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Transcriptional Analysis for Oral Vaccination of Recombinant Viral Proteins against White Spot Syndrome Virus (WSSV) in *Litopenaeus vannamei*

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This study was carried out for the molecular level identification of recombinant protein vaccine efficacy, by oral feeding against white spot syndrome virus infection, with the comparison of viral mRNA transcriptional levels in shrimp cells. For the determination of WSSV dilution ratio for the vaccination experiment by oral feeding, *in vivo* virus titration was carried out using different virus dilutions of virus stock (1×10^2, 2×10^2, and 1×10^3). Among the dilution ratios, 2×10^2 diluted WSSV stock was chosen as the optimal condition because this dilution showed 90% mortality at 10 days after virus injection. Recombinant viral proteins, rVP19 and rVP28, produced as protein vaccines were delivered in shrimps by oral feeding. The cumulative mortalities of the shrimps vaccinated with rVP19 and rVP28 at 21 days after the challenge with WSSV were 66.7% and 41.7%, respectively. This indicates that rVP28 showed a better protective effect against WSSV in shrimp than rVP19. Through the comparison of mRNA transcriptional levels of viral genes from collected shrimp organ samples, it was confirmed that viral gene transcriptions of vaccinated shrimps were delayed for 4~10 days compared with those of unvaccinated shrimps. Protection from WSSV infection in shrimp by the vaccination with recombinant viral proteins could be accomplished by the prevention of entry of WSSV due to the shrimp immune system activated by recombinant protein vaccines.

Keywords: *Litopenaeus vannamei*, protein vaccine, WSSV, RT–PCR, viral protein

White spot syndrome virus (WSSV) is one of the most contagious pathogens infecting shrimps. This virus causes up to 100% mortality within 7 to 10 days in cultured shrimps, resulting in large economic losses to the shrimp culture industry [11]. However, no perfect solutions for WSSV control have been studied.

Viral structural proteins, especially the envelope proteins, are important because of not only virion morphogenesis but also that they are the first molecules to interact with the host cell [3]. The structural proteins often play vital roles in cell targeting, and virus entry, assembly, and budding [4]. Among the structural proteins, viral protein 28 (VP28) expressed in the outer surface of WSSV facilitates the entry of the virion into the cell at the early WSSV infection stage [18]. VP28 is on envelope protein on the surface of the WSSV and reacts with anti-WSSV polyclonal antibodies. This suggests that recombinant VP28 (rVP28) could be a common antigen to control white spot diseases of shrimp [10, 20]. Therefore, rVP28 can be used as a vaccine to prevent the virus entry to the shrimp cell by stimulation of the immune system.

In the previous reports, short-term memory of the specific antigen such as structural proteins of WSSV has been reported in various shrimp species [9]. The results of WSSV neutralization using hemolymphs of surviving shrimps after WSSV infection suggested that an adaptive immune response might exist in the shrimp and protection from WSSV could be induced in shrimp by a vaccination [24].

Reverse transcription–polymerase chain reaction (RT–PCR) technologies have been powerful diagnostic tools for shrimp viral infections and the detection of viral reservoirs in asymptotic carriers [17]. Therefore, this study was carried out to evaluate vaccination effects on mRNA transcription of WSSV genes by the administration of the recombinant viral proteins, rVP19 and rVP28, as oral protein vaccines. The protection effect of protein vaccines against WSSV in shrimps was confirmed by RT–PCR as a molecular biological tool for the determination of the mRNA transcription levels in experimental shrimps. RT–PCR diagnosis methods were used as described in a previous report [16].

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Table 1. Experimental design for *in vivo* titration by injection.

Group	Challenge	Shrimp samples[a]
WSSV I	WSSV stock:PBS=1:100	12×3
WSSV II	WSSV stock:PBS=1:200	12×3
WSSV III	WSSV stock:PBS=1:1,000	12×3
Negative control	PBS (pH 7.4)	12×3

[a]The number of shrimps of each group was 12 and experiments were repeated 3 times under the same conditions.

