Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine

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**Abstract**

Although invertebrates lack a true adaptive immune response, the potential to vaccinate *Penaeus monodon* shrimp against white spot syndrome virus (WSSV) using the WSSV envelope proteins VP19 and VP28 was evaluated. Both structural WSSV proteins were N-terminally fused to the maltose binding protein (MBP) and purified after expression in bacteria. Shrimp were vaccinated by intramuscular injection of the purified WSSV proteins and challenged 2 and 25 days after vaccination to assess the onset and duration of protection. As controls, purified MBP- and mock-vaccinated shrimp were included. VP19-vaccinated shrimp showed a significantly better survival (p < 0.05) as compared to the MBP-vaccinated control shrimp with a relative percent survival (RPS) of 33% and 57% at 2 and 25 days after vaccination, respectively. Also, the groups vaccinated with VP28 and a mixture of VP19 and VP28 showed a significantly better survival when challenged two days after vaccination (RPS of 44% and 33%, respectively), but not after 25 days. These results show that protection can be generated in shrimp against WSSV using its structural proteins as a subunit vaccine. This suggests that the shrimp immune system is able to specifically recognize and react to proteins. This study further shows that vaccination of shrimp may be possible despite the absence of a true adaptive immune system, opening the way to new strategies to control viral diseases in shrimp and other crustaceans.

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**Keywords:** White spot syndrome virus; VP28; VP19; Vaccination; Structural proteins; Penaeus monodon

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1. **Introduction**

Within years after its first discovery in Asia in the early 1990s, white spot syndrome (WSS) has developed into an epizootic disease. Currently, WSSV, the causative virus of the disease, is found in most shrimp farming areas of the world, where it causes large economic losses to the shrimp farming industry. Besides the economic impact of the disease, the natural marine ecology is also threatened as WSSV is able to infect a large number of crustaceans including crabs and crayfish [1,2].

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WSSV virions are ovoid-to-bacilliform in shape and have a tail-like appendage at one end. The virions can be found throughout the body of infected animals, infecting most tissues and circulating ubiquitously in the hemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance. The WSSV virion consists of five major and about 13 minor proteins \([3-6]\). Sequencing of the WSSV genome revealed a circular sequence of 292,967 base pairs (bp) \([7]\), but there is variation in size in geographic isolates of WSSV \([8]\).

Due to the current intensity of aquaculture practices and the broad host range of WSSV, novel control strategies including vaccination against this virus would be highly desirable. However, invertebrates lack a true adaptive immune response system and seem to rely on various innate immune responses \([9]\). Although considered less sophisticated, this innate immune system is able to rapidly and efficiently recognize and destroy non-self material, including pathogens \([10]\). The innate immune response consists of cellular and humoral responses. Hemocytes are responsible for most of the cellular responses, including encapsulation, phagocytosis, melanization, cytotoxicity, cell-to-cell communication, clotting, and the proPO activating system. Humoral response factors originate from granulocytes and include lectins, defensive enzymes, reactive oxygen intermediates and the synthesis of a wide array of antimicrobial peptides \([9-12]\). Immunostimulation of shrimp upon contact with products of microbial origin has already been demonstrated \([13,14]\). Even effective vaccination of \textit{Penaeus monodon} and \textit{Penaeus japonicus} using inactivated \textit{Vibrio} spp. has been reported by several researchers \([15-17]\).

