Journal of Invertebrate Pathology 144 (2017) 97–105

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Fusion of flagellin 2 with bivalent white spot syndrome virus vaccine increases survival in freshwater shrimp

Hansam Cho [a], Na Hye Park [b], Yuyeon Jang [a], Yong-Dae Gwon [a], Yeondong Cho [a], Yoon-Ki Heo [a], Ki-Hoon Park [a], Hee-Jung Lee [a], Tae Jin Choi [b], Young Bong Kim [a,*]

[a] Department of Bio-industrial Technologies, Konkuk University, 120, Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea
[b] Department of Microbiology, Pukyong National University, 45, Yongso-ro, Nam-Gu, Busan 48513, Republic of Korea

ARTICLE INFO

Article history:
Received 27 September 2016
Revised 3 February 2017
Accepted 15 February 2017
Available online 16 February 2017

Keywords:
White spot syndrome virus
DNA vaccine
Baculovirus
FL2
Innate immunity
Shrimp

ABSTRACT

Despite large economic losses attributable to white spot syndrome virus (WSSV), an infectious pathogen of penaeid shrimp and other crustaceans worldwide, no efficient vaccines or antiviral agents to control the virus are available at present. Here, we designed and constructed baculovirus-based vaccines delivering genes encoding the WSSV envelope proteins, VP28 and VP19. To enhance the immunogenicity of the baculovirus-based vaccine, we fused a Salmonella typhimurium flagellin 2 (FL2) gene with VP28 or VP19 gene. Both vaccine constructs elicited similar high titlers of anti-WSSV IgG after oral immunization in mice. The protective effect of oral vaccines upon WSSV challenge was observed in Macrobrachium nipponense. Bivalent vaccine displaying WSSV envelope proteins, VP19 and VP28, led to enhanced more than 10% survival protection against WSSV infection, compared to monovalent vaccine containing WSSV envelope protein, VP19 or VP28. Furthermore, a baculovirus-based WSSV vaccine fused with FL2 gene, Ac-VP28-ie1VP19FL2, efficiently protected mice against WSSV challenge (89.5% survival rate). In support of the efficacy of FL2 in our vaccine, we verified FL2 enhanced survival rate and induced the NF-κB gene in *Palaemon paucidens*. The collective results strongly suggest that our recombinant baculoviral system displaying WSSV envelope protein and delivering FL2-fused WSSV envelope gene effectively induced protective responses, supporting the utility of a potential new oral DNA vaccine against WSSV.

1. Introduction

White spot syndrome virus (WSSV), a highly pathogenic and prevalent virus affecting a variety of crustacean species, is a threat to sustainable aquaculture and potentially damaging to the marine environment (Stentiford et al., 2012). Infection by the virus results in high mortality rates (90–100%) within 3–7 days. Owing to the accompanying enormous economic losses in penaeid shrimp cultures worldwide, WSSV virus has drawn considerable research attention (Johnson et al., 2008; Witteveldt et al., 2004). WSSV was first reported in shrimp aquaculture in the early 1990s in Northeast Asia, and has since spread throughout shrimp culture areas of the Indo-Pacific and Pacific (the Americas) (Lightner, 2011; Rajesh Kumar et al., 2008; Zwart et al., 2010). The distinctive clinical signs of the virus are white spots on the carapace, appendages, and inner surface of the shrimp body (Tang et al., 2007).

The virion consists of an enveloped nucleocapsid containing a circular double-stranded DNA genome of ~300 kbp ellipsoid to bacilliform in shape (Chai et al., 2013; van Hulten et al., 2001a; Verbruggen et al., 2016). Although WSSV was initially included in the *Baculoviridae* family, molecular classification and analysis of the complete genome sequence led to its re-classification into the genus *Whispovirus*, family *Nimaviridae* (Mayo, 2002a, 2002b).

