J. Microbiol. Biotechnol. (2008), **18**(5), 964–967

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Vaccination of Shrimp (*Penaeus chinensis*) against White Spot Syndrome Virus (WSSV)

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Received: August 17, 2007 / Accepted: February 13, 2008

Two structural protein genes, VP19 and VP466, of white spot syndrome virus (WSSV) were cloned and expressed in Sf21 insect cells using a baculovirus expression system for the development of injection and oral feeding vaccines against WSSV for shrimps. The cumulative mortalities of the shrimps vaccinated by the injection of rVP19 and rVP466 at 15 days after the challenge with WSSV were 50.2% and 51.8%, respectively. For the vaccination by oral feeding of rVP19 and rVP466, the cumulative mortalities were 49.2% and 89.2%, respectively. These results show that protection against WSSV can be generated in the shrimp, using the viral structural protein as a protein vaccine.

Keywords: Shrimp, vaccine, WSSV, VP19, VP466, oral feeding

White spot syndrome virus (WSSV) was first discovered in Taiwan in 1992 [8]. 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 and has spread quickly to shrimp-farming areas worldwide, causing major economic damage [13]. However, no perfect WSSV control strategies have been described. Enveloped viruses contain glycoproteins in their viral envelopes, and these often play important roles in the interaction between virus and host, as attachment factor to the receptor and fusion factor to cell membranes [4, 19]. To date, some major structural proteins of WSSV have been identified [6, 17, 18].

Invertebrates do not possess immunoglobulin and the T-cell receptor, and thus do not have a true adaptive immune response [15]. However, specific memory exists in innate immune systems [11, 12]. Although the real mechanisms have not been evaluated, a short memory of antigen as the immune response in shrimp might involve an induced secretion of neutralizing substances or other defense proteins against WSSV invasion [3].

For this study, the VP19 and VP466 genes were used for the development of protein vaccines. A recombinant protein vaccine has an advantage in that animals can be vaccinated with a large amount of specific antigens [7, 14]. The protein vaccine is one of the solutions, especially for shrimp viruses including WSSV, because WSSV cannot be cultured owing to the lack of susceptible cell lines. Therefore, recombinant WSSV envelope proteins, rVP19 and rVP466, were produced for the vaccination of shrimps.

The VP19 and VP466 proteins could be glycosylated or phosphorylated owing to multiple putative *N*- or *O*-glycosylation sites and phosphorylation sites in the amino acid sequences [5]. Therefore, a baculovirus/insect cell expression system could be one of the effective protein vaccine production methods for the shrimp [16].

Shrimps, *Penaeus chinensis* (length 9–11 cm and weight 6–8 g), were purchased from a shrimp farm (Dan-Jang Marine Co.) located in Goheung, Jeonnam, Korea. Shrimps were tested for the presence of WSSV by PCR to ensure they were WSSV-free before the experiments. For challenge experiments, a lethal dose of WSSV was injected into shrimps [20]. The PCR fragments of VP19 and VP466 were cloned into the pFastBac HT vector (Invitrogen, Carlsbad, U.S.A.) tagged with 6-histidine and subcloned into DH10Bac *E. coli* for the transposition into the Bacmid DNA. Recombinant Bacmid DNA fragments were transfected into Sf21 insect cells for the production of rVP19 and rVP466 proteins, as shown in Fig. 1. The concentrations of recombinant proteins were determined using the Bradford assay and adjusted to 50 μg/ml and used as protein antigens.