In contrast to the well-studied effect of microbial immunostimulants on the immune system, there is limited information about the immune response to viral infections. Pan et al. \([18]\) tested tissue extracts from crab, shrimp and crayfish against a variety of viruses for the presence of viral inhibitors. The authors found a 440 kilodalton (kDa) molecule that was able to non-specifically inhibit infection of six types of both RNA and DNA viruses. Furthermore, an upregulation of the lipopolysaccharide and \(\beta\)-1,3-glucan binding protein gene was observed upon infection with WSSV \([19]\). This gene is known to be involved in the proPO cascade, which is upregulated in bacterial and fungal infections. Also, upregulation of protease inhibitors, apoptotic peptides and tumor-related proteins has been observed upon WSSV infection \([20]\). In vivo experiments with \textit{P. japonicus} demonstrated the presence of a quasi-immune response after re-challenging survivors of both natural and experimental infection with WSSV \([21]\). After this re-challenge, the observed mortality of the initial survivors was lower compared to challenged naïve shrimp. Wu et al. \([22]\) observed the presence of WSSV-neutralizing activity of plasma of infected shrimp from 20 days up to well over two months after infection. These results suggest the induction of antiviral responses and suggest that vaccination of shrimp against WSSV may be possible.

To test this hypothesis we have used structural WSSV proteins to vaccinate shrimp. The envelope proteins VP19 and VP28 were selected, as both proteins are likely to be the first to come into contact with the host cells and because the envelope protein VP28 was shown to be involved in the systemic infection of shrimp \([23]\). VP19 and VP28 were fused to the maltose binding protein (MBP) and used, after purification, to vaccinate shrimp by intramuscular injection. The shrimp were challenged with WSSV by the same technique and showed a higher survival when vaccinated with the WSSV proteins.

2. Materials and methods

2.1. Shrimp culture

Healthy \textit{P. monodon} shrimp were imported as post-larvae from Malaysia and maintained in a recirculation system at the facility “De Haar” at Wageningen University. Prior to each experiment, shrimp were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-l aquaria, each fitted with an individual filter system (Eheim, Germany),
heating (Schego, Germany) at 28±1 °C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand.

2.2. WSSV virus stock

The virus isolate used in this study originated from infected *P. monodon* shrimp imported from Thailand in 1996 and was obtained as described before [23]. Crayfish *Orconectes limosus* were injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle (Microfine B&D). After approximately one week, virus was isolated from freshly extracted hemolymph as previously described [23]. Virus samples were examined under a transmission electron microscope for purity and stored in aliquots at −80 °C until further use.

2.3. In vivo titration

Since no crustacean cell lines are available, the WSSV stock was titered by in vivo infection experiments as previously described [23]. In short, shrimp of approximately 1 g were injected intramuscularly with 10 μl of different virus dilutions in 330 mM NaCl in the fourth or fifth abdominal segment of the shrimp using a 29-gauge needle. The mortality was recorded twice a day and dead shrimp 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 experiments (≈ 70%).

2.4. PCR analysis for WSSV

Muscle tissue from the tails of dead shrimp was homogenized and mixed with 200 μl 5% Chelex 100 resin (BioRad) and 16 μl 20 mg/ml proteinase-K. This mixture was incubated overnight at 56 °C followed by 10 min at 95 °C to inactivate the proteinase-K. The samples were tested with two primer pairs. A 16S rRNA primer pair (16S-FW1 5′-GTG CGA AGG TAG CAT AAT C-3′; 16S-RV1 5′-CTG CTG CAA CAT AAG GAT AC-3′), amplifying a 414 bp fragment of ribosomal shrimp DNA, was used as an isolation control. A VP26 primer pair (VP26-FW1 5′-ATG GAA TTT GGC AAC CTA ACA AAC CTG-3′; VP26-RV1 5′-GGG CTG TGA CGG TAG AGA TGA C-3′), amplifying part of the WSSV VP26 gene [4], was used to screen for WSSV-positive animals.

