Experimental Infection for the Neutralization of White Spot Syndrome Virus (WSSV) in Wild Captured Sand Shrimp, *Crangon affinis*

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White spot syndrome virus (WSSV) is one of the most virulent viral agents threatening the penaeid shrimp culture industry. This study was carried out to evaluate the susceptibility of the sand shrimp, *Crangon affinis,* to WSSV as an alternative experimental model. WSSV caused 100% mortality in *C. affinis* within 7 days after experimental infection by immersion. Based on challenge studies, it was confirmed that *C. affinis* could be a potential host in WSSV transmission. Also, the neutralization of WSSV was carried out using an antiserum raised against recombinant envelop protein rVP466 to evaluate the WSSV infection mechanism. A constant amount of WSSV (at 1×10^4 diluted stocks) was incubated with various amounts of antiserum and then mixed to 20 l reservoir for the immersion challenge of *C. affinis* for neutralization. At 5 days post challenge, the shrimp in the positive control immersed in the immersion reservoir containing WSSV stock showed 100% mortality. The shrimps challenged with the 3 different mixtures of WSSV and rVP466 antiserum (1:0.1, 1:0.5 and 1:1) showed 100%, 68.8% and 68.8% mortality at 14 days post challenge, respectively. These results indicated that the antiserum raised against rVP466 could block WSSV infection in *C. affinis*. Therefore, this study confirmed that *C. affinis* can be naturally infected by WSSV as another potential host and that *C. affinis* can be used as an alternative experimental animal instead of penaeid shrimps.

Key words : White spot syndrome virus (WSSV), *Crangon affinis*, VP466, antiserum, neutralization

Introduction

White spot syndrome virus (WSSV) has been reported in cultured shrimp worldwide since early 1990s [2]. Up to date, WSSV is a major disease causing virus to cultured penaeid shrimp such as *Penaeus chinensis, P. indicus, P. monodon, P. stylirostris* and *P. vannamei*. WSSV infection causes 100% mortality within 7 to 10 days in commercial shrimp culture, resulting in large economical losses to the shrimp culture industry [14]. Also, it has a wide host range including marine shrimp such as *Metapenaeus ensis*, freshwater shrimp such as *Macrobrachium rosengergii*, crayfish such as *Procambarus clarkii*, lobster such as *Panulirus* sp. and crab such as *Helice tridens* [12]. Infected animals show the symptoms of lack of movement, reddish discoloration of body and white spots on the carapace.

Sand shrimp *Crangon affinis* is a species similar to a common shrimp *Crangon crangon* belongs to Order Decapoda, Family Crangonoidea. It has an important role for food chain of many seabirds and fishes in the coastal ecosystem.

VP466 is one of identified envelop proteins in WSSV genome (accession no. AF395545). The open reading frame (ORF) of VP466 contained 1381 bp encoding a protein of 466 amino acids with a theoretical molecular mass of 50 kDa [6].

Neutralization assays have been performed to study the role of virion proteins in the infection stage. Antibodies bind to the envelop protein of the virion, thus prevent the attachment of virus to the cell surface and the entry to cell or virus uncoating [1]. *In vivo* neutralization has been widely used for many vertebrate viruses as passive immunization strategies. Musthaq *et al*. [7] used a WSSV envelop recombinant protein, rVP28, to evaluate the potential for the neutralization of shrimp against WSSV.

Therefore, this study was carried out to evaluate the susceptibility of wild captured sand shrimp, *Crangon affinis* to WSSV. The recombinant protein, rVP466 was used for the production of antiserum and the neutralization was carried out to evaluate the role of VP466 in infection of WSSV.

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Materials and Methods

Collection of experimental animals

Healthy sand shrimp *Crangon affinis* were purchased from Dadaepo located in Busan, Korea. Shrimps of approximately 3 to 5 g were used in this experiment. They were kept in reservoirs at 26°C and each shrimp in the experimental reservoirs was placed in individual plastic cages to prevent cannibalism [13].

WSSV stock

Tissues of WSSV infected shrimp (*Penaeus chinensis*) were homogenized in 10-fold volumes of phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4°C. After the centrifugation at 12,000x *g* for 10 min at 4°C, the supernatant was filtered through a 0.45 μm filter. The virus stock after the filteration was stored in aliquots at -80°C for WSSV challenge test.

Experimental infection by immersion

C. affinis were challenged by immersion for the determination of challenge pressure as described in Table 1. Shrimps were immersed in 20 l seawater containing the different doses of WSSV for 8 hr at 28°C, then removed from the immersion reservoir. The shrimps were then rinsed and placed to individual cages. Mortality was recorded daily and dead shrimps were tested for the presence of WSSV by PCR. The time-mortality relationship was used to determine the desired challenge pressure for the neutralization.

