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The Expression Analysis of Innate Immune-related Genes in Kuruma Shrimp *Penaeus japonicus* after DNA Vaccination against Penaeid Rod-shaped DNA Virus

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ABSTRACT—In this study, we have developed a DNA vaccine encoding viral envelope protein VP28 of penaeid rod-shaped DNA virus (PRDV, = WSSV). Protective efficacy of the DNA vaccine against PRDV was confirmed at 7 days post vaccination in kuruma shrimp *Penaeus japonicus*. The VP28 transcript derived from the vaccine was detected in various tissues at 1, 3 and 7 days post vaccination. Moreover, notable up-regulated expression of Rab7, penaeidin, lysozyme and crustin genes was observed upon DNA vaccination. These results suggest that the DNA vaccine significantly increased the protection and innate immune responses in kuruma shrimp against PRDV.

Key words: DNA vaccine, *Penaeus japonicus*, VP28, PRDV, WSSV, innate immune-related genes

Viruses are among the most important pathogens in the crustaceans especially shrimp. Among various viruses affecting shrimp, penaeid rod-shaped DNA virus (PRDV; synonym for WSSV) is currently the most serious viral pathogen affecting the shrimp industry worldwide, resulting up to 100% mortality within 3 to 10 days of infection incurring major economic losses to shrimp farming industry[1,2]. PRDV is extremely virulent with a wide range of host specificity and targets various tissues[3]. The virus is pathogenic to several culture species of penaeid shrimp such as black tiger shrimp *Penaeus monodon*, white leg shrimp *P. vannamei* and kuruma shrimp *P. japonicus.* The presence of PRDV in both wild as well as hatchery reared post-larvae has been reported[4,5].

PRDV is a large DNA, and its particles consist of six major proteins (VPs) with expected sizes of 15, 19, 24, 26, 28 and 664 kDa. VP28 and VP19 are associated with the virion envelope, and the others are associated with nucleocapsid[6]. Moreover, it has been reported that the VP28 envelope protein located on the surface of the virus particle plays an important role in the initial stages of the PRDV infection in shrimp[7]. To date, the subunit vaccine targeting envelope proteins; VP28 synthesized in *E. coli* has been studied, and significant protection against PRDV by oral administration[8] or intramuscular injection[9–11] has been reported. More recently, DNA vaccines encoding envelope proteins were developed in black tiger shrimp[12,13]. The reports suggested that DNA vaccine increased protection against PRDV infection. However, the immune responses in shrimp upon DNA vaccination have not been studied thoroughly to date. This paper describes the efficiency of DNA vaccine in kuruma shrimp, and the expression of innate immune-related genes in vaccinated shrimp.

Materials and Methods

Plasmid DNA construction for vaccine

Viral DNA was extracted from PRDV infected shrimp using a DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen). PCR was performed with PRDV VP28 F1 (5'-ATGGATCTTTCTTT-CAC-3') and R1 (5'-TTACTCGGTCTCAGTGC-3') primer set. The cycle conditions were: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, followed by 1 cycle of 72°C for 5 min. Amplified product of VP28 gene was ligated into the expression vector that contained the human CMV-promoter (pTARGET Mammalian Expression Vector, Promega, USA). The ligated product (pCMV-VP28) and the plasmid vector without VP28 gene (pCMV) were transfected into TAM competent *E. coli* (ActiveMotif, Belgium), and recombinants were identified through red-white color selection on MacConkey agar (Sigma-Aldrich). Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a CEQ8000 Automated Sequencer (Beckman Coulter).

Vaccination and artificial PRDV challenge

PRDV free kuruma shrimp, approximately 15 g, was injected intramuscularly with 10 μg of pCMV-VP28 plasmid DNA dissolved in 100 μL of PBS. A control group of shrimp was injected with 100 μL of PBS and 10 μg of the pCMV dissolved in 100 μL of PBS.

PRDV artificial challenge was carried out by immersion at 7 days post vaccination. Heart and hepatopancreas were collected under sterile conditions from PRDV-infected shrimp. Mixed tissues were homogenized with PBS. DNA was extracted from homogenates using DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions, and copy number of PRDV

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challenge stock (homogenates) was determined by quantitative real-time PCR. Shrimp (n = 25/group) was immersed in 4 L of artificial sea water for 2 h at 20°C contained 5 mL of homogenates (1×10^{10} copies/mL). The survival rate of each group was recorded for 12 days. Assessment of statistical significance was analyzed by a Chi-square test. Relative percent survival (RPS) was calculated according to the method described by Amend (1981)[14].