2.5. Production of recombinant proteins

For bacterial expression, the VP19 ORF was cloned in the pMAL-c2 vector (New England Biolabs) resulting in an N-terminal fusion of VP19 and the maltose binding protein (MBP). The DNA fragment encoding the entire VP19 ORF (WSSV ORF182) was amplified from genomic WSSV DNA by PCR. Using the forward primer VP19-FW1 (3′-CGG GAT CCA TGG CCA CCA CGA CTA A-5′) and reverse primer VP19-R1 (3′-GCC TGC AGC CTG ATG TTG TGT TTC TAT TAT A-5′), a BamHI and a PstI restriction site, respectively, were introduced. The amplified PCR product was ligated in the pGEM-T vector (Promega) and sequenced. The VP19 fragment was removed from the pGEM-T plasmid using the restriction enzymes BamHI and PstI, and after purification from gel (concert nucleic-acid purification system, GIBCOBRL) it was ligated into the pMAL-c2 vector. For VP28, the complete VP28 ORF was cloned in the pMAL-c2 vector, resulting in the N-terminal fusion of VP28 and the maltose binding protein. Using the forward primer VP28-FW1 (3′-CAG AAT TCA TGG ATC TTT TCT TCA C-5′) and reverse primer VP28-RV1 (3′-CAG GAT CCT TAC TCG GTC TCA GTG C-5′), an EcoRI and a BamHI restriction site, respectively, were introduced. After PCR and cloning into the pGEM-T vector, the VP28 insert was removed using the introduced restriction sites and ligated in the pGBK7 vector (Clontech). The insert was
again removed using the restriction enzymes EcoRI and PstI and ligated in the pMAL-c2 vector. Finally, all constructs were electroporated into Escherichia coli DH5α cells. Both the MBP–VP19 and MBP–VP28 proteins and the non-fused MBP protein were overexpressed and purified according to the manufacturer’s instructions by affinity chromatography using amylose resin (New England Biolabs). The resulting E. coli expression samples and the purified proteins were analysed by SDS-PAGE and Western analysis; the protein concentration was determined using the Bradford assay (Bio-Rad).

2.6. Vaccination experiments

For the vaccination experiments, shrimp of approximately 1 g were injected with 4 μg of purified protein in 330 mM NaCl at a final volume of 10 μl. The shrimp of the group vaccinated with a mixture of the two proteins received 2 μg of MBP–VP19 and 2 μg of MBP–VP28 protein (Table 1). Five days after the initial vaccination, the shrimp were boostered by injecting the same amount of protein (Fig. 1). During the vaccination of the groups, the positive and negative control groups were injected with 10 μl of 330 mM NaCl. Two days after the booster, half of the shrimp per group were challenged by injection of a specific WSSV dilution, except for the negative control shrimp that received 10 μl of NaCl. The challenged shrimp were maintained in individual cages to prevent horizontal transmission of WSSV. The other half of the shrimp were challenged in the same way 25 days after the last vaccination and also placed in individual cages.

2.7. Statistical analysis

Statistical analysis of the survival rates among the groups was performed using the χ² test at a 5% confidence level. The protection against WSSV after vaccination was calculated as the relative percent survival (RPS) \((\frac{1}{\text{vaccinated group mortality}} - \frac{1}{\text{control group mortality}}) \times 100\) [24].

3. Results

3.1. Protein production and purification

The WSSV ORFs encoding VP19 and VP28 were overexpressed as MBP fusion proteins in E. coli. Bands corresponding to the two fusion proteins were observed at the expected height (Fig. 2, lanes 1 and 3). The WSSV origin of the bands was confirmed by Western analysis using anti-WSSV polyclonal antiserum (data not shown). Non-fused MBP was overexpressed as a control protein according to the same protocol (Fig. 2, lane 5). The VP19-MBP and VP28-MBP fusion and non-fused MBP proteins were purified by affinity

<p>| Table 1 |
| Names of the groups used in the experiment (first column); the proteins present in the vaccine administered (second column); and the number of shrimp used in the experiments (last column) |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine</th>
<th># Shrimp</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>4 μg MBP</td>
<td>2 × 12</td>
</tr>
<tr>
<td>VP19</td>
<td>4 μg MBP–VP19</td>
<td>2 × 12</td>
</tr>
<tr>
<td>VP28</td>
<td>4 μg MBP–VP28</td>
<td>2 × 12</td>
</tr>
<tr>
<td>MIX</td>
<td>2 μg MBP–VP19 + 2 μg MBP–VP28</td>
<td>2 × 12</td>
</tr>
<tr>
<td>Positive control (C+)</td>
<td>—</td>
<td>2 × 10</td>
</tr>
<tr>
<td>Negative control (C−)</td>
<td>—</td>
<td>2 × 10</td>
</tr>
</tbody>
</table>
purification using amylose resin (Fig. 2, lanes 2, 4 and 6). The concentration of the purified proteins was determined using the Bradford assay kit.