Cloning and expression of recombinant protein VP466

VP466 specific primer set was prepared based on the nucleotide sequences of the VP466 of the WSSV (accession no. AF395545) registered in the Genbank. Forward primer VP466F (5'-GGATCCATGTCTGCATCTTTAATATTGGAC-3') and reverse primer VP466R (5'-AAGCTTGTTATGACACAAACCTATTCCACAA-3') with *Bam*H I and *Hin*dⅢ restriction sites were used to amplify VP466 gene. The DNA fragment encoding the entire VP466 ORF was amplified from WSSV genomic DNA by polymerase chain reaction (PCR).

The VP466 gene was cloned into a constitutive expression vector, pHCE IIB (Bioleaders, Daejeon, Korea). The presence of the insert in the plasmid was confirmed by the digestion with *Bam*H I and *Hin*d III. The transformed *E. coli* strain BL21 (DE3) was cultured at 37°C for 8 hr in LB medium containing 0.1 mg/ml of ampicillin. Cells were disrupted by the sonication with 8 M urea, then centrifuged at 12,000x *g* for 20 min at 4°C. Dialysis was carried out with the supernatant in TE buffer to remove urea using membrane. The obtained protein was analyzed by SDS-PAGE (12% acrylamide gel). The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, USA).

Antiserum production and western blot analysis

Antiserum raised against rVP466 was prepared in white rabbit (2.5 kg) as described by Ha *et al*. [3]. The immunoglobulin (IgG) fraction was purified by protein A-Sepharose (Bio-Rad, Hercules, USA). Enzyme-Linked Immunosorbent Assay (ELISA) was carried out to determine the titers of antiserum against rVP466 as described by Harlow *et al*. [4]. The antiserum was considered positive when the average readings of the triplicate wells were twice higher than the average reading for the negative serum (P/N ratio ≥2).

The western blot analysis was carried out by the method of Ha *et al*. [3]. The result was identified with BCIP/NBT liquid substrate system (Sigma-Aldrich, St. Louis, USA).

Neutralization assay

For neutralization assay, the healthy shrimps without the virus infection were divided into five groups as described in Table 2. The WSSV stocks ($1x10^5$) with 3 different doses of rVP466 antiserum were mixed for 1 hr at 28°C on the shaking rocker. Two ml mixture of WSSV and antiserum was added into the immersion reservoir containing 20 l seawater. At the same time, the mixture of WSSV and PBS as a positive control and PBS as a negative control were included. Shrimps were immersed for 8 hr at 28°C in the immersion reservoir, and then the shrimps were rinsed and

Table 1. Contents of experimental groups for *in vivo* titration by immersion

Experimental group	Contents of immersion reservoir (20 l seawater)	No. of shrimp
Control	2 ml PBS	20
$1x10^6$ WSSV dilution	0.02 ml WSSV stock + 1.98 ml PBS	20
$1x10^5$ WSSV dilution	0.2 ml WSSV stock + 1.8 ml PBS	20
$1x10^4$ WSSV dilution	2 ml WSSV stock	20

Table 2. Contents of experimental groups used in the neutralization assay

Experimental group	Contents of immersion reservoir (20 l seawater)	No. of shrimp
Negative control	2 ml PBS	15x3
Positive control	0.2 ml WSSV stock + 1.8 ml PBS	15x3
WSSV: rVP466 antiserum = 1:0.1	0.2 ml WSSV + 0.02 ml rVP466 antiserum + 1.78 ml PBS	15x3
WSSV: rVP466 antiserum = 1:0.5	0.2 ml WSSV + 0.1 ml rVP466 antiserum + 1.7 ml PBS	15x3
WSSV: rVP466 antiserum = 1:1	0.2 ml WSSV + 0.2 ml rVP466 antiserum + 1.6 ml PBS	15x3

placed to individual cages. After the immersion challenge, the shrimp mortality was monitored daily.

Results and Discussion

Determination of infection pressure by immersion

The experimental infection with immersion challenge was carried out by considering waterborne route of WSSV transmission in natural environment as shown in Fig. 1. No shrimp died in the negative control during the experimental period. The shrimp with low dilution of virus groups ($1x10^4$ and $1x10^5$) showed higher mortality than that with high dilu-
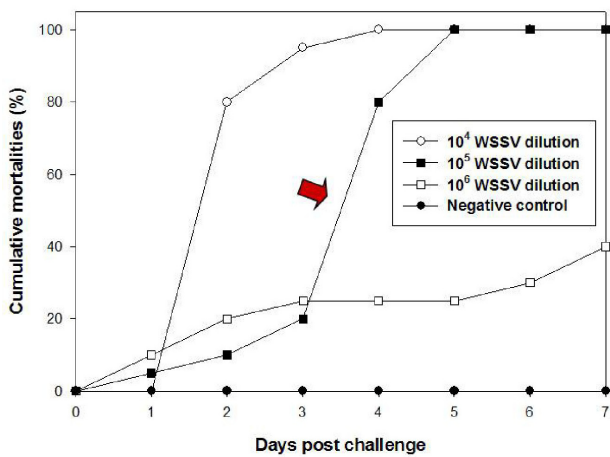

