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Neutralization of white spot syndrome virus (WSSV) for *Penaeus chinensis* by antiserum raised against recombinant VP19

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Abstract: *This study was carried out to neutralize the WSSV, one of the most virulent pathogen causing large economic damage in shrimp culture industry, using the antiserum produced against recombinant WSSV envelope protein VP19 (rVP19) as a tool to evaluate WSSV infection mechanism. A fragment of VP19 was expressed in Sf21 insect cell using baculovirus expression system as fusion protein with 6 His-tag. Then, polyclonal antiserum against rVP19 was raised in white rabbit. A constant amount of WSSV (at 10^4 diluted stock) was incubated with various antiserum concentrations and injected into shrimp, Penaeus chinensis, for the neutralization challenge. At 9 days post injection, the shrimp in the positive control injected with WSSV showed 100% mortality. The shrimps injected with WSSV preincubated with preimmune serum showed 83.3% mortality at 15 days post injection. The shrimps injected with the WSSV preincubated with 1 µl, 5 µl or 10 µl r VP19 antiserum and shrimp mortalities showed 66.6%, 40.0% and 26.6% at 15 days post injection, respectively. The high concentration of antiserum group showed lower mortality than those of the low concentration of antiserum group. This indicates that the WSSV can be neutralized by the rVP19 antiserum in a dose-dependent manner. The neutralization challenge result suggested that VP19 might play an important role in WSSV infection to shrimp.*

Key words: *WSSV, VP19, Sf21, Neutralization, Penaeus chinensis*
PDF of full length paper is available with author ***(*skkim@pknu.ac.kr)***

Introduction

White spot syndrome virus (WSSV) was first discovered in Taiwan in 1992 (Inouye *et al*., 1994). WSSV is an enveloped, rod-shaped, double-stranded circular DNA virus causing high mortality in cultured shrimp. WSSV is a rapidly emerging viral disease agent in shrimp industry and has spread quickly to shrimp-farming areas in Southeast-Asia and Central and Latin-America, causing major economic damage to shrimp culture industry (Rosenberry *et al*., 2000; Sambasivam *et al*., 2003). However, no perfect WSSV control strategies have been described. Enveloped viruses of vertebrates and invertebrates contain glycoproteins in their viral envelopes and those often play important roles in the interaction between virus and host, such as attachment to receptors and fusion with cell membranes (Granof *et al*., 1999). To date, some major structural proteins have been identified. Among those proteins, VP19, VP26/P22, VP28, VP68, VP124, VP281, VP292 and VP466 were identified as viral envelope proteins by immunoelectron microscopy.

Neutralization assays have often been performed to study the role of virion proteins or their domains in the infection stage. Neutralizing antibodies bind to envelope spikes on the virion and prevent the attachment of virus to the cell surface, cell entry or virus uncoating (Burton *et al*., 2000). *In vivo* neutralization experiments have been widely used for many vertebrate viruses and have even led to passive immunization strategies. When combined with the use of monoclonal antibodies, this strategy has been used to identify the virion protein epitope involved in the neutralization (Schofield *et al*., 2000). Syed Musthaq *et al*. (2006) used one WSSV envelope recombinant protein of VP28 to evaluate their potential to neutralization shrimp against WSSV and the results show that neutralization can be generated in shrimp against WSSV using its structural protein.

VP19 protein could be glycosylated or phosphorylated due to multiple putative N- or O-glycosylation and phosphorylation sites in the amino acid sequences (Van Hulten *et al*., 2002). A baculovirus/ insect cell expression system is one of the effective eukaryotic expression tools. This system has been proven as an efficient production system of post translational modified proteins. The baculovirus has a very specific host range and is thus very safe for industrial production and field application (Sumathy *et al*., 1996).

This study was carried out to neutralize the WSSV using the antiserum against recombinant protein VP19 of WSSV.

