Fish & Shellfish Immunology 55 (2016) 401–406

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Injected phage-displayed-VP28 vaccine reduces shrimp *Litopenaeus vannamei* mortality by white spot syndrome virus infection

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ARTICLE INFO

*Article history:*
Received 8 September 2015
Received in revised form
19 May 2016
Accepted 22 May 2016
Available online 27 May 2016

*Keywords:*
WSSV
VP28
Phage display
*Litopenaeus vannamei*
Shrimp vaccine

ABSTRACT

White spot syndrome virus (WSSV) is the most important viral pathogen for the global shrimp industry causing mass mortalities with huge economic losses. Recombinant phages are capable of expressing foreign peptides on viral coat surface and act as antigenic peptide carriers bearing a phage-displayed vaccine. In this study, the full-length VP28 protein of WSSV, widely known as potential vaccine against infection in shrimp, was successfully cloned and expressed on M13 filamentous phage. The functionality and efficacy of this vaccine immunogen was demonstrated through immunoassay and *in vivo* challenge studies. In ELISA assay phage-displayed VP28 was bind to *Litopenaeus vannamei* immobilized hemocyte in contrast to wild-type M13 phage. Shrimps were injected with $2 \times 10^{10}$ cfu animal$^{-1}$ single dose of VP28-M13 and M13 once and 48 h later intramuscularly challenged with WSSV to test the efficacy of the vaccine against the infection. All dead challenged shrimps were PCR WSSV-positive. The accumulative mortality of the vaccinated and challenged shrimp groups was significantly lower (36.67%) than the unvaccinated group (66.67%). Individual phenoloxidase and superoxide dismutase activity was assayed on 8 and 48 h post-vaccination. No significant difference was found in those immunological parameters among groups at any sampled time evaluated. For the first time, phage display technology was used to express a recombinant vaccine for shrimp. The highest percentage of relative survival in vaccinated shrimp (RPS = 44.99%) suggest that the recombinant phage can be used successfully to display and deliver VP28 for farmed marine crustaceans.



## 1. Introduction

For the past 20 years, the white spot syndrome virus (WSSV) has been considered the most important viral pathogen impacting the shrimp farming industry. The danger is due to its high virulence, wide host range, presence in all major shrimp-producing countries, and mass mortality that can reach up to 100% within 3–7 d after the first clinical signs appear, toppling production with huge economic losses. Inside out the virions contain double-stranded circular genome of about 300 kb (Genebank accession no. AF332093), nucleocapsid, tegument, and envelope that conform from an ellipsoid to a bacilliform shaped particle with a distinctive tail-like appendage at one end. Viral proteins VP28, VP19, VP26, and VP15 are the major structural proteins [1–3]. Structural proteins are a rich source for protective immunogens. VP28 is one of the major and most exposed proteins in the WSSV envelope, which is crucial at the first steps of the infection process because it is able to attach to host receptors on the surface of shrimp cells, leading viral entry into the cytoplasm [4]. VP28 has shown to be a potential subunit vaccine, bringing significant protection against WSSV infection to shrimp and crayfish [5,6]. Various expression host systems were used to produce VP28-based vaccine such as gram-negative and positive bacteria, yeast, insect cells, and silkworms with a different degree of success [6–10]. Phage display is a technology, with multiple advantages, not yet explored against WSSV as an expression system of immunogenic recombinant proteins and carrier for those proteins. Some advantages for phage utilization are high capacity to accept exogenous DNA for cloning purpose, less expensive, easy and rapid production, very simple purification with

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http://dx.doi.org/10.1016/j.fsi.2016.05.027
1050-4648/

high purity, resistance to abiotic stressors, adjuvant-like particle properties, high biodegradability avoiding accumulation in tissues, and safety probed per years of testing in animals and humans with no apparent side effects [11].

Genetically engineered phages are widely used in phage display technology to select peptides for binding to target proteins of interest. The most common phage used is M13, which is a filamentous viral particle formed by about 2700 copies of pVIII protein and 3–5 copies of pVII and pXIX on one tip and 3–5 copies of pIII and pVI on other side encoded by gene *VIII*, *VII*, *XIX*, *III*, and *pVI*, respectively. Foreign peptide genes are introduced into the phage genome, usually inserted in gene *III* or gene *VIII*, resulting in foreign DNA product expressed as fusion to phage coat proteins. The number of fusion peptides per viral particle depends on the phage coat protein selected varying from 1 to thousands [11,12]. Beyond the use of phage display as a screening technique, utilization of filamentous phage particles as peptide carriers against cancer [13–15], neurological disorders [16], and infectious diseases is growing as evidenced by numerous publications. Phage-displayed immunogenic peptides from pathogens as human respiratory syncitial virus [17], herpes simplex virus 2 [18], rabies virus [19], HIV [20,21], *Candida albicans* [22] and *Taenia solium* [23,24], among others, were successfully used for immunization of animals.

In aquaculture, the use of recombinant phages as peptide carriers is limited to few papers [25–27]. In this study, the M13 filamentous phage was used to express multiple copies of WSSV envelope VP28 protein on coat surface. Phage-displayed VP28 binding capacity to shrimp hemocyte membrane and its efficacy to protect *Litopenaeus vannamei* shrimp against WSSV infection *in vivo* was evaluated. The results showed VP28 can be expressed on M13 phage and reduce the mortality by experimental viral infection. To our knowledge, our study is the first report of the application of phage-displayed vaccine to farmed marine crustaceans.

