Vaccination of *Penaeus monodon* Against White Spot Syndrome Virus Using Structural Virion Proteins

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ABSTRACT

White spot syndrome virus is currently responsible for significant health problems in shrimp culture and intervention strategies are being seriously sought. Vaccination of shrimp against this disease could be a viable option. However, as very little is known about the shrimp's immune response to viral infections, the potential of shrimp vaccination is uncertain. In this study we performed vaccinations of shrimp using two major structural WSSV proteins, VP19 and VP28, both present in the virion envelope. Recombinant HIS-VP28 and MBP-VP19 fusion proteins were purified and injected into shrimp that were subsequently challenged with WSSV by injection. Results showed that injection with MBP-VP19 or a mixture of MBP-VP19 and HIS-VP28 significantly slowed or reduced mortality caused by WSSV, suggesting a specific role of VP19 in the systemic shrimp defense response. Furthermore, these results also demonstrate that shrimp can specifically recognize proteins and provoke an immune response, opening the way for vaccination against viruses.

INTRODUCTION

Since the discovery of White spot syndrome virus (WSSV) in Asia in 1991/1992, the virus has quickly spread to most shrimp farming areas of the world (Cai *et al*., 1995). Helped by inadequate sanitation and worldwide trade, WSSV has quickly developed into an epizootic disease, causing large economic losses to the shrimp farming industry (Rosenberry, 2002). Besides the economic impact of this disease the natural marine ecology is also threatened, since WSSV is able to infect a large number of crustaceans (Lo *et al*., 1996; Wang *et al*., 1998).

WSSV virions are ovoid-to-bacilliform in shape and have a tail-like appendage at one end. The virions can be found throughout the shrimp body, infecting most tissues and circulating ubiquitously in the haemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance (Loh *et al*., 1997). The virion consists of five major and about thirteen minor proteins (van Hulten *et al*., 2000a, 2000b, 2002; Huang *et al*., 2002).

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Two of the major structural proteins, VP28 and VP19, are located in the envelope and three in the nucleocapsid (VP26, VP24 and VP15) (van Hulten *et al*., 2002). Sequencing of the viral genome revealed a circular sequence of about 300 kb (van Hulten *et al*., 2001a; Yang *et al*., 2001).

Due to current aquaculture practices and the broad host range of WSSV, intervention strategies including vaccination against this virus would be pivotal to save and protect shrimp farming. However, in contrast to the well-studied effects of microbial immunostimulants on the crustacean immune system (Lee and Söderhall, 2002), information on the immune response to viral infections is limited. In the few studies performed so far, antiviral substances were found to be present in tissue extracts of crustaceans, non-specifically inhibiting different viruses (Pan *et al*., 2000). Also upon infection with WSSV, an upregulation of the lipopolysaccharide and (β1,3-glucan binding protein gene (LGBP), known to be involved in the proPO cascade and upregulation of protease inhibitors, apoptotic peptides and tumour-related proteins, have been observed (Roux *et al*., 2002; Rojtinnakorn *et al*., 2002). *In vivo* experiments with *Penaeus japonicus* demonstrated the presence of a quasi-immune response when survivors of both natural and experimental WSSV infections were re-challenged with WSSV (Venegas *et al*., 2000). After re-challenge mortality of the initial survivors was lower than that of challenged naive shrimp. The prospects for shrimp vaccination against WSSV is best supported by the research performed by Wu *et al*. (2002), who found WSSV neutralizing activity in plasma of infected shrimp from 20 days until over two months after infection.

To better understand the mechanism underlying the observed quasi-immune responses and to approach the question whether this mechanism is WSSV specific, vaccination experiments were performed in *Penaeus monodon* shrimp using specific WSSV proteins. Previous research had shown that one of the WSSV structural proteins, VP28, was involved in the systemic infection of WSSV (van Hulten *et al*., 2001b). Since both VP28 and VP19 are associated with the virion envelope and involved in initial interaction with the host, these two structural proteins were used in the vaccination experiments. To have maximum control over the amount of proteins the shrimp are exposed to and to be able to use purified proteins, it was decided to vaccinate via injection. Although a number of potential defense and recognition lines are circumvented this way, it still proved a valuable method for testing the vaccination potential of proteins. To be assured of a constant and reproducible challenge pressure, the challenge was also performed by injection, even though this method also circumvents a number of defense or recognition lines.