Vaccine development for WSSV has focused on the structural components of the nucleocapsid and envelope (Syed Musthaq et al., 2009). Five major structural proteins have been identified, VP28 and VP19 (envelope) and VP26, VP24 and VP15 (nucleocapsid), which are thought to play crucial roles in the infection cycle

Abbreviations: AcMNPV, autographa californica multicapsid nucleopolyhedrovirus; AI, avian influenza; ANOVA, analysis of variance; CMV, cytomegalovirus; DMEM, dulbecco's modified eagle medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FFU, focus-forming units; FL2, Salmonella typhimurium flagellin 2; GFP, green fluorescent protein; HPV, human papillomavirus; HRP, horseradish peroxidase; ie1, WSSV immediate early 1; LD_{50}, lethal dose 50%; MOI, multiplicity of infection; MYD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PBST, phosphate buffered saline with tween® 20; PCR, polymerase chain reaction; pFB, pFastBac1; PO, phenoloxidase; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; Sf9, spodoptera frugiperda 9; SOD, superoxide dismutase; TLR5, toll-like receptor 5; TMB, 3,3′,5,5′-tetramethylbenzidine; WSSV, white spot syndrome virus.

* Corresponding author.
E-mail address: kimera@konkuk.ac.kr (Y.B. Kim).

http://dx.doi.org/10.1016/j.jip.2017.02.004
0022-2011/

comprising viral entry, assembly and budding (Chang et al., 2010; van Hulten et al., 2001b). In particular, VP28 and VP19 are reported to be involved in the initial step of WSSV infection of shrimp (Du et al., 2013; van Hulten et al., 2002; Yi et al., 2004).

Direct injection of recombinant VP28 or VP19 has been shown to increase the survival rate of WSSV-challenge shrimp. However, immersion or oral administration of WSSV VP28-expressing recombinant baculovirus did not show high protection efficacy (Ha et al., 2008; Syed Musthaq and Kwang, 2011, 2015; Syed Musthaq et al., 2009; Witteveldt et al., 2004). Here, we constructed a VP28 and VP19 bivalent DNA vaccine fused with *Salmonella typhimurium* flagellin 2 (FL2) as a novel adjuvant, with a view to improving vaccine efficacy. Flagellin, the major component protein of the flagellar filament (Kodama and Matsui, 2004; McQuiston et al., 2004; Szekely and Simon, 1983), is recognized by the innate immune system of diverse organisms ranging from flies to mammals (Hayashi et al., 2001). Flagellin is initially recognized by Toll-like receptor 5 (TLR5) (Salazar-Gonzalez and McSorley, 2005; Yoon et al., 2012), resulting in adaptor protein MyD88-mediated TLR signaling, which, in turn, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and induces proinflammatory gene expression (Simon and Samuel, 2007). Thus, flagellin has been investigated as a potent activator of a broad range of innate and adaptive immune responses (McDonald et al., 2007; Means et al., 2003; Mizel and Bates, 2010; Salazar-Gonzalez and McSorley, 2005; Zhang et al., 2015).

In this study, we aimed to determine whether the efficacy of WSSV vaccines was affected by FL2-fusion, promoters, and antigen order in the gene construct. Freshwater shrimps (*Macrobrachium nipponense*) were orally administered WSSV DNA vaccine constructs encoding both VP28 and VP19 fused with FL2, and their survival rates against WSSV challenge were evaluated.

2. Material and methods

2.1. Ethics statement

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of Konkuk University (Seoul, Republic of Korea). Animal husbandry and experimental procedures were approved by the Konkuk University Institutional Animal Care and Use Committee (IACUC approval No.: KU14082). Throughout the study, the condition of animals was monitored twice a day. In this study, no mice exhibited symptoms of illness or appeared to be close to death. Moreover, no mice died during the monitoring phase. After final monitoring, mice were humanely euthanized via cervical dislocation according to the AVMA guidelines for euthanasia of animals.

2.2. Cells

Sf9 (spodoptera frugiperda 9, Invitrogen, CA, USA) cells were maintained at 27 °C in Sf-900 medium (Invitrogen, CA, USA) supplemented with 1% Antibiotics-Antimycotics (Gibco-BRL, CA, USA). 293TT cells (embryonal kidney cell line transformed with sheared adenovirus type 5 DNA and simian virus-40, kindly donated by Dr. Schiller, National Cancer Institute, NIH, USA) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 400 μg/mL hygromycin B (Invitrogen).