## 2. Materials and methods

### 2.1. Phagemid construction

The *VP28* gene (GenBank accession No. DQ681069) was amplified from genomic WSSV DNA using the primers *VP28*-FW 5′-CGCGGATCCGATGGATCTTTCTTTCACTCTTTC-3′ and *VP28*-RV 5′-CCGGAATTCTTACTCGGTCTCAGTGCCA G-3′ and inserted into pG8SAET phagemid vector in fusion with pVIII phage gen. *Escherichia coli* TG1 was electrotransformed with pG8SAET-*VP28* and pG8SAET. Positive transformants were selected from LB agar plates supplemented with 100 μg/ml ampicillin and cultured in LB-Amp. Colony PCR analysis was performed using specific phagemid primers. The DNA from PCR positive and negative bacterial colonies were sequenced using the same primers.

### 2.2. Expression and display of VP28

To produce M13 phage-displayed recombinant VP28 (VP28-M13) and M13 control phage particles (M13), *VP28*-pG8SAET and pG8SAET were rescued, respectively, from transformed TG1 cells by infection with M13K07 helper phage and cultivated in LB containing 100 μg/ml ampicillin and 50 μg/ml kanamycin overnight at 37 °C. Phages extruded through the bacterial membrane to the medium were purified by double PEG/NaCl precipitation. VP28-M13 was titering in LB-Amp agar plates by infecting TG1 cells and stored at 4 °C until the experiment began.

To evaluate VP28 expression, total RNA was extracted from a sample of superinfected cells by TRIZOL reagent (Invitrogen, USA). RT-PCR was performed using ImProm-II Reverse Transcription System (Promega, USA) and synthesized cDNA was used as PCR template to amplify *VP28*.

### 2.3. ELISA

Hemolymph was collected from the shrimp heart with shrimp isotonic solution (SIC) containing 20 mM ethylene diamine tetraacetic acid (SIC-EDTA) as described by Vargas-Albores [28] at a ratio of 1:2 and centrifuged at 800×*g* at 4 °C for 10 min. The pellet was suspended with SIC half original volume and centrifuged again thrice. Shrimp hemocytes were coated in a 96-well microplate allowing cell adhesion at room temperature (100 μl per well); then 20% acetone was added. After fixing in −20 °C for 10 min wells were washed thrice with PBS and blocked with 3% BSA in PBS for 1 h at 37 °C. After washes with PBS-T, VP28-M13 ($1 \times 10^{12}$ cfu $ml^{-1}$) in Tris-buffered saline (TBS) was added and incubated overnight at 4 °C and washed with PBS-T. Wells were incubated with HRP/anti-M13 antibody (1:2000) for 1 h at 37 °C and washed with PBS-T. The horseradish peroxidase reaction was developed using OPD substrate, and absorbance values were measured at 450 nm. Some wells in the 96-well plate incubated with M13 and BSA served as negative control. All the experiment was made in triplicate.

### 2.4. Shrimp culture

*Litopenaeus vannamei* shrimp, approximately 12 g body weight, were received from a local shrimp farm nursery (Sonora, Mexico) and tested by PCR to ensure they were WSSV-free. The organisms were acclimated to experimental conditions two days before initiating the trial and fed *ad libitum* twice a day with commercial feed.

### 2.5. Virus stock

WSSV was obtained from PCR positive-infected shrimp muscle. For virus replication, 50 μl of inoculum previously prepared in our lab was injected into healthy *L. vannamei*. When clinical signs of infection but no mortalities were observed, 100 μl hemolymph was extracted with 400 μl of SIC-EDTA. Hemocyte was separated from plasma by centrifugation at 800*g* at 4 °C for 5 min and used for DNA extraction. Positive PCR WSSV-infected shrimp were sacrificed and used for viral inoculum preparation. The abdominal muscle was homogenized with six parts of PBS (1:6 w/v) and centrifuged at 3000*g* at 4 °C for 20 min. The supernatant was recovered and centrifuged again at 13,000*g* at 4 °C for 20 min. Supernatant was filtered through 0.2 μm membrane filter (Millipore, USA) and stored at −80 °C.

### 2.6. Vaccination

A static experimental system was used. Shrimps were separated in plastic covered tanks with filtered and UV-sterilized seawater at 35 PSU and 29 °C. An air stone individually aerated each tank. VP28-M13, M13, and TBS were delivered intramuscularly to three shrimp groups. The first two groups ($n = 30$) were injected with 50 μl TBS containing $2 \times 10^{10}$ cfu $shrimp^{-1}$ of VP28-M13 or M13, respectively. The third group ($n = 30$) received 50 μl TBS as control. After injection, treated- and untreated-shrimp continued receiving the pelletized commercial feed. Shrimps were sampled at 8 and 48 h post-vaccination (hpv). Fifty microliters of hemolymph of each shrimp were extracted with SIC (1:10 v/v) for individual immunological parameter analysis.

### 2.7. Challenge study

For WSSV challenge dose determination, the virus stock was