MATERIALS AND METHODS

Shrimp culture

Healthy *P. monodon* were imported as post-larvae from Malaysia and maintained in a recirculation system at the aquaculture facility "De Haar" at Wageningen University. Each shipment was tested for the presence of WSSV, monodon baculovirus, yellow head virus, taura syndrome and infectious hypodermal and hematopoietic necrosis virus by PCR. Prior to each experiment, shrimp were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180-liter aquariums. Each

aquarium was fitted with an individual filter system (Eheim, Germany) containing pre-conditioned filter material, heating (Schego, Germany) to 28°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20 parts per thousand (ppt).

WSSV stock

The virus isolate used in this study originated from an infected *P. monodon* shrimp imported from Thailand in 1996 and was obtained as described before (van Hulten *et al*., 2001b). Crayfish *Orconectes limosus* was 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 haemolymph as described by van Hulten *et al*. (2001b). Virus samples were examined under the transmission electron microscope for integrity and purity, and stored in aliquots at -80°C until further use.

***In vivo* titration**

Since no crustacean cell lines are available, the WSSV stock was titered by *in vivo* infection experiments as described by van Hulten *et al*. (2001b). In short, shrimp of approximately one gram were injected intramuscularly with 10 µl of various virus dilutions in 330 mM NaCl (10^3-10^8 times diluted) in the 4^{th} or 5^{th} abdominal segment of the shrimp using a 29 gauge needle (Microfine B&D). After injection, the shrimp were maintained in individual housing to prevent horizontal transmission of WSSV by predation. Mortality was recorded twice a day and dead shrimp were tested for the presence of WSSV by PCR. The obtained time-mortality data were used to determine the desired challenge pressure of 70-90% final mortality for the vaccination experiments.

PCR analysis for WSSV

Muscle tissue retrieved from the tail of dead and surviving 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 minutes 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 a control to verify DNA integrity. 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 (van Hulten *et al*., 2000b), was used to screen for WSSV positive shrimp.

Expression of recombinant proteins

For bacterial expression of VP19, the entire 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, van Hulten *et al*., 2001a) 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-RpMAL (3'-GCC TGC AGC CTG ATG TTG TGT TTC TAT A-5') *Bam*HI and *Pst*I

restriction sites respectively, were introduced. The amplified PCR product was ligated into the pGEM-T easy vector (Promega) and sequenced. The VP19 fragment was removed from the pGEM-T easy plasmid and ligated into the pMAL-c2 vector and electroporated into *E. coli* DH5 cells for protein expression.

The full length ORF encoding the major WSSV envelope protein VP28 (WSSV ORF1, van Hulten *et al*., 2001a) was first expressed using the pET28a vector which fuses a $(HIS)_6$-tag to VP28 for detection and purification purposes. Expression levels were very low, probably due to the presence of a strong N-terminal hydrophobic region. Therefore, a new construct without the N-terminal hydrophobic region, was constructed and used for expression. The partial VP28 fragment was amplified from genomic WSSV DNA by PCR using the forward primer VP28PF (3'-AAG GAT CCC ACA ACA CTG TGA CCA AG-5') and reverse primer VP28PR (3'-TAG CGG CCG CAA AAG CAC GAT TTA TTT AC-5') which introduced *Bam*HI and *Not*I restriction sites respectively. This fragment was ligated into the pGEM-T vector and sequenced. After digestion with *Bam*HI and *Not*I, the fragment was ligated at the *Bam*HI and *Not*I site of the pET28a vector (Novagen). The pET28a-VP28 construct was electroporated into BL21 electrocompetent cells for protein production.

Protein production and purification

The MBP-VP19 fusion protein was purified by affinity chromatography using amylose resin (New England Biolab) and the HIS-VP28 fusion protein using TALON metal affinity resin (CLONTECH). The resulting *E. coli* expressions and the purified proteins were analysed by SDS-PAGE and Western-blot. Protein concentration was determined using the Bradford protein assay (Bio-Rad).