2.3. Experimental animals

WSSV-free freshwater shrimps, Palaemon paucidens (~0.3 g body weight), and Macrobrachium nipponense (~3 g body weight) from Haman Lake in Korea were maintained separately in 300 L freshwater tank with airlift filters at an ambient temperature of 20–24 °C, pH 6.5–7.5, and fed with artificial shrimp feed (Korea Feed Co., Ltd., Republic of Korea). Light cycle was kept almost night state because shrimps are nocturnal. After acclimation to the new environment for 2–3 days, shrimps (n = 10, n refers to the number of freshwater shrimp) were randomly collected and reevaluated for WSSV infection via PCR using VP28-specific primers (Table 1). Preparation of shrimp samples were homogenized and centrifuged at 2000*g* at 4 °C for 20 min. The supernatant was re-centrifuged at 8000*g* at 4 °C for 20 min and filtered using a 0.45 μm filter unit (Pall co., USA). The resulting filtrate was stored at −80 °C. Viral DNA was extracted from the supernatant using a NucleoSpin RNA Virus kit (Macherey-Nagal, Germany).

Table 1
Primers used in this study.

Target	Primer	Nucleotide Sequence
WSSV	WSSV F	5′-AGGGAACATTCAAGGTGTGG-3′
	WSSV R	5′-GGTGCCAACTTCATCCTCAT-3′
WSSV VP28	VP28 F	5′-AAAAGTCGACATGGATCTTTCTTTCAC-3′
	VP28 R	5′-AAAAGTCGACTTACTCGGTCTCAGTG-3′
WSSV VP19	VP19 F	5′-AAAAGTCGACATGGCCACCACGACTA-3′
	VP19 R	5′-AAAAGTCGACACTGCCTCCTCTTGGG-3′
WSSV ie1 promoter	ie1 F	5′-AAAAGCGGCCGCGAATTTTTGGACAGTA-3′
	ie1 R	5′-AAAAGCGGCCGCAGAGAGAGAGCTAGTT-3′
Salmonella typhimurium Flagellin 2 (FL2)	STF2 F	5′-AAAAGTCGACATGGCACAAGTAAT-3′
	STF2 R	5′-AAAAGTCGACTTAAACGTAACAGAGA-3′
VP28 fused FL2 (overlapping PCR)	VP28-FL2 F	5′-GAGACCGAGGCACAAGTAATCAACACTA-3′
	VP28-FL2 R	5′-TACTTGTGCCTCGGTCTCAGTGCCAGA-3′
VP19 fused FL2 (overlapping PCR)	VP19-FL2 F	5′-AGGAGGCAGGCACAAGTAATCAACACTA-3′
	VP19-FL2 R	5′-TACTTGTGCCTGCCTCCTCTTGGGGTAA-3′
NF-κB	NF-κB F	5′- ATGAGTTCCCCATACAGTGGAGAGTC-3′
	NF-κB R	5′- ACTCTTGTAGCGGAATCGGAATTTAGAC-3′

Six-week-old female BALB/c mice (21 ± 2 g) purchased from Orient Bio (Kyonggi-do, Republic of Korea) were housed in filter-top cages with water and food provided ad libitum. Mice were maintained in a Bio-safety Level 2 facility.

2.4. Preparation of WSSV viral stock

The laboratory WSSV stock was purified from WSSV-infected *Penaeus vannamei* collected in Korea. Infected shrimp samples were homogenized and centrifuged at 2000*g* at 4 °C for 20 min. The supernatant was re-centrifuged at 8000*g* at 4 °C for 20 min and filtered using a 0.45 μm filter unit (Pall co., USA). The resulting filtrate was stored at −80 °C as viral stock. Viral DNA was extracted from the stock solution using a NucleoSpin RNA Virus kit according to the manufacturer's instruction (Macherey-Nagal, Germany), and viral DNA copy number estimated via quantitative real-time PCR by reference to a standard curve prepared using serial dilutions of pGEM-T easy vector (Promega, USA) containing a partial ORF of the VP28 gene. The DNA copy number of the WSSV stock was determined as $\sim 3.22 \times 10^5$ copy/ml.

2.5. Construction of baculovirus vector expressing WSSV structural proteins

Recombinant baculoviral WSSV DNA vaccines were constructed by inserting VP28/19 genes and the WSSV ie1 promoter amplified from WSSV viral DNA. The 3′-end of the VP28/19 gene was fused with the FL2 gene (kindly supplied by Dr. Manki Song, International Vaccine Institute, Republic of Korea) via overlapping PCR