Materials and Methods

Shrimp Culture

Healthy whiteleg shrimps, *Litopenaeus vannamei*, were purchased from a shrimp farm located in Muan, Jeonnam, Korea. Shrimps of approximately 6 to 8 g were used in experiments. Shrimps were tested for the presence of WSSV by PCR using primer sets encoding each viral protein to ensure that they were virus free before the experiments. They were kept in tanks at 26°C with continuous aeration and fed with commercial shrimp pellet feed. Each shrimp for experiments was placed in individual plastic cages to prevent cannibalism [22].

Preparation of Viral Inoculums

A lethal dose of WSSV was injected intramuscularly to *L. vannamei*. Frozen dead shrimps with clear white spots were thawed and muscle tissues of the infected shrimp were homogenized in phosphate-buffered saline (PBS) at 4°C and centrifuged twice at 6,500 ×*g* for 10 min at 4°C. The supernatant was filtered through a 0.45-μm membrane filter. Virus samples were stored in aliquots at −80°C for WSSV titration and challenge.

In Vivo Titration

Fifty μl of different virus dilutions as shown in Table 1 were injected intramuscularly in the second abdominal segment of the shrimp using a 29G needle [7]. PBS was injected to a negative control group. For each group, 12 shrimps were used. Mortality was recorded twice 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 vaccination.

Production of Recombinant Proteins VP19 and VP28

The recombinant plasmids pHCE VP19 and pHCE VP28 were obtained from a previous study [12]. Both VP19 and VP28 from the *E. coli* BL21 (DE3) transformed with pHCE VP19 and pHCE VP28 were produced by culturing at 37°C for 8 h in LB medium containing 100 μg/ml ampicillin, respectively.

For the harvest of recombinant protein, cultured bacterial cells were centrifuged at 6,000 ×*g* for 10 min at 4°C. Bacterial pellets were thoroughly resuspended in 1 ml of PBS and then centrifuged at 9,800 ×*g* for 4 min at 4°C. The harvested cell was disrupted by sonication in PBS buffer. The expression of VP19 and VP28 proteins was further confirmed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Most of the expressed proteins were solubilized from inclusion bodies. The protein concentration was determined using the Bradford assay (Bio-Rad).

Vaccination by Oral Feeding

Commercial pellets weighing approximately 36 g were mixed with 18 ml (200 μg/ml of protein) of recombinant proteins VP19 and VP28. The feed pellets were mixed with recombinant proteins and coated with 2.8 μl of Freund's Complete Adjuvant (FCA; Sigma, St. Louis, MO, USA) per gram of commercial pellet and incubated on ice to allow the absorption of suspension with FCA to prevent dispersion of the recombinant protein suspension in the water.

In the vaccination experiments, group of 12 shrimps were vaccinated by feeding feed pellets at 5% of body weight for 14 days, as indicated in Table 2. During the vaccination of the test groups, the commercial pellets without protein vaccine were fed to the positive and negative control groups. After the vaccination, the shrimps were challenged by the injection of specific WSSV dilution, except for the negative control that was mock infected.

Infected Organs Collection from Moribund Shrimps

L. vannamei was infected with WSSV for the titration of WSSV dosage and vaccination experiments. Infected organ samples of the moribund shrimps were collected through random sampling at specific day of post-infection. After sampling, the hemolymph was separated from the shrimp through cephalothoraxes using a 3-ml syringe with 23G needle. The shrimp organs including stomach, heart, hepatopancreas, and gut were collected. The collected hemolymph and organs were stored at −80°C until RNA extraction.

Total RNA Isolation and RT–PCR

Total RNA was extracted from collected shrimp organs at different infection stages using a FastPure RNA isolation kit (Takara, Shiga, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) was generated in a 20-μl reaction volume using PrimeScript reverse transcriptase (Takara, Shiga, Japan), and the reaction was conducted at 45°C for 40 min. The obtained cDNA was quantified at 260 and 280 nm using a UV–Vis spectrophotometer. Diluted cDNA (200 ng/μl) was used as the template for the PCR

Table 2. Set-up for vaccination experiments by oral feeding.

