NOTE

Increased tolerance of *Litopenaeus vannamei* to white spot syndrome virus (WSSV) infection after oral application of the viral envelope protein VP28

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ABSTRACT: It has been generally accepted that invertebrates such as shrimp do not have an adaptive immune response system comparable to that of vertebrates. However, in the last few years, several studies have suggested the existence of such a response in invertebrates. In one of these studies, the shrimp *Penaeus monodon* showed increased protection against white spot syndrome virus (WSSV) using a recombinant VP28 envelope protein of WSSV. In an effort to further investigate whether this increased protection is limited to *P. monodon* or can be extended to other penaeid shrimp, experiments were performed using the Pacific white shrimp *Litopenaeus vannamei*. As found with *P. monodon*, a significantly lower cumulative mortality for VP28-fed shrimp was found compared to the controls. These experiments demonstrate that there is potential to use oral application of specific proteins to protect the 2 most important cultured shrimp species, *P. monodon* and *L. vannamei*, against WSSV. Most likely, this increased protection is based on a shared and, therefore, general defence mechanism present in all shrimp species. This makes the design of intervention strategies against pathogens based on defined proteins a viable option for shrimp culture.

KEY WORDS: White spot syndrome virus · VP28 · Vaccination · Structural proteins · *Litopenaeus vannamei* · Immunology

INTRODUCTION

It has been assumed that it is not possible to protect invertebrates against pathogens using an induced (e.g. vaccination) or primed (i.e. past experiences with a pathogen) immune response. In recent years, however, many phenomenological data have suggested the existence of such adaptive immune mechanisms in invertebrates (Kurtz 2005). In the last decade, viral diseases have caused major problems in the expanding shrimp culture industry. With the increase in both the social and economical importance of shrimp culture (Lundin 1996), much research has been aimed at designing disease intervention strategies and has resulted in expanded knowledge of crustacean immunology.

In vivo experiments using *Penaeus japonicus* have demonstrated the presence of a quasi-immune response to white spot syndrome virus (WSSV) in which survivors of both natural and experimental infections re-challenged with WSSV showed higher survival compared to naive shrimp (Venegas et al. 2000). Moreover, haemolymph of the survivors collected 17 d post-challenge showed some virus neutralisation activity. Further evidence of a specific immune response in crustaceans has been found using *Daphnia magna* (Little et al. 2003). In the present study, maternal transmission of strain-specific immunity was demonstrated, as progeny were less likely to be infected by parasite strains to which their mothers had been challenged. The presence of immunological memory has also been

demonstrated for the copepod *Macrocyclops albidus*, individuals of which were less likely to be re-infected with antigenically related lines of a natural parasite compared to less related lines (Kurtz & Franz 2003).

However, as the above examples involve whole parasites, the enhanced immunological status might be explained by the recognition of general 'pathogen-associated molecular patterns' (e.g. lipopolysaccharide, lipoteichoic acid, mannans, glycans) by 'pattern-recognition receptors' (PRRs), the effect of which has been shown in numerous studies (Duvik & Söderhäll 1990, Söderhäll et al. 1994, Kim et al. 2000, Romeo-Figueroa et al. 2004). A recent study using the over-expressed WSSV envelope protein VP28 as a subunit vaccine in *Penaeus monodon* shrimp was the first to show that a VP28-specific antiviral response could be triggered with a protein lacking patterns that can be recognised by known PRRs, suggesting the presence of an unknown type of immune system (Witteveldt et al. 2004a,b).

To further investigate whether this increased protection is strictly species specific or based on a more general mechanism, experiments were performed using the Pacific white shrimp *Litopenaeus vannamei* as an alternative host for WSSV. This species is of increasing importance in shrimp farming, as it is reported to have a lower susceptibility to WSSV compared to *Penaeus monodon* (Briggs et al. 2004). Nevertheless, the culture of *L. vannamei* is still greatl hampered by mass mortalities due to WSSV (International Office of Epizootics, 2005; available at: www.oie.int/eng/info/hebdo/a_dsum.htm). In the present study, *L. vannamei* shrimp were orally fed with inactivated bacteria overexpressing VP28 and found to be tolerant to WSSV following challenge by immersion.