Vaccination experiments

For the vaccination experiments, shrimp of approximately one gram were injected intramuscularly in the 4^{th} or 5^{th} abdominal segment with 4 µg of purified protein diluted in 330 mM NaCl in 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 HIS-VP28 proteins. Five days after the initial vaccination, the shrimp were boosted by injecting the same amount of protein. The positive and negative control groups were injected with 10 µl of 330 mM NaCl in the same regime. Two days after the booster the shrimp were challenged by injection of a WSSV dilution aimed at 70-90% mortality in experiment I and 100% mortality in experiment II, except for the negative control shrimp that received 10 µl of 330 mM NaCl. After the challenge, the shrimp were maintained in individual housing to prevent cannibalism and horizontal transmission of WSSV. In experiment I, four experimental groups of 15 individuals each (Table 1) were injected with either VP19, VP28 or PBS for the positive and negative controls. In experiment II, the same groups as experiment I, but with the addition of a MIX group, injected with both VP19 and VP28 were used. After the challenge of both experiments, the shrimp were placed in individual cages in 180 liter aquaria with heating to 28°C and continuous aeration.

Table 1. Group names, types of vaccine administered and number of shrimp used in experiments I and II.

	Group name	Vaccine	Number
Experiment I	MBP-VP19	MBP-VP19	15
	HIS-VP28	HIS-VP28	15
	Positive control	-	15
	Negative control	-	15
Experiment II	MBP-VP19	MBP-VP19	15
	HIS-VP28	HIS-VP28	15
	MIX	MBP-VP19+HIS-VP28	15
	Positive control	-	15
	Negative control	-	15

Statistical analysis

Statistical analysis on the mortality curves was performed using the Kaplan-Meier survival analysis (Bull and Spiegelhalter, 1997). After analysis, the significance, hazard ratio and 95% confidence interval were obtained.

RESULTS

Protein production and purification

WSSV VP19 and VP28 ORFs were overexpressed in *E. coli* as MBP and $(HIS)_6$-tag fusion proteins, respectively. Bands corresponding to the two fusion proteins were observed at the expected positions (Figure 1, lanes 1 and 3). The viral origin of the bands was confirmed by Western blot analysis using an anti-WSSV polyclonal antiserum. After sonication and centrifugation the supernatant of both expressions was used for purification using affinity chromatography. MBP-VP19 was purified using amylose resin and yielded a highly enriched preparation of VP19-MBP fusion protein. Purification of HIS-VP28 using TALON metal affinity resin also resulted in a highly enriched preparation of purified HIS-VP28. (Figure 1, lanes 2 and 4). The final concentration of the purified proteins was determined using the Bradford assay.

Vaccination with VP19 and VP28 (experiment I)

Shrimp vaccinated with purified MBP-VP19 protein showed a significantly slower mortality and a reduced final mortality when compared to the positive control ($p < 0.05$) (Figure 2). By contrast, vaccination with HIS-VP28 gave no significant difference in mortality when compared with the positive control. The final mortality in the positive control reached about 60%, which was slightly below the percent anticipated from the dilution used. Random samples of surviving shrimp were tested for WSSV by PCR and found negative.