Materials and Methods

Shrimp culture: Shrimps, *Penaeus chinensis* (length of 9-11 cm and weight of 6-8 g), were purchased from a shrimp farm (Dan-Jang marine) located in Goheung, Jeonnam, Korea. Shrimps were tested for the presence of WSSV by PCR to ensure WSSV free before the experiments. They were kept in tanks at 25 ± 1°C with continuous aeration and fed a commercial pelleted feed at 5% of body weight per day during the experiments (Huang *et al*., 2001). Each shrimp in the experimental tanks was placed in individual plastic cages to prevent cannibalism.

Virus purification and stock: Shrimps were injected intramuscularly with a lethal dose of WSSV using a 30-gauge needle. Dead shrimps were stored at -80°C until use in the experiments and tested for the presence of WSSV by PCR using VP19 primers.

Deep-freezed WSSV infected shrimps were thawed and muscle tissue was homogenized using a homogenizer in 10-fold volumes of phosphate buffered saline (PBS) at 4°C and centrifuged at 12,000 × rpm for 10 min at 4°C. The supernatant was filtered through a 0.45 µm membrane and used for WSSV challenge experiments (Xie *et al*., 2004). Virus samples were stored in aliquots at -80°C.

WSSV detection: Oligonucleotide primer set (forward primer 5' GAATTCATGGCCACCACGACTAACACT 3' and reverse primer 5' GTCGACCTTACTGCCTCCTCTTGGGGTA 3') was prepared based on the nucleotide sequence of the VP19 of the WSSV registered in the Genbank.

For the isolation of WSSV genomic DNA, 20 mg samples of tissues obtained from *Penaeus chinensis* infected by WSSV were prepared, and the genomic DNA of WSSV was extracted with Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). PCR amplification VP19 gene was carried out with a Takara thermal cycler (Takara Shuzo, Japan).

PCR products were eluted from the agarose gel with Gel Extraction Kit (Nucleogen, Korea) and inserted in pGEM-T Easy vector system (Promega Co., USA). *E. coli* DH5α strain was transformed with the ligated DNA and the transformed cells were cultured using Luria-Bertani (LB) broth containing ampicillin (100 µg/ml). The plasmid DNA was extracted with Plasmid Purification Kit (Nucleogen, Korea). The plasmid DNA was sequenced at Macrogen Ltd. (Seoul, Korea). The nucleotide sequence of VP19 was confirmed with GenBank databases using BLAST.

Expression of rVP19 protein using baculovirus/insect cell expression system: The PCR fragment of VP19 was subcloned into pGEM-T Easy vector and then cloned into the pFastBac HT vector (Invitrogen, Carlsbad, USA) tagged with 6-histidine. Recombinant pFastBac HT-VP19 was transformed into DH10Bac *E. coli* for the transposition into the Bacmid DNA. Recombinant Bacmid DNA was confirmed by PCR using the M13 forward primer (5' GTTTTCCCAGTCACGAC3') and M13 reverse primer (5' CAGGAAACAGCTATGAC 3'). Recombinant Bacmid DNA VP19 was isolated and transfected into Sf21 insect cells for the production of VP19 protein in high concentration. Culture was carried out with the cell density of 1 × 10^6 cells/ml for 5 days at 27°C. Multiplicity of infection (MOI) of 0.5 to 20 were used for the determination of the optimal MOI.

The expressions of the WSSV antigen VP19 was confirmed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of rVP19 and antibody production: Overexpressed histidine-tagged rVP19 was purified using a Ni Sepharose High Performance Resin (GE Healthcare Bio-science, Wikstroms, Sweden) open column. Cells were sonicated in binding buffer (20 mM Sodium phosphate, 500 mM NaCl, 30 mM Imidazole, pH 7.4) and then centrifuged at 12,000 × rpm for 20 min at 4°C.