Fig. 1. Titration of WSSV stock in *Crangon affinis* for the determination of optimal challenge pressure. The arrow shows the appropriate virus dose for the neutralization experiment.

tion group ($1x10^6$). The shrimp immersed in 20 l seawater containing WSSV dilution of $1x10^5$ showed 100% mortality in 5 days. Therefore, the dilution of $1x10^5$ was chosen as the virus dose for further experiments with *C. affinis* because this dilution of WSSV could give the optimal response to the neutralization in terms of mortality reduction. Also, this result showed that the sand shrimp, *C. affinis*, was highly susceptible to WSSV infection. Several studies have confirmed that many suspected decapods carriers of WSSV can transmit the virus to penaeid shrimps [12]. Therefore, this study suggests that *C. affinis* can be naturally infected by WSSV as a host. Also, *C. affinis* can be a decapods carrier of WSSV transmitting penaeid shrimps.

Production of recombinant protein VP466

The expression of VP466 was carried out using pHCE IIB vector. The pHCE vector, using a constitutively expressing HCE promoter derived upstream from the D-amino acid aminotransferase (D-AAT) gene of *Geobacillus toebii*, was used for the high-level expression of protein vaccine without IPTG induction [8].

In this study, DNA fragment encoding WSSV structural protein VP466 was amplified by PCR and cloned into pHCE vector. As shown in Fig. 2, agarose gel showed bands at expected positions of insert gene, VP466 and the vector.

After the expression of rVP466, the protein was analyzed on SDS-PAGE (12% acrylamide gel) as shown in Fig. 3A. The band was observed at the expected position of 50 kDa corresponding to VP466 protein.
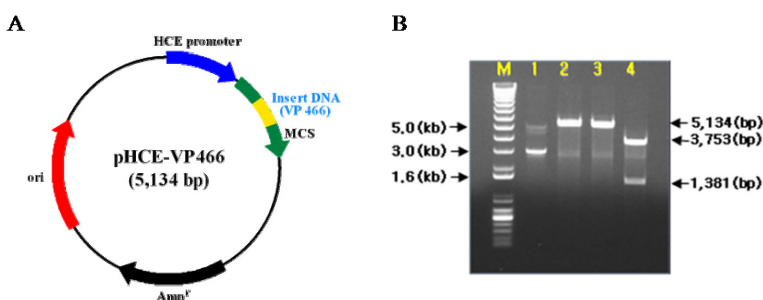

Fig. 2. The map of recombinant plasmid pHCE-VP466 and restriction patterns. (A) pHCE-VP466 with the size of 5,134 bp, (B) restriction map using *Bam*H I and *Hin*d III. M: 1 kb DNA ladder marker, lane 1: Uncut recombinant plasmid pHCE-VP466, lane 2: One cut fragment with *Bam*H I, lane 3: One cut fragment with *Hin*d III, lane 4: Two cut fragments with *Bam*H I and *Hin*d III.

In the western blot analysis, rVP466 antiserum showed a clear reaction with recombinant protein VP466 as shown in Fig. 3B. This result indicates that VP466 was correctly expressed and the antiserum had a proper immunoreactivity with the antigen, rVP466.

In vivo neutralization of WSSV in *C. affinis*

The rVP466 antiserum was used for *in vivo* neutralization assay with *C. affinis*. As shown in Fig. 4, the shrimps in the positive control showed 100% mortality at 5 days of post challenge by immersion. When the shrimps challenged with the mixture of WSSV and low concentration of rVP466 antiserum (1:0.1), the shrimps mortalities showed similar with the positive control group. The shrimps challenged with the mixtures of WSSV and high concentrations of rVP466 anti-
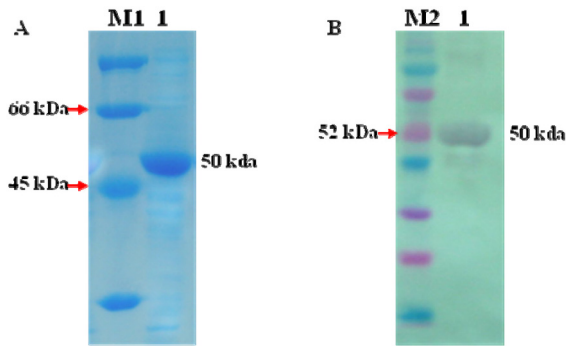

