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Protection of *Procambarus clarkii* against white spot syndrome virus using recombinant oral vaccine expressed in *Pichia pastoris*

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Abstract

The potential for oral vaccination of crayfish against white spot syndrome virus was investigated. The envelope proteins VP19 and VP28 were expressed in yeast (*Pichia pastoris*). The expressed proteins were used as oral vaccines in different forms viz., in whole culture form, whole culture sonicated form, whole culture centrifuged supernatant form, and cell residue form. The recombinant proteins were mixed with food pellets and fed to crayfish for 25 days. The vaccinated groups were divided into two even groups and challenged on the 3rd and 21st day of post vaccination. Among different vaccine groups the relative percent survival (RPS) values of sonicated form and supernatant form vaccines were found the best and met the criterion (>RPS 60%) of effective vaccine even after 21st day of post vaccination. Development of vaccine by using recombinant proteins VP19 and VP28 in yeast as expression vector was feasible with significant effects.

Keywords: WSSV; Oral vaccine; Yeast; VP19; VP28

1. Introduction

White spot syndrome is the most hazardous and devastating disease spreading from penaeids to other crustaceans as an alternate host [1–4] in natural as well as cultured aquatic habitat. WSSV virions belong to a new family of *Nimaviridae* (http://www.ncbi.nlm.nih.gov/ICTvdb/Ictv/Index.htm) and genus *Whispovirus* with single species. It is enveloped, ovoid (120 nm) to bacilliform (~275 nm) in shape [5,6] and have a tail-like appendage at one end. The double stranded viral DNA is 305 Kb with 181 open reading frames [7,8]. It consists of at least five major proteins with estimated sizes of 15 kDa (VP15), 19 kDa (VP19), 24 kDa (VP24), 26 kDa (VP26) and 28 kDa (VP28). In addition to it 13 minor proteins with the nucleocapsid also present [5,6,9,10]. The WSSV virions have circular sequence of 292,967 base pairs in Thailand [8] but variation in size is possible, 305,107 bp (AX151396) in China and

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307,287 bp (AF440570) in Taiwan among different geographic isolates, which are mainly due to several small insertions and one large 12 kb deletion [4,11,12].

The defence system in crustaceans depends mostly on innate immunity which is carried out through the phagocytic, encapsulating and agglutinating activities of the circulating haemocytes as well as by anterior orbital factor haemolymph [13–15]. The proPO system that is synthesised in haemocytes and localised in granules [11,16] participates in host-defence by enhancing phagocytosis, initiating nodule or capsule formation, mediating coagulations and producing fungal false substances [16]. WSSV inhibits the proPO system in haemocytes [17]. In addition to this the presence of antimicrobial peptides and lectins has also been reported [16,18–21] but these factors are mainly aimed at bacteria, fungi or parasites and not on virus [11]. Peroxinectin, an adhesion molecule, which is important for the development of immune memory in vertebrates, has also been reported in crayfish (*Pacifastacus leniusculus*) [22].

Use of various immunostimulants has been reported against WSSV infection, such as lipopolysaccharide in *Penaeus japonicus* [23,24], and glucan in *P. monodon* [25–28]. Quasi immune system is a new concept reported by Venegas et al. [29] in *P. japonicus*. Various vaccine agents' i.e. inactivated WSSV vaccines [5,28]; antibacterial components [30] and subunit recombinant vaccines [31–36], etc. have been tried so far against white spot syndrome virus with notable results. Use of recombinant vaccines is applicable as being well defined, non-infectious and simple, as well as inexpensive to produce in large quantities.

Keeping these facts in mind the most exposed WSSV envelope proteins VP19 and VP28 were expressed in methylotrophic yeast (*Pichia pastoris*) a eukaryotic expression system. The expressed recombinant proteins were used as oral vaccines and their efficacy was measured.

2. Material and methods

2.1. WSSV virus stock production

WSSV infected *Penaeus monodon* were collected from a farmer's ponds in Xiang Shan, China. The haemolymph were withdrawn from the hearts by using sterile syringes and then followed by centrifugation at 3000 × *g* for 20 min at 4 °C. The supernatant liquid was taken from the centrifuged haemolymph and the supernatant was re-centrifuged at 8000 × *g* for 20 min at 4 °C and the final supernatant was filtered through a 0.4-μm filter.

The filtrate was then injected to Crayfish *Procambarus clarkii* intramuscularly at a lethal dose (200 μl of viral supernatant) of WSSV for in vivo stock production of the virus. Haemolymph was withdrawn from moribund crayfish and the virus was purified by centrifugating at 80,000 × *g* for 1.5 h at 4 °C on a 20–45% continuous sucrose gradient in TN (20 mM Tris, 400 mM NaCl, pH 7.4). The visible virus bands were removed and the virus particles were subsequently pelleted by centrifugating at 45,000 × *g* at 4 °C for 1 h. The virus pellets were re-suspended in TE (pH 7.5) and the integrity of the virus was checked by transmission electron microscopy. The virus stock was stored at −80 °C for further use.

2.2. PCR amplification of viral structural genes

Viral structural genes VP19 and VP28 were amplified using cloning primers designed in accordance with the already published sequence [33]. The forward and reverse cloning primers for VP19 gene were VP19C-FW (5′ GACG TACCTCTTCATCAAACAG 3′) and VP19C-R (5′ ATTTTTGTCCCTGATGTTGTGTT 3′) respectively with a product size of 429 bp and an annealing temperature of 53.6 °C. Similarly the forward and reverse cloning primers for VP 28 gene were VP28C-FW (5′ GTTCGATAAAGAAAAAAACTCG 3′) and VP28C-R (5′ CCCTATCTATA TAAAAAGCACG 3′) respectively with a product size of 668 bp and an annealing temperature of 53.1 °C. The reaction conditions were 1 μl of template DNA, 1 μM of each primer, 1 μM of dNTPs and 0.5 U of *Taq* DNA Polymerase in PCR buffers (Shanghai Bioasia, China). The PCR reaction for VP19 was carried out for 35 cycles in an automatic thermal cycle programmed for 2 min at 94 °C, 50 s at 94 °C, 50 s at 54 °C, 1 min at 72 °C, 10 min at 72 °C and hold temperature at 4 °C. The PCR reaction for VP28 was carried out for 35 cycles in an automatic thermal cycle programmed for 2 min at 94 °C, 50 s at 94 °C, 50 s at 53 °C, 1 min at 72 °C, 10 min at 72 °C and hold temperature at 4 °C. The PCR products were run on agarose gel electrophoresis, the specific bands were recovered using DNA purification kit (Shanghai Bioasia, China). The purified DNA were sequenced (Invitrogen Biotechnology Co. Ltd. China) and compared with the original sequence [5].