For the dosage determination of WSSV for the infection of shrimp, various dilutions of virus stock were injected intramuscularly in the second abdominal segment of the

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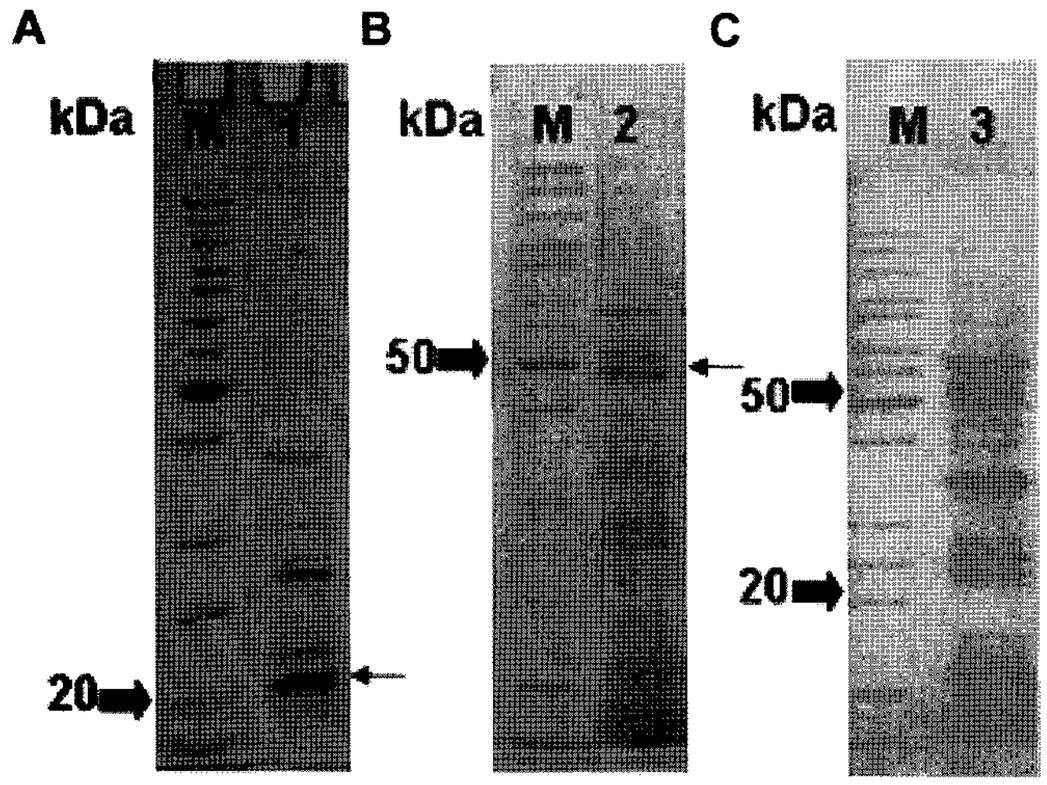

Fig. 1. SDS-PAGE of expressed proteins of VP19 and VP466 using the Baculovirus/insect cell expression system. (**A**) rVP19 protein (DH10Bac/pFastBac HT-VP19, 19 kDa), (**B**) rVP466 protein (DH10Bac/pFastBac HT-VP466, 50 kDa), and (**C**) negative control. M, PageRuler protein ladder marker (Fermentas); lane 1, DH10Bac/pFastBac HT-VP19, 19 kDa; lane 2, DH10Bac/pFastBac HT-VP466, 50 kDa; lane 3, negative control.