Fig. 3. Identification of expressed recombinant VP466 protein using (A) SDS-PAGE and (B) western blot analysis. M1: Protein molecular weight standard marker, M2: ProSieve-Color protein ladder marker, lane 1: product of pHCE-VP466.
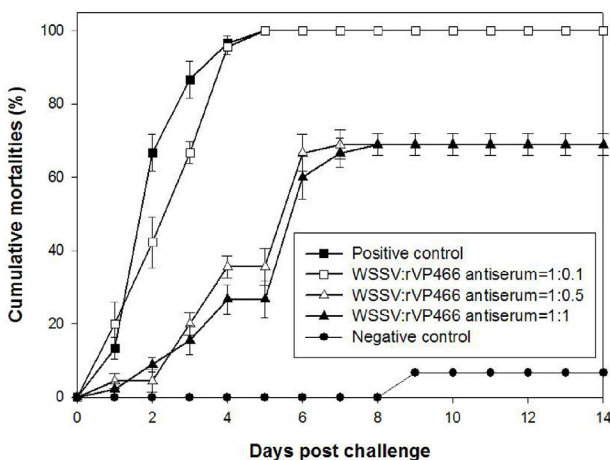

Fig. 4. *In vivo* neutralization of WSSV infection in *Crangon affinis* using antiserum raised against recombinant VP466 protein.

serum (1:0.5 and 1:1) showed shrimp mortalities of 68.8% at 14 days of post challenge. And shrimps in the negative control showed 6.7% of mortalities without WSSV infection at 14 days of post challenge.

Neutralization assay with antibodies against the individual structural proteins was used to identify the proteins involved in virus entry during infection [11]. Until now, 39 structural proteins in WSSV have been identified and 22 of the structural proteins are envelop proteins [9]. Envelop proteins play a crucial role in viral infections and are often involved with viral entry, assembly and budding. VP28 is a representative protein of the major envelop proteins in WSSV and is involved in the systemic infection of shrimp [7]. However, VP28 is not the only virus protein related attachment to the host cell, as it has been reported that many attachment proteins are involved in infection mechanism for the large DNA virus [5]. Therefore, the further studies with other envelop proteins are required for the correct analysis of WSSV attachment, entry and budding in shrimps.

In this study, *in vivo* neutralization assay with the antiserum against rVP466 showed 31.2% of survival ratio slightly less than that of VP28 [7]. However, the antiserum raised against VP466 could access to the viral antigen by the independent or coordinate manners [15]. Therefore, results suggest that envelop protein VP466 is involved in the initial step during WSSV infection similar to VP28 reported by van Hulten *et al*. [10].

In conclusion, wild captured sand shrimp, *Crangon affinis*, was susceptible to WSSV infection. It was confirmed that the antiserum raised against rVP466 blocks WSSV infection. *In vivo* experiments using WSSV can be conducted stably in winter because *C. affinis* can be collected regardless of seasons. Therefore, this study confirmed that *C. affinis* can be naturally infected by WSSV as another potential host and *C. affinis* can be used as an alternative experimental animal instead of penaeid shrimps.

Acknowledgment

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초록 : 자연산 자주새우(*Crangon affinis*)에서 흰반점바이러스(WSSV)의 neutralization을 위한 인위감염(experimental infection)

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흰반점바이러스(WSSV)는 새우양식산업에 막대한 경제적 손실을 일으키는 가장 치명적인 바이러스성 질병 원인체 중 하나이다. 본 연구는 인위적인 실험모델로서 WSSV에 대한 자주새우(*Crangon affinis*)의 감수성을 확인하고자 실험을 수행하였다. WSSV가 희석된 수조 내에서 침지법으로 감염된 새우는 감염 후 7일째에 100% 누적폐사율을 보여 자주새우가 WSSV에 대해 매우 높은 감수성을 갖고 있음을 확인하였다. 또한 재조합단백질인 rVP466에 대한 항혈청의 중화효과를 확인하기 위해 항혈청과 반응시킨 바이러스액(1×10^4 배로 희석된 WSSV)을 이용하여 침지법으로 자주새우에 대해 공격실험(challenge test)을 수행하였다. 실험 결과, WSSV로 challenge한 감염대조구(positive control)의 새우들은 감염 후 5일째에 100% 폐사하였으며, WSSV와 rVP466 항혈청을 1:0.01, 1:0.1, 1:1로 혼합한 액으로 challenge한 새우들은 감염 후 14일째에 각각 100%, 68.8%, 68.8%의 누적폐사율을 보였다. 따라서 본 연구 결과는 연안서식종인 자주새우가 양식장으로부터 배출된 WSSV에 의해 자연상태에서 감염될 수 있는 가능성과 함께 항혈청에 대한 중화효과를 나타냄으로써 겨울철 저수온기에 WSSV 감염을 위한 대체 실험생물로서의 유용성을 확인하였다.