VP28 50 µg/shrimp, challenge 3 days post last feeding</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2wks feeding with shrimp commercial pellets mixed with BSCM, challenge 3 days post last feeding</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2wks feeding with shrimp commercial pellets mixed with PBS, challenge 3 days post last feeding</td>
<td>116</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2wks feeding, 1 feeding contain rN-VP28 50 µg/shrimp, challenge 3 days post last feeding</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2wks feeding with shrimp commercial pellets mixed with BSCM, challenge 3 days post last feeding</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2wks feeding with shrimp commercial pellets mixed with PBS, challenge 3 days post last feeding</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Intramuscular injection with 50 µg of rN-VP28, challenge 7 days post injection</td>
<td>100</td>
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</tbody>
</table>
in this experimental run. Similar to the first experimental run, fifty shrimps in the 1st, 2nd and 3rd treatments were individually fed pellets mixed with rN-VP28, BSCM and PBS, respectively. All orally administered shrimp were fed for 7 days followed by virus challenge at 3 days post last feeding. Shrimp in the 6th treatment were IM injected with 50 μg of rN-VP28 before virus challenge at 7 days post injection.

**Challenge test**

In the first experimental run, the challenge consisted of IM-injection of 50 μl of either one-tenth or one-hundredth lethal dose 50 (LD50) of WSV. For the second experimental run, the challenge consisted of IM-injection of 50 μl of either LD50 or one-tenth LD50 of WSV. Dead shrimps were observed and collected twice a day to avoid cannibalism. Mortality was monitored for 12 days. Protection against WSV was assessed by calculating the relative percentage survival (RPS) (Amend 1981). The statistical analysis was calculated using χ² test at the 0.05 level of significance.

**Results**

**Recombinant VP28 expression**

Several *B. choshinensis* transformants expressed rN-VP28 and rNC-VP28. Fig. 2a showed rN-VP28 at about 20 kDa serially diluted at 2x dilution (lane 2-6) while rNC-VP28 was at about 18 kDa serially diluted also at 2x dilution (lane 7-11). The secretion levels of each VP28-derivative plateau after 3 days of incubation, reached approximately 250 mg/l. Fig. 1b, on the other hand, showed anti-VP28 antibody recognizing both recombinant proteins, although rN-VP28 was marked more strongly than rNC-VP28 (Fig. 2b).

**Challenge tests**

1. Immunostimulation with rN-VP28 and rNC-VP28

When challenged with 100-fold LD50 WSV, shrimps fed with rN-VP28 and rNC-VP28 and had cumulative mortalities of 76.3% and 97.6%, respectively (Fig. 3a). The control treatments, commercial pellets mixed with BSCM and PBS, had cumulative mortalities of 93.7% and 100%, respectively. N-VP28 oral administration resulted in the highest relative percent survival (RPS) (23.6%), followed by BSCM (6.3%) and rNC-VP28 (2.4%) (Table 2). The χ² test showed non-significant difference between rNC-VP28 and control group, while rN-VP28 and BSMC showed significant different from the control group.

When challenged with 10-fold LD50, shrimps fed with rNC-VP28 and rN-VP28 had cumulative mortalities of 80.0% (14.9 RPS) and 58.0% (38.3 RPS), respectively (Fig. 3b).
Recombinant VP28 Administration Inhibits WSV in Shrimp

2. Comparison of oral administration and intramuscular injection

At a challenge of 10-fold LD_{50}, shrimps fed with rN-VP28 protein had cumulative mortalities of 42.0% (46.2 RPS), while rN-VP28-injected shrimp had a cumulative mortality of 32% (59.0 RPS) (Fig. 4a). The control treatments, commercial pellets mixed with BSCM and PBS, had cumulative mortalities of 52.0% (33.3 RPS) and 78.0%, respectively. For the challenge with 1x LD_{50}, the highest mortality was observed in the PBS treatment with 52.0% mortality (Fig. 4b). The shrimp injected with rN-VP28 had the lowest cumulative mortality at 14.0% (73.1 RPS) followed by the shrimp fed with rN-VP28 at 16.0% (69.2 RPS). In contrast, mortality of the BSCM-fed shrimp was 28.0% (46.2 RPS) (Table 2). The χ² test showed significant difference when oral administration of rN-VP28, BSMC and injected rN-VP28 was compared with control group in both challenge doses experiment.