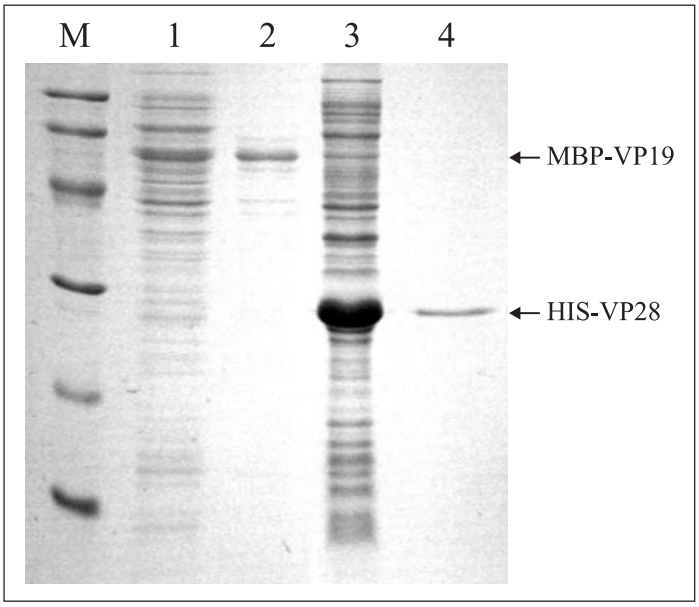

Figure 1. Coomassie stained SDS-PAGE gel of MBP-VP19 and HIS-VP28 expressions in *E. coli* cells. M: Low molecular weight marker; Lane 1: total MBP-VP19 expression; Lane 2: purified MBP-VP19; Lane 3: total HIS-VP28 expression; Lane 4: purified HIS-VP28. Numbers on left side indicate the size (in kDa.) of low molecular weight protein markers.

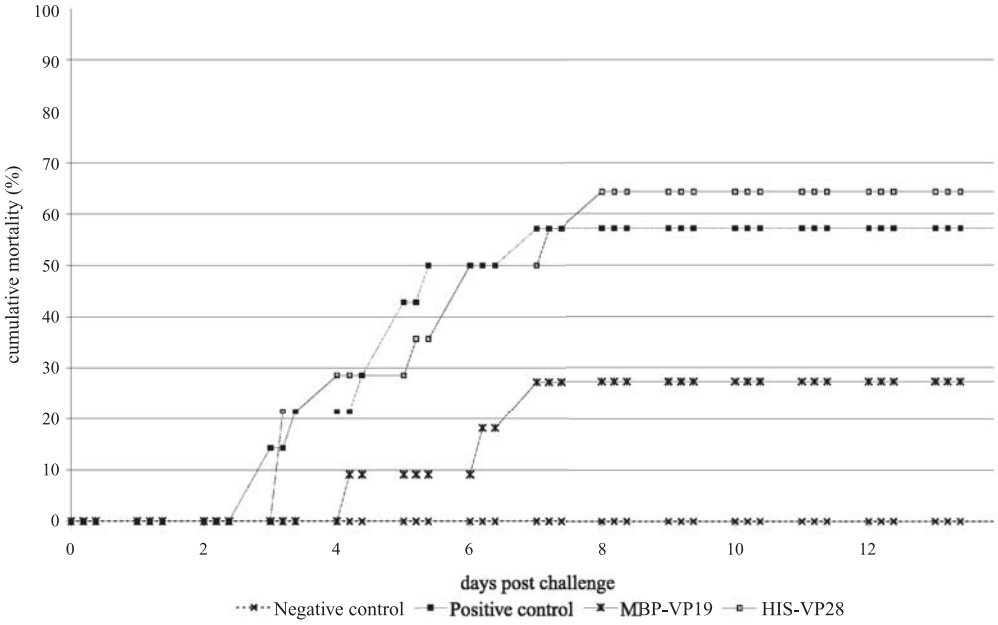

Figure 2. Time-mortality relationship of vaccination experiment I. Cumulative mortality rates of shrimp vaccinated with different proteins and challenged by injection of WSSV are plotted against days after challenge. Positive and negative controls are injected with NaCl prior to challenge with WSSV or NaCl, respectively, to exclude injection effects.

Vaccination with VP19, VP28 and a mixture of VP19 and VP28 (experiment II)

The increased challenge pressure in this experiment, gave 100% mortality in 5 days for the positive control group (Figure 3). All experimental groups, except the negative control group reached 100% mortality after 10 days. There was no significant difference between the positive control group and the groups injected with either MBP-VP19 ($p = 0.16$) or HIS-VP28 alone even though the rate of mortality for MBP-VP19 appeared reduced. However, when both MBP-VP19 and HIS-VP28 are administered, a significant delay in mortality was observed when compared to the positive control ($p < 0.02$). There was no significant difference between this group and the one treated with MBP-VP19 alone ($p = 0.44$). Random samples of surviving shrimp were tested for WSSV by PCR and found negative.