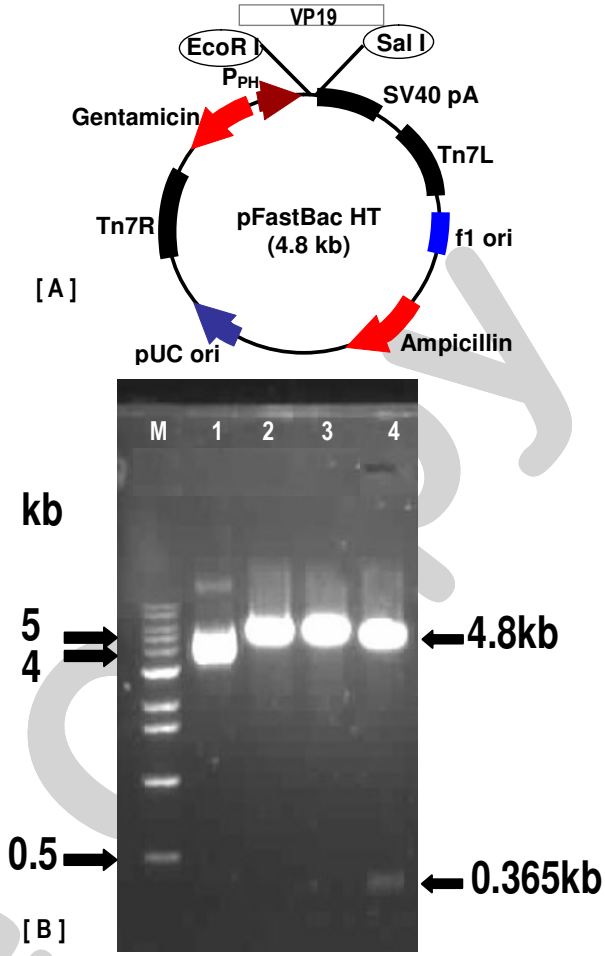


Fig. 1: The map of the recombinant plasmid and agarose gel electrophoresis of the cloned gene. The map of the recombinant vector pFastBac HT-VP19 (A) and electrophoresis patterns of the cloned gene (B) using baculovirus/insect cell expression system. [M: 1kb DNA ladder marker, lane 1: Uncut fragment, lane 2: One cut fragment (EcoR I), lane 3: One cut fragment (SalI), lane 4: Two cut fragments (EcoR I / Sal I)]

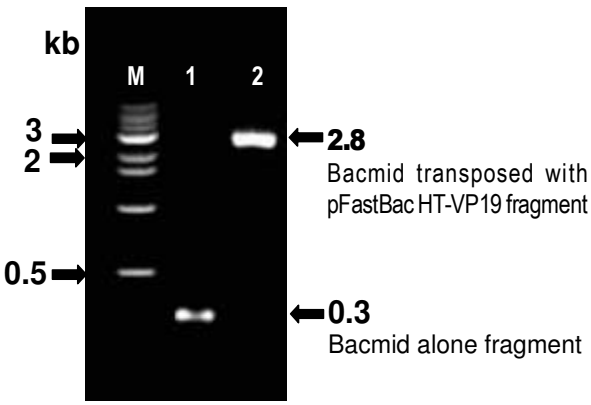


Fig. 2: Recombinant Bacmid DNA by PCR using agarose gel electrophoresis [M: 1kb DNA ladder marker, Lane 1: Bacmid alone fragment (~300 bp), Lane 2: Bacmid transposed with pFastBac HT-VP19 fragment (M13 primer region of Bacmid DNA (~300 bp) + Tn7R to Tn7L size (~2,130 bp) + size of VP19 (365 bp)]