Discussion

VP28 became the focus of vaccine development against WSV because of its abundance

![Fig. 3. Time-cumulative mortality 1. Shrimps were fed with recombinant rN-VP28 and rNC-VP28 expressed in Brevibacillus choshinensis for 7 days, BSCM and PBS treatments were added as control treatments. Three days after the last oral administration, shrimps were challenged with WSV. A) Challenge with hundred-fold LD_{50} dose. B) Challenge with ten-fold LD_{50} dose.](image1)

![Fig. 4. Time-cumulative mortality 2. Shrimps were fed with recombinant rN-VP28 expressed in Brevibacillus choshinensis for 7 days, BSCM and PBS treatments were added as control treatments. One more treatment is IM injection with rN-VP28. Three days after the last oral administration and 7 days after IM injection shrimps were challenged with WSV. A) challenge with ten-fold LD_{50} dose. B) challenge with LD_{50} dose. (IM: injection with rN-VP28, OA: oral administration with rN-VP28, BSCM: oral administration with BSCM, and PBS: oral administration with PBS.](image2)
Table 2. Cumulative mortality and corresponding Relative Percentage Survival (RPS) of shrimp in the different experimental runs

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<tr>
<td></td>
<td></td>
<td>Mortality (%)</td>
<td>RPS (%)</td>
<td>Mortality (%)</td>
</tr>
<tr>
<td>1 1</td>
<td></td>
<td>97.6</td>
<td>2.4</td>
<td>80.0</td>
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<tr>
<td>2 2</td>
<td></td>
<td>76.3</td>
<td>23.6</td>
<td>58.0</td>
</tr>
<tr>
<td>3 3</td>
<td></td>
<td>93.7</td>
<td>6.3</td>
<td>76.0</td>
</tr>
<tr>
<td>4 4</td>
<td></td>
<td>0</td>
<td>-</td>
<td>94.0</td>
</tr>
<tr>
<td>2 1</td>
<td></td>
<td>42.0</td>
<td>46.2</td>
<td>16.0</td>
</tr>
<tr>
<td>2 2</td>
<td></td>
<td>52.0</td>
<td>33.3</td>
<td>28.0</td>
</tr>
<tr>
<td>3 3</td>
<td></td>
<td>78.0</td>
<td>-</td>
<td>52.0</td>
</tr>
<tr>
<td>4 4</td>
<td></td>
<td>32.0</td>
<td>59</td>
<td>14.0</td>
</tr>
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on the surface of the WSV envelope and its strong reaction with anti-WSV polyclonal antibodies (van Hulten et al. 2000). Several years and numerous reports later, it has been proven to be indeed an effective vaccine agent against WSV infection. Its efficacy appears to be related with other molecules such as PmRab7, which was found to be a VP28 binding protein involved in WSV infection in the shrimp, *P. monodon* (Srituyalucksana et al. 2006).

Our results showed that the rN-VP28 protected Pacific white shrimp against WSV, in agreement with recent reports of Witteveldt and co-workers (2004 and 2006). The difference in our study is that the rN-VP28 was produced in the Gram-positive *B. choshinensis* and not from Gram-negative *E. coli*. This offers some advantage since using a Gram-positive bacterium, such as *B. chosinensis*, is safer because they do not produce endotoxins like lipopolysaccharides (LPS) (Yuki et al. 2005). *B. chosinensis* also produces a functional non-glycosylated recombinant protein that is directly secreted into the culture medium, making it easier to be purified and produced in a large-scale commercial level.