Tissue distribution of VP28 transcript post vaccination

The hemolymph, muscle (injected part), gill, intestine, stomach, heart, hepatopancreas, lymphoid organ and were isolated from three individual shrimp with PBS and pCMV-VP28 at 1, 3 and 7 days post injection. Prior to the isolation of RNA, each tissue at all the time period post injection was mixed together only in PBS injected group. Total RNA was isolated using ISOGEN (Nippon Gene) following the manufacturer's instructions and the contaminating DNA was digested by the treatment with DNase I (Takara Bio, Shiga Japan) at 37°C for 30 min. cDNA was synthesized from 2 μg of total RNA using ReverTra Ace qPCR Kit (Toyobo).

Nested PCR was performed with PRDV VP28 F1 and R1 (1st PCR), PRDV VP28 F2 (5'-TGGATCAGGCTACTTCAAGAT-3') and R2 (5'-AAAGGTGGTACCACACACAAA-3') (2nd PCR) primer set as above condition. Shrimp β-actin gene (F 5'-ATGACACAGATCATGTTCGA-3'; R 5'-GTAGCACAGCTTCTCCTTGA-3') was used as internal control for RT-PCR. PCR products were separated on 2.0% agarose gels and visualized by staining the gels in TBE buffer containing 100 ng/mL ethidium bromide (Sigma-Aldrich).

Expression analysis of innate immune-related genes by semi-quantitative RT-PCR analysis

The intestine and lymphoid organ were isolated from shrimp at 1, 3 and 7 days post injection with PBS, pCMV or pCMV-VP28. Each tissue was extracted from three individual shrimp in each group and mixed together prior to the RNA extraction. RNA extraction and cDNA synthesis was carried out using kits described above. PCR was conducted with primer combinations; Pj (kuruma shrimp) Rab7 F (5'-CTCGCAAGAAGATTCTCCTG-3') and R (5'-CTTCGTTGATACCGCCCTAT-3'), Pj lysozyme F (5'-TCCTAATCTAGTCTGCAGGGA-3') and R (5'-CTAGAATGGGTAGATGGA-3')[15], Pj crustin F (5'-CACCTTCAGGGACCTTGAA-3') and R (5'-GTAGTCGTTGGAGCAGGTTA-3'), Pj penaeidin F (5'-GCTGCACCCACTATAGTCTTT-3') and R (5'-CTACCATGGTGATGAAACAAA-3'), Pj β-actin F and R. In order to have a semi-quantitative approach of gene expression, both kuruma shrimp innate immune-related and β-actin genes were amplified using a series of cycle numbers (21–35) following the condition described above. After specific PCR was conducted with optimal cycle number, the expression ratio of innate immune-related (35 cycles)/β-actin (25 cycles) was determined by densitometry using Science Lab99 Image Gauge software (Fujifilm). The expression analysis was conducted in triplicates Assessment of statistical significance was analyzed by one-way ANOVA, followed by a Tukey's test.

Results and Discussion

Prior to the construction of DNA vaccine, we considered the selection of promoter inserted in the expression vector for the study. In the commercial protein expression system using insect cell, p10 or polyhedron promoters derived from baculovirus is generally used to synthesize the interested protein. It has been confirmed that CMV-promoter derived from human cytomegalovirus functions in insect (Fall armyworm, *Spodoptera frugiperda*) cell[16] and black tiger shrimp[17] using luciferase and β-galactosidase reporter assays. More recently, the expression of VP28 protein in the muscle of black tiger shrimp injected with CMV-promoter driven expression vector was confirmed by immunohistochemistry[13]. Therefore, we selected the CMV-promoter for the construction of DNA vaccine for this study.

The protective immunity against PRDV was increased by immunization with constructed DNA vaccine in kuruma shrimp at 7 days post vaccination. The RPS value between vaccinated and control groups; PBS and pCMV was 70.0 and 62.4%, respectively. The efficacy of constructed DNA vaccine was confirmed, and the result was similar with that of previously published reports[12,13]. And the injection of empty vector also showed a slightly increased protection to PRDV infection when compared to that of control (PBS). To date, it is known that DNA vaccine themselves possess their own adjuvant activity in vertebrates because of the presence of unmethylated cytosine-guaninedinucleotides (CpG) motifs in particular base contents[18]. Also in shrimp, it was reported that CpG oligodeoxynucleotides activate the innate immune responses such as phenoloxidase activity[19] and respiratory burst[20]. Therefore, this increasing protection would depend on the CpG motif in the expression vector. However, the survival rate of vaccinated group was significantly higher than that of control groups. Thus, the constructed vaccine could be considered as an effective tool to combat PRDV.