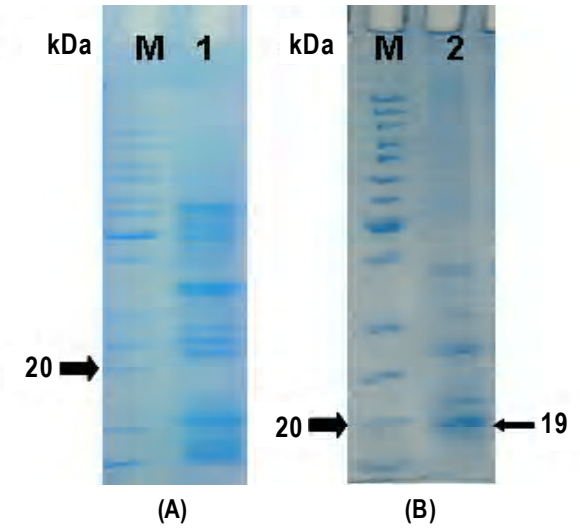


Fig. 3: SDS-PAGE of expressed recombinant VP19 protein (DH10Bac/pFastBac HT-VP19, 19 kDa) [M: PageRuler Protein ladder marker (Fermentas), lane 1: Negative control; lane 2: DH10Bac/pFastBac HT-VP19, 19 kDa]

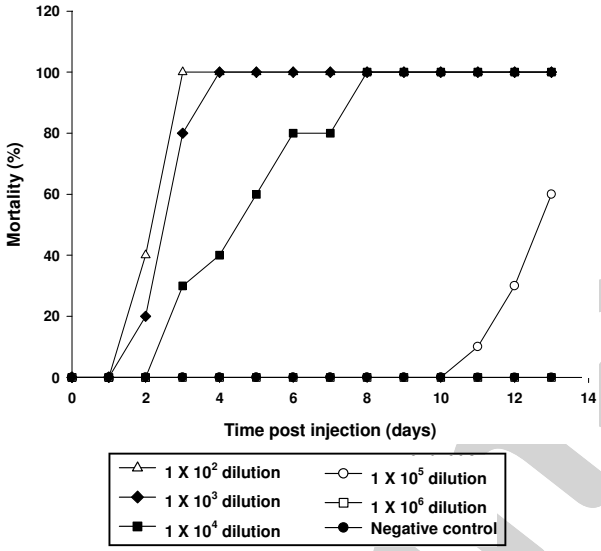


Fig. 4: Titration of WSSV stock in *P. chinensis* for the determination of optimal challenge dilution. [Ten microliters of a 1×10^2, 1×10^3, 1×10^4, 1×10^5 and 1×10^6 diluted stock was injected intramuscularly to *P. chinensis*]

Then the supernatant was passed through a 0.45 μm syringe filter before purification. After equilibrated with binding buffer, the column was washed with binding buffer and then eluted with elution buffer (20 mM Sodium phosphate, 500 mM NaCl, 500 mM Imidazole, pH 7.4). The purified protein was further concentrated by Amicon Ultra-4 (Millipore, Bedford, USA). The protein concentration was determined by Bio-Rad protein assay solution (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. The proteins were separated and analyzed by SDS-PAGE.

Polyclonal antiserum against rVP19 was raised in white rabbit (2.5 to 3.0 kg). The purified rVP19 protein (150 μg) in 800 μl of Freund's complete adjuvant (Sigma) was injected into a rabbit and