3.2. Vaccination experiment

Vaccination was performed using the WSSV envelope proteins VP28, VP19 or a mixture of both proteins. Six experimental groups were set up; four groups of 24 individuals receiving a protein vaccination and two control groups of 20 individuals each receiving a mock-vaccination treatment (Table 1). The four groups of 24 shrimp received two injections of 4 mg of purified proteins with a five-day interval; the positive and negative control groups received two injections, also with a five-day interval, of 330 mM NaCl. Half of the shrimp of each group were challenged two days after the second protein injection. The other half was challenged 25 days after the second protein injection, to test the duration of the WSSV protection. All groups were challenged with a titered WSSV stock, except for the negative control shrimp, which were

Fig. 1. Vaccination and challenge scheme of the experiment. Shrimp are kept in groups and receive vaccinations on days 0 and 5. Two days after the second vaccination, half of the shrimp per experimental group are challenged, placed in individual cages and observed. The remaining half of the shrimp are kept together for another 25 days after the second vaccination, challenged, placed in individual cages and observed.

Fig. 2. Coomassie stained SDS-PAGE gel of MBP–VP19, MBP–VP28 and MBP expressions in E. coli cells. M: Protein Molecular mass marker; Lane 1: total MBP–VP19 expression; Lane 2: purified MBP–VP19; Lane 3: total MBP–VP28 expression; Lane 4: purified MBP–VP28; Lane 5: total MBP expression; Lane 6: purified MBP. Numbers on the left indicate molecular mass (kDa) of the Protein Molecular mass marker.
injected with 330 mM NaCl. After the challenge, the shrimp were kept in individual cages and checked for mortality twice a day.

The resulting time–mortality relationship of the shrimp challenged two days after protein injection is shown in Fig. 3A. The challenge pressure resulted in a final cumulative mortality of 90% for the positive control. The MBP control group showed a final cumulative mortality of 75%, which is significantly lower than that of the positive control ($p < 0.05$). The three groups receiving MBP–VP19, MBP–VP28 or both,

![Graph A](image1)

![Graph B](image2)

Fig. 3. Time–mortality relationship of vaccinated shrimp, challenged two days (A) and 25 days (B) after the last vaccination. Cumulative mortality rates of shrimp from the experimental groups as indicated in Table 1 are plotted against the days after challenge ($n = 12$ vaccinates, $n = 10$ controls).
all showed a final cumulative mortality of approximately 45%. This is significantly different from both the positive control and the MBP group. The RPS values for the MBP–VP19, MBP–VP28 and MIX groups, compared to the positive control and non-fused MBP groups, are shown in Table 2. Vaccination with MBP–VP28 gives the highest RPS value and also shows a low initial mortality compared to the other groups. Diseased shrimp were invariably tested positive for the presence of WSSV by PCR. Randomly selected survivors from every group were taken on day 16 and all tested negative for WSSV.

The challenge at 25 days after the last vaccination was carried out in a manner similar to the first challenge after two days. The resulting time–mortality relationships of this experiment can be found in Fig. 3B. Although the applied challenge pressure was identical to the first challenge, the positive control showed a final cumulative mortality of 60%. The non-fused MBP group showed a final mortality of 65%, followed by MBP–VP28 with 55%, MIX with 40% and MBP–VP19 with 30%. The χ² test only showed a significant difference in survival rates (p < 0.05) between the MBP–VP19 and the control groups (positive control and non-fused MBP group). The RPS values for the MBP–VP19, MBP–VP28 and MIX groups, compared to the positive control and non-fused MBP groups, are shown in Table 2. Randomly selected survivors from every group were taken at day 16 and all tested negative for WSSV.