Group	Feed composition	Challenge	Shrimp samples[a]
Negative control	Commercial feed	PBS (pH 7.4)	12×3
Positive control	Commercial feed	WSSV stock:PBS=1:200	12×3
FCA control	Commercial feed+FCA	WSSV stock:PBS=1:200	12×3
Host control	Commercial feed+*E. coli* BL21 protein+FCA	WSSV stock:PBS=1:200	12×3
rVP19	Commercial feed+rVP19 protein+FCA	WSSV stock:PBS=1:200	12×3
rVP28	Commercial feed+rVP28 protein+FCA	WSSV stock:PBS=1:200	12×3

[a]The number of shrimps of each group was 12 and experiments were repeated 3 times under the same conditions.

Table 3. Oligonucleotide primer sequences of VP19, 28, 26, and β-actin of WSSV for RT–PCR.

Genomic region	Primer	Oligonucleotide sequence (5' to 3' direction)	Location	Expected size of fragment
VP19	Forword Reverse	CGGAATTCATGGCCACCACGAC GGGATCCCTGCCTCCTCTTG	Envelope	365 bp
VP28	Forword Reverse	CGGGATCCATGGATCTTTATTTCAC ACGCGTCGACTTACTCGGTCTCAGTG	Envelope	614 bp
VP26	Forword Reverse	CGGGATCCATGGATCTTTATTTCAC ACGCGTCGACTTACTCGGTCTCAGTG	Tegument	614 bp
β-actin	Forword Reverse	ATGGARAARATYTGGCAYCAYACHTTYTACAARAT CCACATCTGYTGGAAKGT	Control	892 bp

reaction. The oligonucleotide primer sequences listed in Table 3 were designed based on published *L. vannamei* cDNA sequences [3, 8, 12]. PCR amplification was carried out in a 20-μl reaction mixture containing the cDNA of WSSV-infected shrimp organs. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing EtBr (0.5 μg/ml) in TAE buffer and visulized with a UV transilluminator.

RESULTS

Virus Titration

For the determination of dilution ratio with 100% mortality of shrimps in the desired period of experiments, *in vivo* virus titration was performed. The virus stock was diluted from 1×10^2 to 1×10^3 times in PBS, as indicated Fig. 1. All shrimps survived in the negative control, whereas the mortality due to virus infection occurred in all groups with virus dilutions. As shown in Fig. 1, the administration of virus dilution of 1×10^2 to the shrimp showed 100% mortality at less than 6 days post-injection. However, virus dilution of 2×10^2 and 1×10^3 showed final mortalities of 90% and 50% at 10 days post-injection. Therefore, the dilution of 2×10^2 was chosen as the virus dose for further experiments because this dilution could give the optimal response for the vaccination in terms of mortality reduction.

Vaccination of Shrimp Using Recombinant Viral Proteins

Vaccinations of shrimps were carried out using rVP19 and rVP28 as protein vaccines by oral delivery. rVP28, rVP19, FCA control, host control, and positive control groups were challenged by the injection of 2×10^2 WSSV dilutions. The negative control was mock challenged using PBS. During the challenge, shrimps were fed with commercial feed pellets. As shown in Fig. 2, the positive and FCA control groups showed 100% cumulative mortalities at 10 days after the challenge. The host control group showed 100% mortality at 7 days later than that of positive and FCA control groups owing to the adjuvant effect of bacterial host cells. The cumulative mortalities at 21 days after the challenge with WSSV were determined and groups vaccinated with rVP19 and rVP28 showed cumulative moralities of 66.7% and 41.7%, respectively. The cumulative