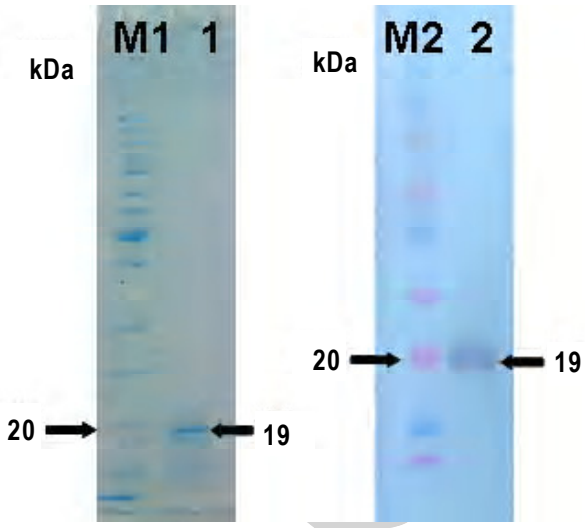


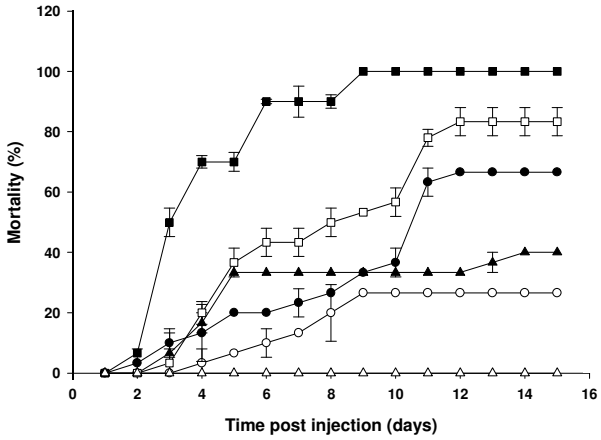
Fig. 5: SDS-PAGE gel (a) and Western blot analysis (b) of purified rVP19 protein [M1: PageRuler Protein ladder marker, lane 1: Purified rVP19 protein, M2: ProSieve-Color Protein Markers (Cambrex, Rockland, USA), lane 2: Transferred protein after antigen-antibody reaction. The nitrocellulose membrane was probed with antibody of rabbit against the purified rVP19 protein]

then given two booster injections with purified rVP19 (500 μg) in 600 μl of Freund's incomplete adjuvant (Sigma) at 2 week intervals. The antiserum was prepared after 2 weeks. ELISA (Enzyme-Linked Immunosorbent Assay) was performed to determine the titers of antiserum against rVP19.

Western blot: Expressed protein was separated by SDS-PAGE. After gel running, one loaded gel was stained with Brilliant Blue G (Sigma, USA). The other gel was transferred onto a 0.45 μm pore nitrocellulose membrane (BioTrace, Pensacola, USA) at 100 V for 1 h in a Bio-Rad mini Trans-Blot electrophoretic transfer cell for Western blot analysis. The western blot analysis was carried out by the method described by Park *et al*. (2005).

Dosage determination of WSSV for shrimp infection: Ten μl of different virus stock dilutions were injected intramuscularly in the second abdominal segment of the shrimp using a 30-gauge needle. For each group, 10 shrimps were used. The mortality was recorded once a day and dead shrimps were tested for the presence of WSSV by PCR. The obtained time-mortality relationship was used to determine the desired challenge pressure for the neutralization assay.

Neutralization assay: For neutralization challenge, the healthy shrimps collected from stock were divided into six groups as described in Fig. 6. The preimmune serum was included as a control for the effect of the serum on shrimp mortality. The WSSV virions were incubated with rVP19 antiserum or rabbit preimmune serum as a control for 1 hr at room temperature on the rocker of continuous shaking. The 50 μl mixture of WSSV and antiserum or PBS was intramuscularly injected into shrimp using a syringe with 30-gauge



Group		Constitution of injection solutions (50 µl / shrimp)	# Shrimp
-■-	Positive control	10 µl WSSV + 40 µl PBS	15 × 3
-□-	Preimmune serum control	10 µl WSSV + 10 µl preimmune antiserum + 30 µl PBS	15 × 3
-●-	VP19 I	10 µl WSSV + 1 µl rVP19 antiserum + 39 µl PBS	15 × 3
-▲-	VP19 II	10 µl WSSV + 5 µl rVP19 antiserum + 35 µl PBS	15 × 3
-○-	VP19 III	10 µl WSSV + 10 µl rVP19 antiserum + 30 µl PBS	15 × 3
-△-	Negative control	50 µl PBS	15 × 3

Fig. 6: Neutralization of WSSV infection in *P. chinensis* using antiserum raised against rVP19 protein of WSSV. The composition and experimental factors are summarized in the legend

needle. At the same time, a negative (PBS) and a positive control were included in the injections. For each treatment group, 15 shrimps were used. After the injection, the shrimp mortality was monitored daily.