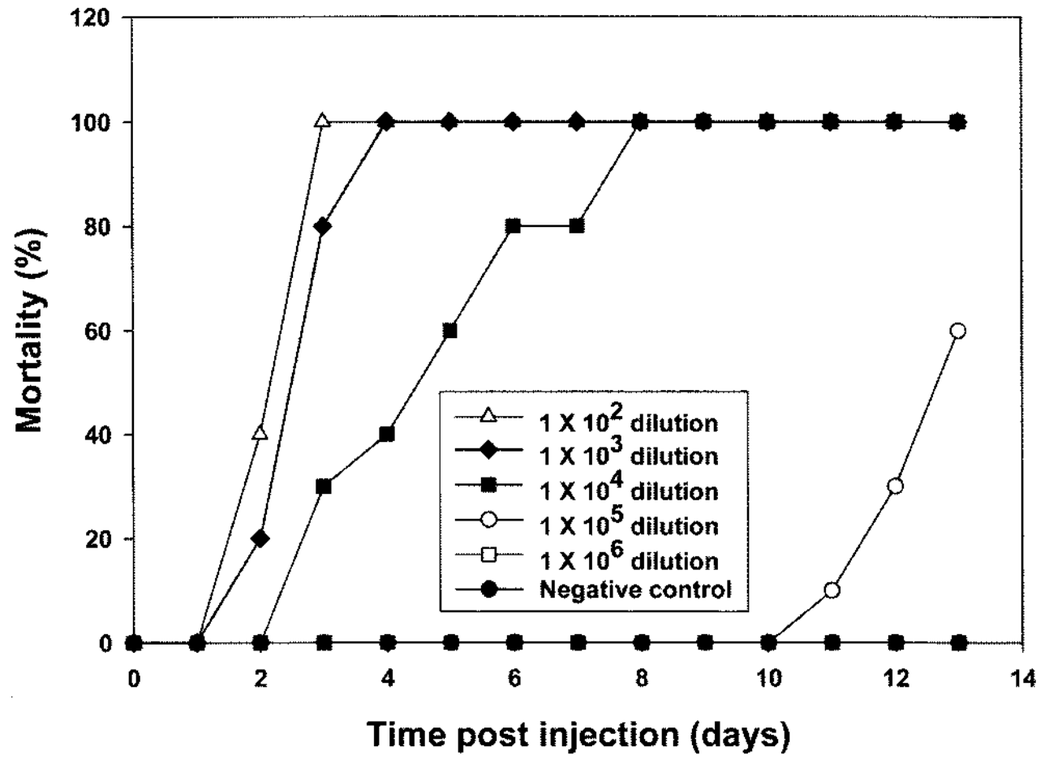

Fig. 2. Titration of WSSV stock in *P. chinensis*.
Ten μl each of 1×10^2, 1×10^3, 1×10^4, 1×10^5, and 1×10^6 diluted stocks was injected intramuscularly to *P. chinensis*.

shrimp. The time-mortality relationship obtained was used to determine the desired challenge pressure for the vaccination experiments.

For the evaluation of the specific WSSV dilution, the virus stock was diluted stepwise from 1×10^2 to 1×10^6 times in PBS. Shrimps injected with PBS served as the negative control for the infection. Shrimps in the negative control group and those injected by 1×10^6 virus dilution survived, whereas mortality due to virus infection occurred in all other groups, as shown in Fig. 2. 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 the challenge because this condition could give the optimal response to the challenge in terms of mortality reduction.

After the acclimatization of shrimps for a week, recombinant antigens were injected as antigens to shrimps, as described in Table 1(a). At a week after the injection, all the shrimps were challenged by the intramuscular injection of WSSV, except for the negative control group. The mortality was recorded for 15 days after the challenge. For the vaccination by oral feeding, feed pellets were prepared. Commercial pellets weighing approximately 25 g were mixed with 10 ml (50 μg/ml of protein) of recombinant proteins from 5×10^6 transfected insect cells. The mixed feed pellets were incubated on ice to allow absorption with 10 ml of Freund's Complete Adjuvant (FCA; Sigma) to prevent the dispersion of the recombinant proteins in the water. Shrimps were vaccinated be feeding prepared feed pellets at 5% of body weight per day for 10 days, as indicated in Table 1(b). During the vaccination of the test groups, the positive and negative control groups were fed the commercial pellets. The FCA control group was fed the commercial pellets mixed with FCA. Ten days after the initial vaccine feeding, shrimps were challenged by the injection of WSSV, except for the negative control shrimp that was injected with 50 μl of PBS. All measurements were made in triplicate. The protection against WSSV was calculated as the relative percent survival (RPS=(1-mortality of protection group/control group mortality)×100) [2].

Table 1. Preparation for vaccination by injection and oral feeding.