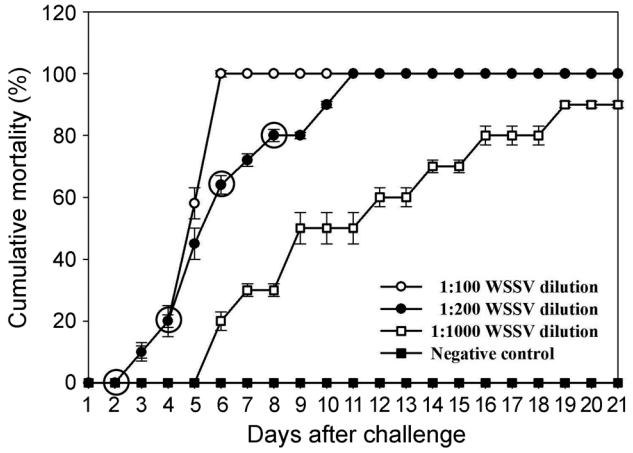

Fig. 1. WSSV titrations in *Litopenaeus vannamei* by injection.
Fifty μl of 1×10^2, 2×10^2, and 1×10^3 diluted WSSV stock was injected intramuscularly to *L. vannamei.* Circle points indicate the sampling dates.

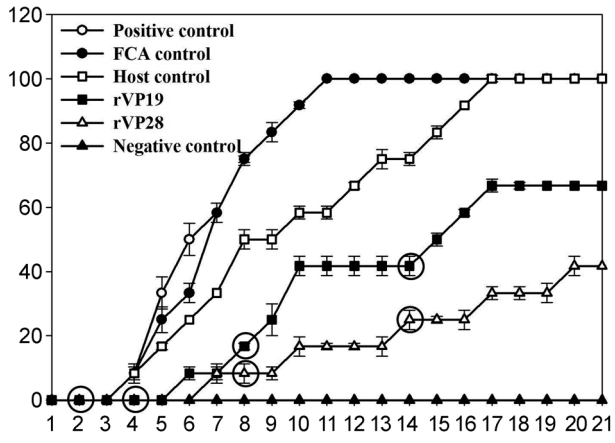

Fig. 2. Cumulative mortalities of *L. vannamei* for the determination of the efficacies of rVP19 and rVP28 as protein vaccines.
Recombinant WSSV structural proteins rVP19 and rVP28 were vaccinated by oral feeding and challenged with WSSV on 14 days after the vaccination. Circle points Marks indicate the sampling dates.

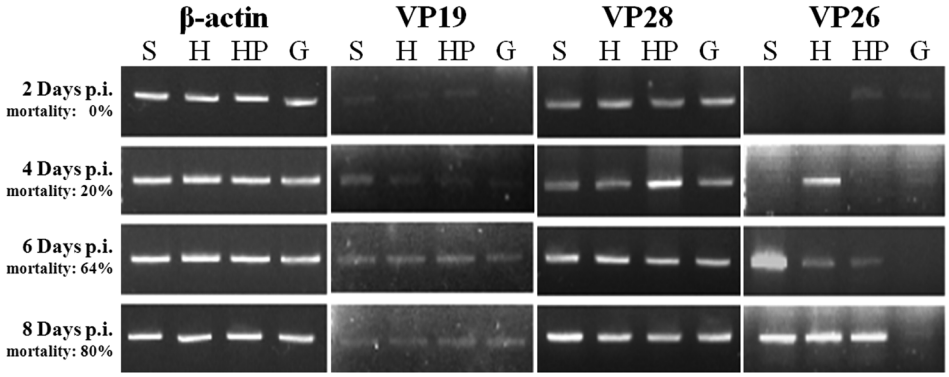

Fig. 3. RT–PCR for mRNA extracted from infected shrimp organs from the titration of WSSV (S: Stomach; H: Heart; HP: Hepatopancreas; G: Gut).
Transcriptions of the envelope proteins (VP19 and VP28) having a transmembrane domain were detected earlier than other viral proteins.

mortalities for vaccinated groups were significantly lower as compared with control groups. rVP28 showed a better protective efficacy against WSSV in shrimps than did rVP19.

RT–PCR Analysis

The RT–PCR of the transcripts from organs of WSSV-infected shrimps showed a difference in the quantity of mRNA transcriptional level. As shown in Fig. 3, mRNA of β-actin from the host was included as an internal control for RNA expression and was detected at a similar level in every sample. Transcripts of VP19 were detected in every sample at low transcriptional levels after WSSV infection compared with those of VP28. Transcripts of VP28 were detected in higher transcriptional levels than those of other viral proteins. After the transcription of VP19 and VP28, transcripts of VP26 were detected in the heart and then observed in the stomach and hepatopancreas of shrimps.