The inactivity of rNC-VP28 shows that the C-terminus of VP28 (about 36 a.a.) is needed to induce protection in shrimp. This is not surprising because a crystallographic study has shown that the C-terminus of VP28 is the part that protrudes from the viral envelope (Tang et al. 2007). We speculate that the deletion decreases ability of WSV VP28 to bind to shrimp cells, thereby decreases its ability to act as an immunostimulant.

There was a noted difference in the molecular weight between our recombinant VP28 protein and that of Witteveldt et al. (2004) even though both proteins’ hydrophobic N-terminal regions were deleted, fused with six His-tag then expressed in bacterial systems. Our N-terminal truncated protein showed about 20 kDa, which is about the same size as the prediction, while their protein was about 28 kDa. Added to this confusion is the reference weight of VP28 from its first report, which is 28 kDa (van Hulten et al. 2000). In this first study, the difference between the actual weight (28 kDa) and the predicted weight (22 kDa) was attributed to the use of an insect expression system that may be the result of post-translational modifications (e.g. glycosylation, phosphorylation) (van Hulten et al. 2000). The discrepancy in sizes among these studies is not clear at this point, but it is possible that it is due to the different expression systems used. However, using prokaryotic system to express and purify recombinant VP28 has been demonstrated to protect shrimp against WSV (Witteveldt et al. 2004; Namikoshi et al. 2004; Caipang et al. 2008). Hence, modification of recombinant VP28 seems not necessary for conferring protective ability.

When shrimps fed or injected with rN-VP28 were challenged with 10-fold LD$_{50}$, the RPS was less than 60, which is the minimum value for highly effective vaccines (Chang et al. 2003). This may have been because the dose was too high. In the second experiment, the RPS was significantly improved for both delivery methods (intramuscular injection and oral delivery). This suggests that both delivery methods can
be used effectively to administer rN-VP28 in shrimp culture farms perhaps even at a commercial scale. Interestingly, BSCM treatment also conferred protection to some extent, possibly because of some extracellular products from Gram-positive bacteria that stimulated the shrimp innate immune system. Peptidoglycan, which is a bacterial cell wall component, has been shown to enhance resistance of shrimp against vibriosis and WSV infection (Itami et al. 1998).

In conclusion, we have demonstrated that the N-terminal-truncated recombinant VP28 protein (rN-VP28) can be produced in large quantities using low toxicity Gram-positive bacteria, *B. choshinensis*. Further studies are needed to determine the dose of recombinant VP28, feeding duration, duration of the protection on a commercial scale to increase the applicability of these results to the shrimp industry. Furthermore, there is a need to improve protein-based vaccines to make it more convenient and effective. Plant-based expression systems instead of bacterial expression systems may be one way to address this problem.

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peptide hybrid protein by Brevibacillus choshinensis 
expression system as a nasal vaccine against autoim-

Brevibacillus choshinensis より作製したN末端欠損組換え
VP28タンパク質を投与したホワイトシュリンプの
ホワイトスポットウイルス感染に対する抵抗性

MAVICHAK RAPEEPA·近藤秀裕・廣野育生・青木 宙・清野 宏・幸 義和

Brevibacillus choshinensis を用いて、N 末端のみ (rN-VP28) または N および C の両末端 (rNC-VP28) 
を欠失させたホワイトスポットウイルス (WSV) の組換え VP28 タンパク質を作製した。これらの組 
換え VP28 タンパク質を Pacific white shrimp に投与し、WSV 感染に対して抵抗性を示すかどうかを 
調べた。毎日、1 尾あたり 50 μg の組換え N-VP28 を 7 日間経口投与した試験区では有意な感染防御 
効果を示したが、50 μg の rNC-VP28 を投与した試験区では感染防御効果が認められなかった。また、 
注射法で 50 μg の rN-VP28 を接種した場合には、経口投与よりも高い感染防御効果が見られた。B. 
choshinensis を用いることで組換え VP28 タンパク質を大量に作製することができ、さらに、WSV に 
対する免疫賦活剤として有効であることが分かった。