Group		Injection	Challenge	No. Shrimp
(a)	VP19	50 μl rVP19	10 μl WSSV+40 μl PBS	15×3
	VP466	50 μl rVP466	10 μl WSSV+40 μl PBS	15×3
	Positive control	50 μl PBS	10 μl WSSV+40 μl PBS	15×3
	Negative control	50 μl PBS	50 μl PBS	15×3
(b)	VP19	Commercial feed+rVP19+FCA	10 μl WSSV+40 μl PBS	15×3
	VP466	Commercial feed+rVP466+FCA	10 μl WSSV+40 μl PBS	15×3
	Positive control	Commercial feed	10 μl WSSV+40 μl PBS	15×3
	Negative control	Commercial feed	50 μl PBS	15×3
	FCA control	Commercial feed+FCA	10 μl WSSV+40 μl PBS	15×3

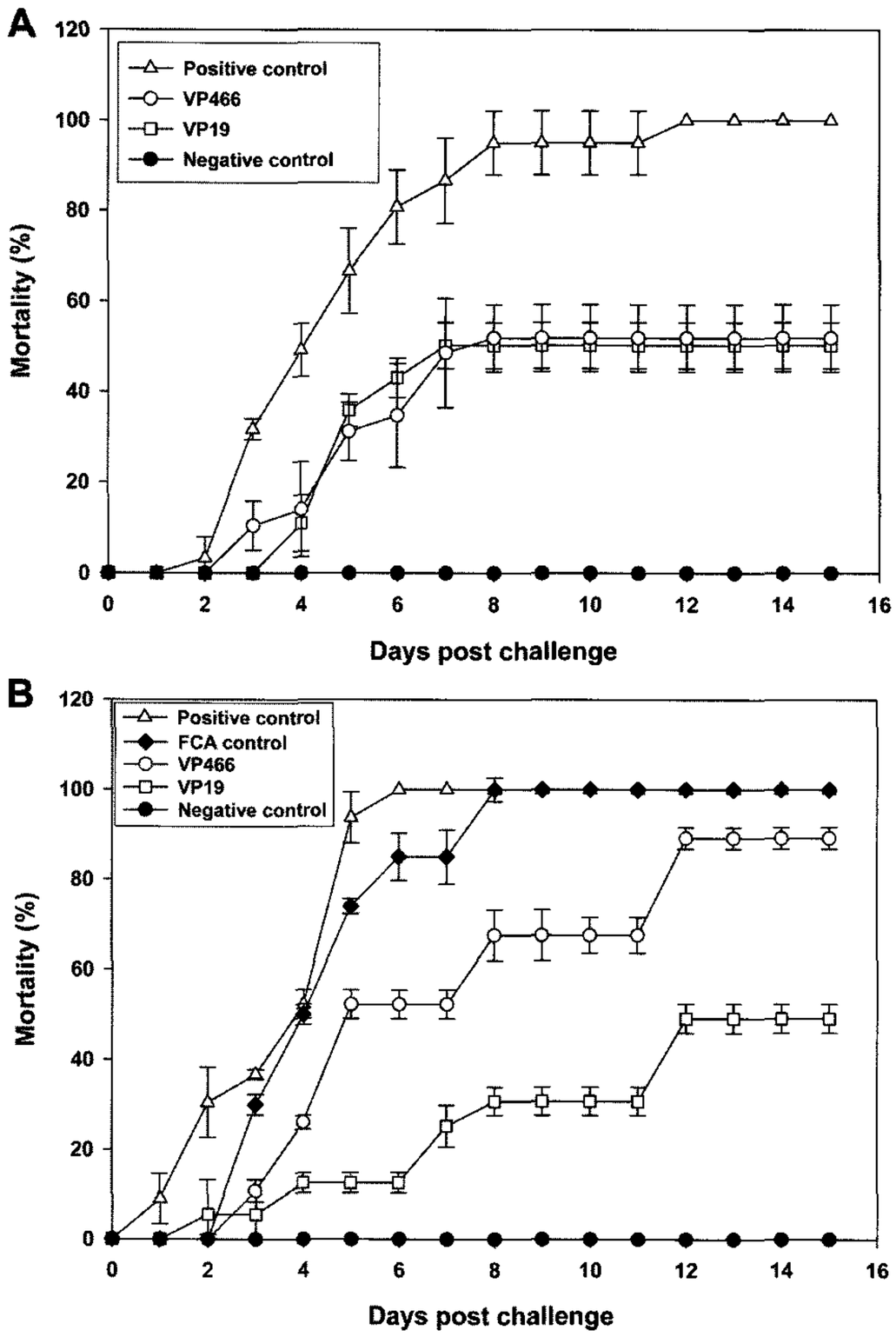

Fig. 3. Challenge tests of *P. chinensis* vaccinated by injection and oral feeding of rVP19 and rVP466.
Recombinant WSSV structural proteins, rVP19 and rVP466, were vaccinated and (**A**) challenged with WSSV at 1 week after the vaccination by injection and (**B**) challenged with WSSV at 10 days after the vaccination by oral feeding.

Vaccination by injection was performed using the recombinant proteins, as shown in Fig. 3A. The cumulative mortalities of the shrimps vaccinated with rVP19 and rVP466 at 15 days after the challenge with WSSV (22 days post vaccination) were 50.2% and 51.8%, respectively. RPS values of shrimp groups vaccinated by rVP19 and rVP466 were 49.8% and 48.1%, respectively. The cumulative mortalities for vaccinated groups were significantly lower than that for the positive control group.

For the vaccination by oral feeding, shrimps were divided into five groups, as shown in Fig. 3B. The cumulative mortalities of the shrimps vaccinated with rVP19 and rVP466 at 15 days after the challenge with WSSV (25 days post vaccination) were 49.2% and 89.2%, respectively. RPS values of shrimp groups vaccinated by rVP19 and rVP466 were 50.9% and 10.8%, respectively. The cumulative mortalities for vaccinated groups were significantly lower than those for the positive control groups.