The transcriptional pattern of viral genes from organs of vaccinated shrimps with recombinant viral proteins of rVP19 and rVP28 showed a similar trend to that of unvaccinated shrimps, as shown in Fig. 4. In the case of vaccinated shrimps with rVP19 (Fig. 4A), the results clearly showed that the transcription of tegument protein VP26 was delayed 4 days compared with that of unvaccinated shrimps. The transcription of VP26 for vaccinated shrimp with rVP28 (Fig. 4B) was only detected in the heart at 14 days post-challenge, and no transcripts were detected in the other organ samples. These results indicated that the vaccination of shrimp with rVP28 delayed the transcription of tegument protein VP26 about 10 days compared with that of unvaccinated shrimps.

DISCUSSION

The feasibility of oral vaccination using the prokaryotic system to express and purify recombinant VP28 has been tested, because VP28 protein is not glycosylated [19] and the system is commercially established [2, 12]. Significant protection against WSSV has been achieved using VP28 expressed from *E. coli* [12, 22].

Vaccination by recombinant viral proteins significantly increased the survival ratio of shrimps compared with that of control groups. rVP28 showed a better protective effect against WSSV in shrimps than did rVP19, because the level of VP28 transcription was higher than VP19 and other structural proteins in the WSSV envelope [26]. These results were confirmed by RT–PCR (Fig. 3 and 4). The transcriptions of envelope proteins such as VP19 and VP28 initiated earlier than that of tegument protein such as VP26, as shown in the RT–PCR in Fig. 3 from WSSV-infected shrimps.

VP19 and VP28 of WSSV are major structural proteins that play key roles for virus attachment and penetration into host cell in the life cycle of the virus [27]. These structural proteins form a multiprotein complex with other viral proteins such as tegument protein VP26, nucleocapsid protein VP24, and envelope protein VP51A for virus assembly and penetration into host cell [25, 27]. In this study, VP26 acts as the linkage protein between structural proteins [3]. Linkage by VP26 also agreed with a previous study mentioning the essential complex formation between VP26 and VP28 acting as an anchor on the envelope membrane through N-terminal transmembrane regions in virus propagation [15].

Shrimp, like other invertebrates, does not produce antibodies and relies on the innate immune system to recognize and react to pathogens or environmental antigens. However, protective immune responses in the shrimp induced by DNA vaccines against intramuscularly infected white spot syndrome virus have been reported in recent papers [5, 13]. The studies indicated that shrimp might have their own protective immune system [21]. Concerning immune defense, crustaceans are able to recognize self from non-self by means of defense processes involving cellular