The transcript of PRDV VP28 gene derived from expression vector was confirmed in hemolymph, muscle, gill, intestine, stomach, heart, hapatopancreas and lymphoid organ of shrimp at 1, 3 and 7 days post vaccination (Fig. 1). The expression levels among individual tissues were different. Although transcript of VP28 gene was not detected by the 1st PCR (data not shown), it was detected in various tissues by the nested PCR. We conducted immunohistochemistry and Western blotting to detect the expressed VP28 protein from

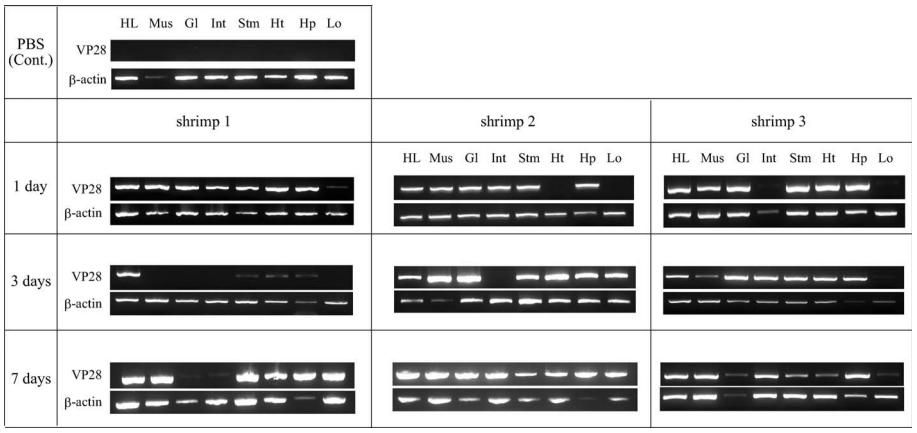

Fig. 1. Analysis of the tissue expression of VP28 mRNA in DNA vaccinated shrimp (3 individuals) and control (PBS). RT-PCR was performed using primers specific for VP28 and *β*-actin genes with cDNA synthesized from a variety of tissues; HL: hemolymph, Mus: muscle, Gl: gill, Int: intestine, Stm: stomach, Ht: heart, Hp: hepatopancreas, Lo: lymphoid organ of shrimp at 1, 3 and 7 days post vaccination. The accession numbers of *β*-actin and VP28 genes are AB055975 and AJ551447, respectively.

the internal organs of shrimp, however, the protein was not detected (data not shown). Therefore, these suggest that transcription level of VP28 gene in shrimp was low. Previous reports on DNA vaccine in black tiger shrimp showed the transcript of VP28 gene in muscle tissue after vaccination, and the expression last for 30−50 days[12,13]. Moreover, they analyzed the tissue distribution of injected plasmid DNA, and showed the persistence in various tissues such as muscle tissue, pleopods, telson, gill, gut (up to 60 days post vaccination), hepatopancreas (45 days) and hemolymph (15 days)[12]. Therefore, it suggests that injected plasmid DNA was circulated to the other tissues from the muscle via hemolymph.