Two different trials of vaccination, injection and oral feeding, were carried out to evaluate the immunization of *P. chinensis* against WSSV. Vaccination trials by injection enhanced the resistance of shrimp against WSSV. Results showed that survival from WSSV infection could be obtained in shrimp by using WSSV structural protein as a protein vaccine. In addition, vaccination trial by oral feeding showed a significantly low cumulative mortality in shrimp vaccinated by VP19 compared with control groups. However, the protection of the shrimps vaccinated by oral feeding of rVP466 was not satisfactory. This result suggests that rVP466 might not be transferred to the circulation system of shrimp. In the case of the vaccination by injection, both rVP19 and rVP466 were effective in maintaining survival of shrimp, which indicates that the protein vaccine could be circulated in the body and act as antigens. In the case of the vaccination by oral feeding, rVP19 was effective as a protein vaccine, but rVP466 was not effective, probably owing to the degradation of rVP466 in the gastrointestinal tract, or an absorption barrier of the gastrointestinal absorption system for high molecular mass rVP466 (50 kDa) [1]. Therefore, further study should be carried out on the gastrointestinal absorption system and delivery system of recombinant protein vaccine in the gastrointestinal tract of the shrimp [10].

The presence of the FCA (Freund's Complete Adjuvant) might provide a positive effect on shrimp survival upon WSSV challenge [9]. The FCA in feed mixed with rVP19 and rVP466 could act as an effective adjuvant for the vaccination by oral feeding.

This study showed that the shrimp defense system was able to recognize WSSV structural proteins, and thus the vaccination of shrimp against WSSV could be possible by the oral feeding of the protein vaccine, rVP19, and the injection of rVP19 and rVP466. This opens the way for the design of practical strategies to control WSSV.

Acknowledgments

This research was supported by a grant (P-2004-04) from the Marine Bioprocess Research Center of the Marine Bio 21 program funded by the Ministry of Maritime Affairs and Fisheries, Republic of Korea. Ms. Soo-Jung Gong and Mr. Chae Hun Ra received financial support from the fund from the Brain Busan 21 project of the metropolitan city of Busan, Republic of Korea.

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