Results and Discussion

Overexpression of rVP19 in Sf21 insect cells: The DNA fragments encoding of WSSV structural proteins VP19 was amplified using PCR. The PCR fragment of VP19 was subcloned into pFastBac vector (Fig. 1a) and confirmed by the electrophoresis pattern of cloned gene as shown in Fig. 1b. Recombinant Bacmid DNA was prepared by the transposition of pFastBac HT-VP19 in DH10Bac *E. coli* and the transformation was determined. Transformation was confirmed by PCR and analyzed using agarose gel electrophoresis. The sizes of Bacmid alone fragment DNA and Bacmid transposed with pFastBac HT-VP19 fragment DNA were approximately 300 bp and 2,800 bp, respectively (Fig. 2).

Recombinant Bacmid DNA VP19 was expressed under control of the polyhedrin promoter in insect cells using recombinant baculovirus. The growth of infected Sf 21 ceased with increased cell diameter and granular viral buddings could be observed.

The recombinant proteins were soluble and easily recovered from infected cells at late stage. The rVP19 was optimally expressed at MOI of 3. Optimal culture duration was 5 days. Recombinant VP19 from Sf21 cells infected with recombinant baculovirus were analyzed on a 12% SDS-PAGE gel and the expressed proteins had molecular weights of approximately 19 kDa (Fig. 3).

WSSV titration: The virus stock was diluted stepwise from 1×10^2 to 1×10^6 times in PBS. Ten µl of diluted virus solution was injected intramuscularly into 10 shrimps. Shrimps injected with PBS served as negative control for the infection. All shrimps serving as negative control and those having the 1×10^6 virus dilution survived, whereas mortality due to virus infection occurred in all other groups with a low virus dilutions as shown in Fig. 4. Administration of virus dilution from 1×10^2 and 1×10^3 resulted in almost 100% mortality in 5 days. The dilution of 1×10^4 was chosen as the virus dose for further experiments because this condition could give the optimal response to the neutralization experiments in terms of mortality reduction.

Neutralization assay with rVP19 antibody: In the Western blot analysis, the polyclonal antiserum against rVP19 showed a clear reaction with the purified rVP19 (Fig. 5). The rVP19 polyclonal antiserum was used in *in vivo* neutralization assay with *P. chinensis*. A constant amount of WSSV was incubated with various antiserum concentrations. The mixture was injected into shrimps as shown in the contents of each group (Fig. 6). No shrimp died in the negative control injected with PBS as shown in Fig. 6. The shrimp in the positive control injected with WSSV (at 1×10^4 diluted WSSV) showed 100% mortality at 9 days post injection. The WSSV preincubated with preimmune serum resulted in 83.3% mortality at 15 days post injection. When the WSSV was preincubated with 1 µl or 5 µl or 10 µl rVP19 antiserum, shrimp mortalities decreased to 66.6%, 40.0% and 26.6% at 15 days post injection, respectively (Fig. 6). The high concentration of antiserum group showed lower mortality than that of the low concentration of antiserum group. This indicates that the WSSV could be neutralized by the rVP19 antiserum in a dose-dependent manner.

Neutralization of virus with antiserum have been performed to study the role of virion proteins or their domains in the infection process. However, standardized (primary) shrimp cell culture was not available and therefore, *in vivo* challenge was performed. The *in vivo* neutralization challenge showed that WSSV infection was neutralized by the rVP19 polyclonal antiserum. WSSV neutralization using the VP19 polyclonal antiserum was concentration dependent. The preimmune serum control resulted in a small positive effect on shrimp survival. This could be due to compounds present in the serum stimulating the shrimp defense system as reported by Witteveldt *et al.* (2004). A specific polyclonal antibody has been generated against the major envelope protein VP28 using a baculovirus expression

vector system and this antiserum was able to neutralize WSSV infection of *P. monodon* in a concentration-dependent manner upon intramuscular injection (Van Hulten *et al*., 2001). From the result of neutralization challenge, it could be concluded that the envelope protein, VP19 of WSSV plays a critical role in WSSV infection to shrimp. Therefore, it can be possible to develop protein or gene based vaccines using VP19 against WSSV such as oral feeding vaccination by feed-supplement. This study showed that the neutralization challenge was able to recognize WSSV structural proteins. This opens the way for the design of practical strategies to control WSSV.

Acknowledgments

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