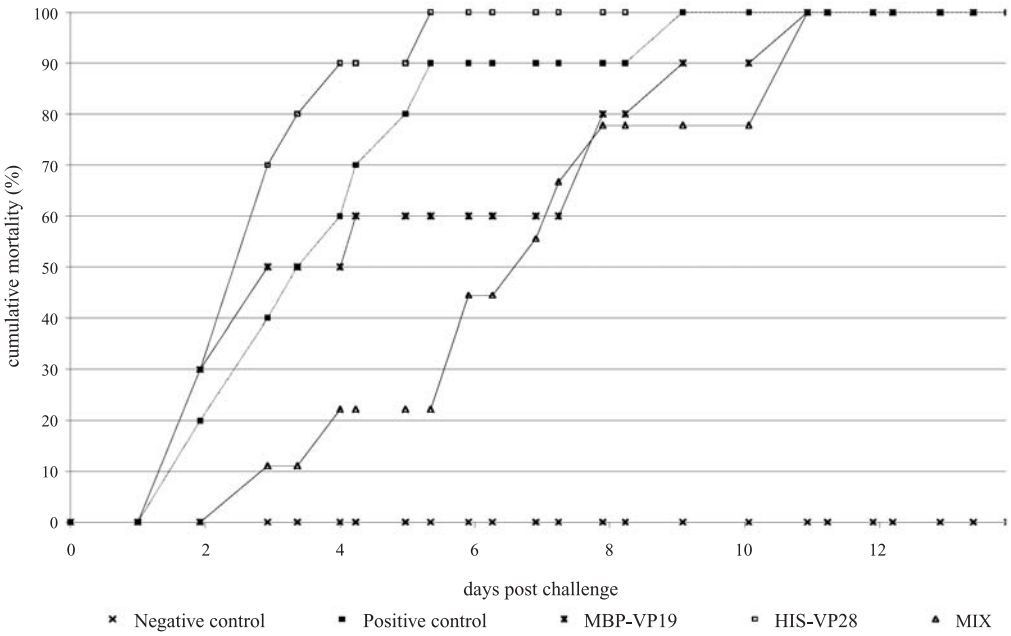

Figure 3. Time-mortality relationship of vaccination experiment II. Cumulative mortality rates of shrimp vaccinated with different proteins and challenged by injection of WSSV are plotted against days after challenge. Positive and negative controls are injected with NaCl prior to challenge with WSSV or NaCl, respectively, to exclude injection effects.

DISCUSSION

Invertebrates constitute ninety-five percent of all animal species and rely on defence mechanisms primarily based on a broad range of cellular innate immune responses. Because of the lack of a known adaptive immune response, the potential for vaccination against viral pathogens is uncertain. However, a few reports suggested the presence of such a response in crustaceans (Venegas *et al*., 2000; Wu *et al*., 2002) and this has opened up the possibility of vaccination as an intervention strategy to combat viral diseases in shrimp.

In this study we explored the possibility of shrimp vaccination using the two major WSSV envelope proteins, VP28 and VP19. These proteins are likely candidates to interact with the

host, triggering a potential defense response. Systemic infection of WSSV upon injection into shrimps could be neutralized with a VP28-specific antiserum (van Hulten *et al*., 2001). So it may be possible to use VP28 and VP19 as a subunit vaccine. VP28 and VP19 were successfully overexpressed in *E. coli*, purified and used as subunit vaccines.

Since vaccination with MB-VP19 and in particular a mixture of MBP-VP19 and HIS-VP28 were able to induce a delay or decrease in mortality upon WSSV challenge, it appears that MBP-VP19 is more important in the shrimp systemic immune response than VP28. It was somewhat surprising that VP28 on its own did not have an effect in view of earlier neutralization experiments (van Hulten *et al*., 2001b). It is possible that the HIS-VP28 construct is less stable than the MBP-VP19 construct or that MBP on its own may have a protective effect, however, preliminary experiments suggest the latter is not the case (J. Witteveldt, personal communication). Another explanation for its lack of effect may be the removal of its hydrophobic domain during the fusion protein construction. It is interesting that HIS-VP28 did not provoke a protective response on its own but did in consort with MBP-VP19. Possibly, VP19 and VP28 interact, as they may do in the WSSV virion, to provoke a more effective response.

It is not clear whether the partial protection we observed is the result of a genuine immune response of the shrimp or the consequence of a coverage of WSSV entry sites by MBP-VP19. As the time between the booster and challenge was only 2 days, the possibility of competition between receptor sites occupied by the injected structural proteins and WSSV virions cannot be excluded.