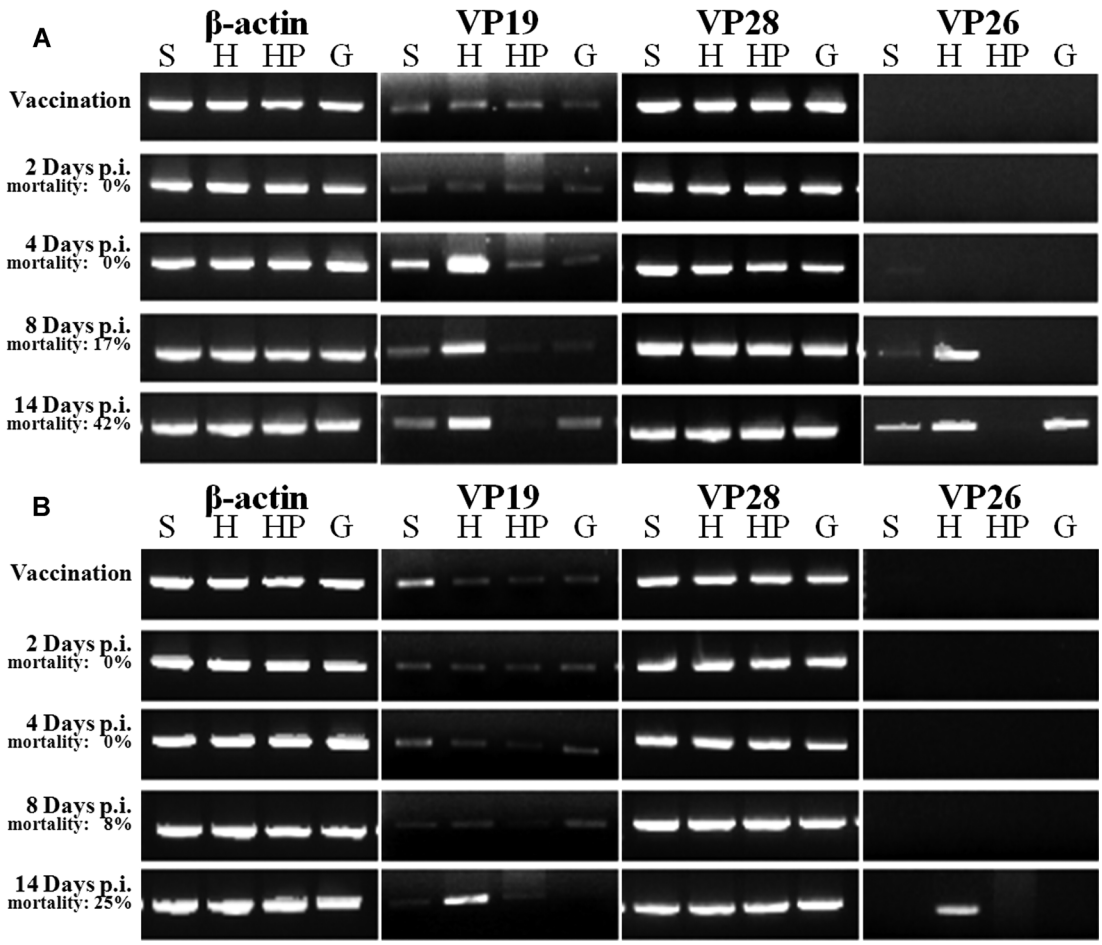

Fig. 4. RT–PCR analysis of viral gene transcription in vaccinated shrimps with recombinant viral proteins of rVP19 and rVP28 as protein vaccines.
(S: Stomach; H: Heart; HP: Hepatopancreas; G: Gut). (**A**) The vaccinated shrimp with rVP19 delayed gene transcription about 4 days comparing to that of unvaccinated shrimps. (**B**) The vaccination of shrimp with rVP28 delayed viral gene transcription about 10 days comparing to that of unvaccinated shrimps. However, the general mRNA transcriptional pattern of vaccinated shrimps showed similar trend to those of unvaccinated shrimps.

responses [14]. In recent reports, some invertebrates such as *Penaeus monodon* and *Penaeus japonicus* have shown some level of a memory response against bacteria [1].

Through the comparison of mRNA transcriptional levels of genes in various organs by WSSV infection to shrimp, it was confirmed that the vaccination to shrimp delayed viral gene transcription for 4~10 days compared with those of unvaccinated shrimp. The protection by vaccination with recombinant viral proteins could be obtained by the prevention of entry of WSSV due to the shrimp immune system being activated by recombinant protein vaccines. Moreover, these results could explain the decrease of cumulative mortalities by the vaccination with recombinant viral proteins.

RT–PCR analysis from infected shrimps indicated that VP28 was not suitable for the analysis of infection stage owing to the early and high level of transcription in every shrimp organ sample. However, VP26 could be a useful marker of infection stage owing to the infection stage-related RT-PCR transcriptional pattern. RT–PCR of VP26 could provide information on the shrimp mortality and virus propagation.

The results suggested that the immune system of shrimps stimulated by recombinant proteins was caused by the blocking of host cell receptors for envelope proteins of WSSV, thus preventing viral infection and confirming the efficacy of VP28 protein as a protective antigen in shrimp [2, 6, 22, 23].

In conclusion, the shrimp immune system was able to recognize WSSV structural proteins, and thus the vaccination of shrimp against WSSV could be made possible by the oral feeding of the protein vaccines rVP19 and rVP28.

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