MATERIALS AND METHODS

Shrimp culture. Specific pathogen-free (SPF) *Litopenaeus vannamei* of the Kona strain (Wyban et al. 1992) were used for the experiments. Postlarvae (PL 8 to 12) from Ceatech farms (USA) were maintained at the Laboratory of Aquaculture & Artemia Reference Centre, Ghent University. Each shipment was tested by PCR (polymerase chain reaction) for the presence of WSSV, *Monodon baculovirus*, yellow head virus, Taura syndrome virus and infectious hypodermal and haematopoietic necrosis virus. Prior to each experiment, shrimp of approximately 5 g were transferred to an experimental system located at the Laboratory of Virology at Wageningen University and stocked in 180 l aquariums, each fitted with an individual filter system (Eheim), heating (Schego) to 28 ± 1°C and continuous aeration. All experiments were performed in artificial seawater (Instant Ocean, Aquarium Systems) at a salinity of approximately 20‰.

Feeding experiments. Groups of 10 shrimp were vaccinated by feeding food pellets coated with either VP28-expressing bacteria, bacteria containing the empty pET28a vector, or PBS (phosphate-buffered saline) for the positive and negative controls. The shrimp were vaccinated for 7 d, followed by 7 d of normal food, as this incubation period showed the highest response in previous studies (Witteveldt et al. 2004b). Subsequently, all shrimp were challenged by immersion, except for 5 shrimp that received PBS-coated food pellets, which were mock infected.

Statistical analysis. Statistical analysis of the obtained time–mortality relationships was performed using chi-squared tests at a 5% confidence level. The protection against WSSV after feeding bacteria-coated food pellets was calculated as a relative percent survival (RPS) calculated as: $(1 -$ vaccinated group mortality/control group mortality$) \times 100$ (Amend 1981).

Unless stated otherwise, the materials and methods were similar to the procedures previously described in Witteveldt et al. (2004b).

RESULTS AND DISCUSSION

The major WSSV envelope protein VP28 was selected for use, as this protein showed an increase in shrimp survival after oral application in *Penaeus monodon* (Witteveldt et al. 2004b). VP28 was expressed fused to a $(HIS)_6$-tag and without its N-terminal hydrophobic domain. An empty pET28a vector was used as negative control. Expression of the proteins was checked through SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and confirmed by Western analysis using anti-WSSV polyclonal antiserum to be of viral origin (Witteveldt et al. 2004b).

The cumulative mortalities of the titration of WSSV in *Litopenaeus vannamei* are shown in Fig. 1. A final cumulative mortality of 100% was observed when shrimp were incubated with 9 µl WSSV stock shrimp^{-1}. Incubation with 3 and 6 µl stock shrimp^{-1} resulted in final cumulative mortalities of 75%, although 3 µl showed a delay in the onset of mortalities. Incubation with 1 µl resulted in a slightly lower cumulative mortality (67%). Based on this titration, a challenge pressure of 6 µl of WSSV stock shrimp^{-1} was selected for the experiment.

Two groups of *Litopenaeus vannamei* shrimp were vaccinated by feeding for 7 d with food pellets coated with bacteria containing pET28a-VP28 and pET28a (control). Two other groups of shrimp received PBS-coated food pellets. After the 7 d period, all shrimp received normal food for another period of 7 d. Subse-
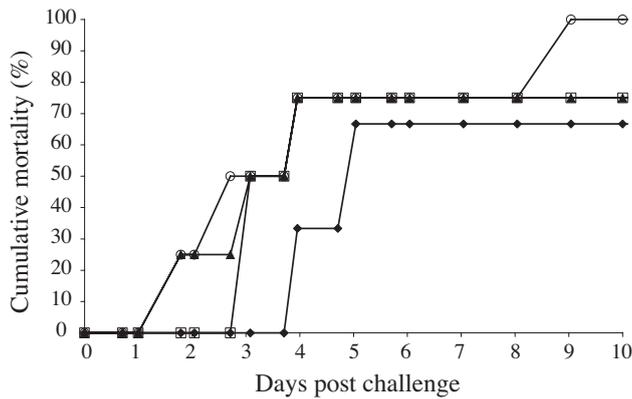
Fig. 1. *Litopenaeus vannamei*. Time–mortality relationship of *L. vannamei* challenged with 4 different white spot syndrome virus (WSSV) dilutions. Cumulative mortality rates of shrimp challenged with 1 µl (◆–), 3 µl (⊟–), 6 µl (▲–) and 9 µl (⊖–) of WSSV stock shrimp^{-1} are plotted against the days after challenge