Venegas *et al*. (2000) and Wu *et al*. (2002) have demonstrated that shrimp, previously exposed to WSSV show antiviral activity in their haemolymph. The trigger needed to obtain this type of protection was, however, unknown. Here we have shown that vaccination with WSSV envelope proteins can induce a similar improved survival. A question still to be answered is whether the observed effect on the WSSV infection is WSSV-specific or whether it is based on a broader antiviral activity. This study further shows which viral proteins might be involved in this process and demonstrates that the shrimp immune system is able to specifically recognize WSSV structural proteins. Oral vaccination with VP28 and VP19 constructs should indicate whether this strategy is viable and practically feasible. If successful, it would open the way to new control strategies for WSSV and other pathogens.

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REFERENCES

Bull, K. and Spiegelhalter, D.J. 1997. Tutorial in biostatistics: survival analysis in observational studies. Statistical Medicine 16, 1041-1074.

Cai, S.L., Huang, J., Wang, C.M., Song, X.L., Sun, X., Yu, J., Zhang, Y. and Yang. C.H. 1995. Epidemiological studies on the explosive epidemic disease of prawn in 1993-1994. Journal China Fisheries 19, 112-117.

Huang, C., Zhang, X., Lin, Q., Xu, X., Hu, Z.H. and Hew, C.L. 2002. Proteomic analysis of shrimp white spot syndrome viral proteins and characterization of a novel envelope protein VP466. Molecular and Cellular Proteomics 1, 223-231.

Lee, S.O. and S_derhall, K. 2002. Early events in crustacean innate immunity. Fish and Shellfish Immunology 12, 421-437.

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, Y.L., Chiu, H.C., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H. and Kou, G.H. 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Diseases of Aquatic Organisms 27, 215-225.

Loh, P.C., Tapay, L.M., Lu, Y. and Nadala, E.C. 1997. Viral pathogens of the penaeid shrimp. Advances in Virus Research 48, 263-312.

Pan, J., Kurosky, A., Xu, B., Chopra, A.K., Coppenhaver, D.H. Sigh, I.P. and Baron, S. 2000. Broad antiviral activity in tissue of crustaceans. Antiviral Research 48, 39-47.

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y. and Aoki, T. 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. Fish and Shellfish Immunology 13, 69-83.

Rosenberry, B. (ed.). 2002. World Shrimp Farming 2002. Shrimp News International. 276p.

Roux, M.M., Pain, A., Klimpel, K.R. and Dhar., A.K. 2002. The lipopolysaccharide and (β1,3-glucan binding protein gene is upregulated in white spot syndrome virus-infected shrimp (*Penaeus stylirostris*). Journal of Virology 76, 7140-7149.

Van Hulten, M.C.W., Goldbach, R.W. and Vlak, J.M. 2000a. Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. Journal of General Virology. 81, 2525-2529.

Van Hulten, M.C W., Westenberg, M., Goodall, S.D. and Vlak, J.M. 2000b. Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology 266, 227-236.

Van Hulten, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Klein Lankhorst, R. and Vlak, J.M. 2001a. The white spot syndrome virus DNA genome sequence. Virology 286, 7-22.

Van Hulten, M.C.W., Witteveldt, J., Snippe, M. and Vlak., J.M. 2001b. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology 285, 228-233.

Van Hulten, M.C.W., Reijns, M., Vermeesch, A.M.G., Zandbergen, F. and Vlak, J.M. 2002. Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation of the WSSV major structural proteins. Journal of General Virology 83, 257-265.

Venegas, C.A., Nanaka, L., Mushiake, K., Nishikawa, T. and Murog, K. 2000. Quasi-immune response of *Penaeus japonicus* to penaeid rod-shaped DNA virus (PRDV). Diseases of Aquatic Organisms 42, 83-89.

Wang, Y.C., Lo, C.F., Chang, P.S. and Kou, G.H. 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture. 164, 221-231.

Wu, J.L., Nishioka, T., Mori, K., Nishizawa, T. and Muroga, K.A. 2002. Time-course study on the resistance of *Penaeus japonicus* induced by artificial infection with white spot syndrome virus. Fish and Shellfish Immunology 13, 391-40.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X. and Xu, X. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. Journal of Virology 75, 11811-20.