4. Discussion

Virus research with WSSV has shown the existence of a quasi-immune response as survivors of a WSSV infection showed an increased survival compared to naïve shrimp after a re-challenge [21,22]. In this study we have performed experiments to gain more insight into the proteins responsible for this observed quasi-immune response. Shrimp were vaccinated using the WSSV envelope proteins VP19 and VP28 fused to MBP, and non-fused MBP was included as a control. As the general assumption is that shrimp do not have an adaptive immune response, one challenge was performed shortly after the last vaccination (two days) to ensure that short-term effects of injection with WSSV proteins would not be missed. As Wu et al. [22] have shown that shrimp become resistant to WSSV between three and four weeks post-initial exposure, a second challenge was performed 25 days after the last vaccination to test the duration of the induced protection. Vaccination was performed via intramuscular injection to ensure the application of a consistent amount of protein per shrimp. Even though this technique is far from practical under shrimp farming conditions, it is very suitable in determining the vaccinating potential of proteins. The amount of injected proteins was based on previous unpublished experiments. During the entire period before the challenge, the shrimp were kept together in a large aquarium. After the challenge, the shrimp were placed in individual cages to prevent horizontal transmission by cannibalism [25], as this increases the applied challenge pressure on the shrimp and affects the reproducibility of the results.

Both the challenges 2 and 25 days after vaccination resulted in a significantly higher survival when shrimp were vaccinated with the MBP–VP19 fusion protein as compared to shrimp injected with MBP alone (RPS of 33% and 57%, respectively). A significant difference was also found between the groups

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Positive control</th>
<th>MBP control</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>VP19</td>
<td>44</td>
<td>52</td>
</tr>
<tr>
<td>VP28</td>
<td>53</td>
<td>13</td>
</tr>
<tr>
<td>MIX</td>
<td>44</td>
<td>34</td>
</tr>
</tbody>
</table>
vaccinated with either MBP–VP28 or MIX and the MBP group when challenged two days after the last vaccination. However, this effect was reduced at 25 days after vaccination and the $\chi^2$ test showed that the effect was no longer significant. The experiments also show that injection of shrimp with non-fused MBP results in a small positive effect on shrimp survival when challenged two days after the booster. This suggests that besides the generation of a more specific immune response (long term protection provided by injection of virus specific proteins like VP19), a small general immune response can be provoked by injection of a foreign protein like MBP. This immune response may also have long term effects by enhancing the response to WSSV.

These data show that vaccination of shrimp with MBP–VP19 has a positive effect on shrimp survival after challenge with WSSV up to 25 days after vaccination. Most interestingly, the experiments show that the effect is VP19-specific and therefore suggests that the shrimp immune system is capable of specifically recognizing foreign protein subunits. Using the developed vaccination and challenge set-up, experiments aimed at locating the VP19 domain(s) responsible for this immune response could be performed. Furthermore, the required amount of proteins necessary for eliciting the observed immune response can be optimised using this experimental set-up.

Although the amount of virus injected in both challenges was equal, the second challenge showed generally lower mortality rates compared to the first challenge. This might be due to an increase in mean body weight during the 23 extra days before challenge compared to the first challenge. An interesting question to be answered is whether the observed effect is WSSV-specific or whether it is based on a more broad antiviral activity, possibly showing cross immunity to other shrimp viruses. Because vaccination via injection is neither a viable nor a practical strategy under current shrimp farming conditions, oral vaccination experiments should be performed to see if the same vaccinating potential as observed in this study could be found.

This study is the first to show that the shrimp immune system is able to recognize WSSV structural proteins and that vaccination of shrimp against WSSV might be possible. It opens the way to the design of new strategies to control WSSV and other invertebrate pathogens.

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