quently, the VP28, pET28a and positive control groups were challenged by immersion using 6 µl WSSV stock shrimp^{-1}. At the same time, the negative control was mock challenged. The resulting time–mortality relationship is shown in Fig. 2. The shrimp vaccinated with VP28 showed a significantly lower cumulative mortality (RPS value of 50%) compared to both the pET28a-fed shrimp and the positive control. All shrimp that died during the experiment tested positive, and all surviving shrimp tested negative for WSSV by 1-step PCR (Witteveldt et al. 2004b).
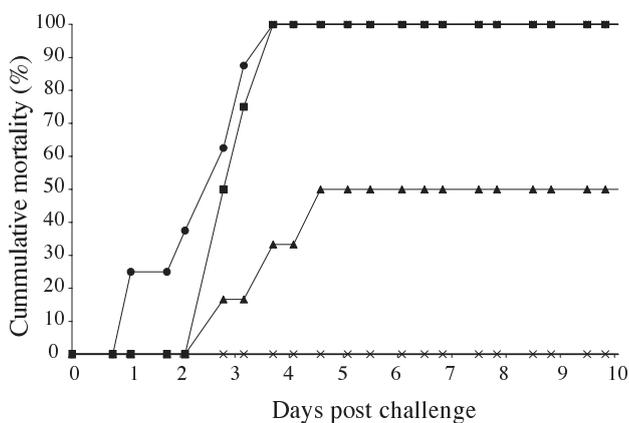

Fig. 2. *Litopenaeus vannamei*. Time–mortality relationship after feeding bacteria-coated food pellets and challenge of *L. vannamei.* Cumulative mortality rates of shrimp from the experimental groups VP28 (▲–), pET28a (■–), positive control (●–) and negative control (✕–) are plotted against the days after challenge. The VP28-vaccinated group shows a significantly lower (p < 0.05) cumulative mortality compared to both the pET28a and positive control groups

The 7 d interval period between the last bacteria-coated pellet feeding and the challenge was chosen as this resulted in the highest level of protection of *Penaeus monodon* (Witteveldt et al. 2004b). The level of protection in *Litopenaeus vannamei* was lower compared to *P. monodon* (RPS values of 50% in *L. vannamei* and 77% in *P. monodon* when comparing the VP28 to the pET28a groups), but was comparable to other trials in *P. monodon* using a similar, high, challenge pressure (authors' unpubl. results).

This study has shown that the enhanced tolerance of *Penaeus monodon* to WSSV upon oral application of VP28 can also be induced in *Litopenaeus vannamei*. Although the experiments do not address the mechanism of the observed protection, they do suggest that the effect is based on a general and probably novel type of immunological mechanism conserved among shrimp species. Molecular analysis of the host response to VP28 application, as a first step in revealing this mechanism, is in progress.

Increased protection using the WSSV envelope protein VP28 has now been demonstrated in 2 of the most important shrimp species in aquaculture, *Penaeus monodon* and *Litopenaeus vannamei*. Most likely, similar results could be obtained for other shrimp species, underlining the value of vaccination strategies to assist the shrimp farming industry in the battle against viral and bacterial diseases. Furthermore, these results make the design of intervention strategies against pathogens based on defined proteins a viable option for a healthy shrimp culture.

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