The expression of innate immune-related genes was analyzed in intestine and lymphoid organ since the PRDV attaches on the epithelial cells of digestive tract at the initial infection stage[1] and causes severe infection at the later stages of the viremia. The expression of Rab7 gene involved in PRDV infection[21] was significantly increased in the intestine compared with that of control shrimp at all the time periods after vaccination (Fig. 2). And the peak increased expression was recorded at 7 days post vaccination. A previous report showed the increased protection against PRDV infection by the injection of recombinant Rab7 protein, and this molecule has important role for the attachment of PRDV at early infection stage[21]. Therefore, the increase of Rab7 gene expression might be related to the increased protection against PRDV. However, the difference of induction mechanism of Rab7 gene activation in PRDV infection or DNA vaccination is not clear. Shrimp does not possess an adaptive immune system; however, has a rapid and efficient innate immune system that is sufficient to protect themselves from foreign pathogens. Innate immune-related genes such as lysozyme, penaeidin and crustin are known to be a member of antimicrobial peptides. The activities of these peptides against bacteria and fungi have been well defined[15, 22−24], but their potential involvement to the antiviral responses is still unknown. Recent studies have shown the up-regulation of antimicrobial peptides as a response to viral infection in shrimp[25] and *Drosophila*[26]. These suggest the overlap of the responses induced with viral or bacterial infection[25]. The factors (like interferon/Mx known in vertebrates) directly related to the antiviral responses is still unknown in shrimp. Thus, the expression of these genes was analyzed to understand the immune response in shrimps after vaccination. The expression of lysozyme, penaeidin and crustin genes was significantly increased in intestine and lymphoid organ after vaccination (Fig. 2). The conspicuous increased expression of these genes was confirmed in lymphoid organ at 7 days after vaccination. Also in pCMV injected group, the up-regulation of penaeidin (intestine and lymphoid organ) and lysozyme (lymphoid organ) genes was confirmed compared to the control shrimp. However, the expression level of these genes was lower than that of vaccinated shrimp. It is unclear whether the increased expression of penaeidin and lysozyme genes in pCMV injected group indicates elevated levels of protection against PRDV; our results indicate that these antimicrobial peptide genes might be involved in the protective immunity to PRDV infection.

The results of this study emphasize the role of DNA vaccine, not only as a combat tool against particular pathogen but also as an activator of innate immune responses in shrimp. Detailed investigation will be required in the future to understand the mechanisms of DNA vaccine and their interaction with the immune responses (factors).

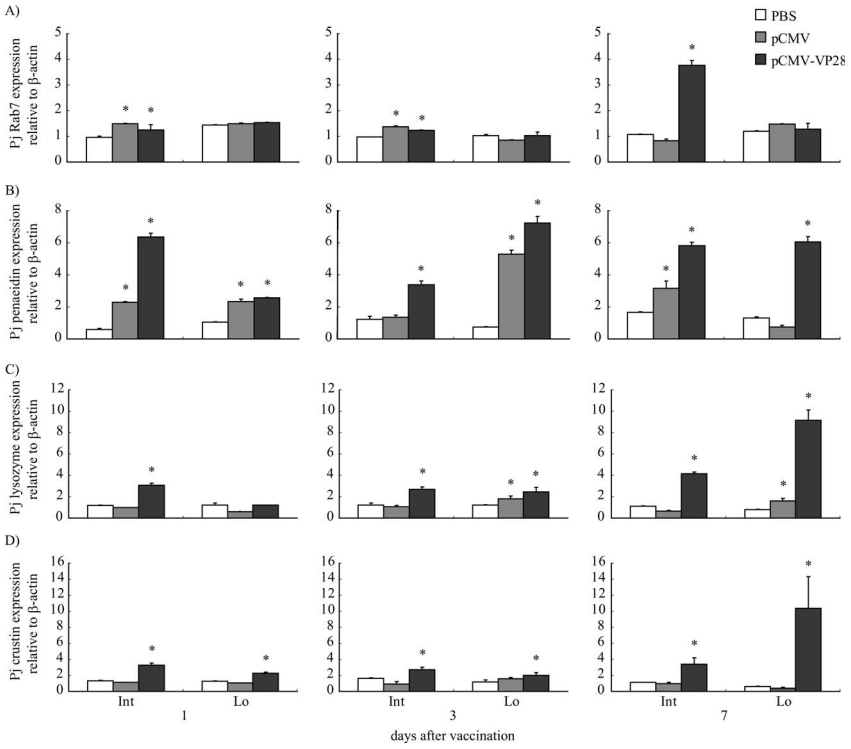

Fig. 2. Effects of DNA vaccination on the expression of shrimp innate immune-related genes in intestine and lymphoid organ at 1, 3 and 7 days post vaccination. Data are presented as shrimp innate immune-related gene PCR products after normalizing against products β-actin gene. The X-axis indicates the tissues tested (Int, intestine; Lo, lymphoid organ) and relative expression of the shrimp innate immune-related gene is on the Y-axis. Data are presented as mean ± S.D. of triplicate samples. Asterisks indicate the significant difference ($p < 0.05$) compared to the control. Graphs indicate the expression pattern of innate immune-related genes; A) Rab7 (Acc. No. AB379643), B) penaeidin (Acc. No. AU175636), C) lysozyme (Acc. No. AB080238) and D) crustin (Acc. No. AB121740).

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