**Adjuvant effects of poly I:C and imiquimod on the immunization of kuruma shrimp
(*Marsupenaeus japonicus*) with a recombinant protein, VP28 against white spot
syndrome virus**

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Abstract

The adjuvant effects of poly I:C and imiquimod during immunization with a recombinant protein, rVP28 derived from white spot syndrome virus (WSSV) was investigated in kuruma shrimp (*Marsupenaeus japonicus*). Shrimps were injected intramuscularly with different doses of rVP28, poly I:C and imiquimod, and combined rVP28 + poly I:C or imiquimod, and challenged with WSSV. Expression of innate immune-related genes was examined in heart and lymphoid organ of combined rVP28 + poly I:C or imiquimod immunized shrimps at 1, 3 and 7 days after WSSV challenge. Shrimps received rVP28 + poly I:C and rVP28 + imiquimod had significantly higher survivals of 52 and 58%, respectively compared to the rVP28 alone or PBS injected control groups ($P<0.05$). A significant up-regulation of innate immune-related genes, such as Rab7, lysozyme, penaeidin, crustin, Toll and TNF was noticed in combined rVP28 + poly I:C or imiquimod immunized shrimps. Our results indicate that injection administration of poly I:C or imiquimod + sub-unit protein (rVP28) provides a significant protection and induces immune response in kuruma shrimps against WSSV. Therefore, poly I:C and imiquimod have potentials to be used as adjuvants or immunostimulants in shrimp immunization.

Keywords: Poly I:C; Imiquimod; *Marsupenaeus japonicus*; WSSV; Innate immune-related genes; VP28.

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68 **1. Introduction**

69 The global crustacean aquaculture industry with shrimp as a major product is worth
70 in excess of US\$30 billion annually (FAO, 2014), but continues to be beset by endemic
71 viral diseases (Johnson et al., 2008). Farmed shrimps are susceptible to a wide variety of
72 pathogens, including viruses, bacteria, fungi and protozoa. Losses resulted from diseases
73 have had a devastating impact on shrimp aquaculture during its 30-year lifespan. The
74 main contributors to these losses were viral diseases (Lotz, 1997), of which white spot
75 syndrome virus (WSSV) is the most important one due to its epizootic spread and
76 economic impact. Kuruma shrimp (*Marsupenaeus japonicus*), the highest priced shrimp
77 species among the farmed crustaceans and widely cultured in Japan, China, Australia and
78 Southeast Asian countries (Rosenberry, 2001) is also infected by WSSV including other
79 penaeid shrimps, such as black tiger shrimp (*Penaeus monodon*), Chinese shrimp
80 (*Fenneropenaeus chinensis*), crayfishes, crabs and lobsters (Otta et al., 1999;
81 Supamattaya et al., 1998). Control of viral diseases is, therefore, of paramount necessity
82 in sustaining this important primary production industry.

83 Adjuvants are helper substances that are added to vaccine formulations in order to
84 improve immune response to the incorporated antigen (Bowden et al., 2003). In the
85 conventional vaccines, adjuvants are used to elicit an early, high and long-lasting
86 immune response. Numerous compounds are under evaluation as immunological
87 adjuvants and peptide-carriers to improve the immune response. The double-stranded
88 synthetic RNA, poly I:C has been widely used as a viral mimic to examine immune
89 responses in vertebrates (Huang et al., 2006). In shrimps, antiviral immunity is induced
90 by double-stranded RNA (dsRNA) that confers protection against WSSV infection
91 (Robalino et al., 2004). Although the exact mechanism of action is unknown, imiquimod,
92 a nucleoside analog of imidazoquinoline is an agonist for toll-like receptor (TLR) 7 and 8
93 (Schön and Schön, 2007). In mammalian models, imiquimod acts on induction of
94 cytokines, such as interferon alpha (IFN- α), interleukin-12 (IL-12), and tumor necrosis
95 factor alpha (TNF- α) (Gupta and Khandelwal, 2004). The desire for new and improved
96 adjuvants stems not only from the need to make existing inactivated vaccines more
97 potent, but also to gain features such as antigen-spreading ability, stimulation of T-cell
98 immunity, and longer-lasting protective immunity. Therefore, the benefits of
99 incorporating any adjuvant into vaccines must be balanced against any increased risk of

adverse reactions.

Modern practices in finfish aquaculture include highly effective routine vaccinations against multiple pathogens that have dramatically reduced impact of diseases. It has been widely assumed that true adaptive systems do not exist in invertebrates (Kimbrell and Beutler, 2001), thus vaccines have not been routinely developed and used in shrimp aquaculture. In particular, invertebrate immune response to viruses is poorly understood. Within the last decade, a body of literature has been building that indicates that shrimp and other crustaceans can be immunized with either inactivated virus or protein “sub-unit” vaccines and thereby protected from mortality induced by WSSV (Johnson et al., 2008). Recently, the protective efficacy of oral delivery and injection of a recombinant protein, rVP28 derived from WSSV envelope and synthesized using wheat germ cell-free technology was demonstrated in kuruma shrimp (Kono et al., 2014).

The aim of the present work was to investigate the adjuvant effects of poly I:C and imiquimod during immunization with rVP28 protein in kuruma shrimp and to use them as conjugative carriers of viral proteins.

2. Materials and methods

2.1. Experimental shrimps

Healthy kuruma shrimps (mean body mass 10 ± 1 g) without any overt disease symptoms were obtained from Matsumoto Fisheries, Miyazaki, Japan. Shrimps were maintained in an indoor system facilitated with running artificial seawater at 20°C and fed with a commercial diet (Higashimaru, Tokyo, Japan) once a day. Before challenge test, shrimps were sampled randomly and screened for WSSV by PCR (Kim et al., 2007).

2.2. Preparation of rVP28 sub-unit protein

Recombinant VP28 (rVP28) was synthesized using the ENDEXT Wheat Germ Expression H Kit (CellFree Sciences, Ehime, Japan). Protein synthesis and purification were performed as described in our previous report (Kono et al., 2014). Total protein, wheat-GST-rV28 was used for shrimp immunization. The concentration of rVP28 in total protein was measured by SDS-PAGE using α -lactalbumin (Sigma-Aldrich, St. Louis, MO, USA) as standard.

2.3. Immune challenge and sample collection

In the first experiment, the effective dose of the recombinant VP28 to protect the shrimp against WSSV disease and the onset of protection were determined. Individual shrimp from three groups was immunized with an intramuscular (i.m.) injection of 100 µg of total protein containing 5.0, 2.5 or 1.0 µg of rVP28 dissolved in 100 µL phosphate buffered saline (PBS). A control group received an i.m. injection of 100 µL PBS. Injection was made in the second abdominal segment. An artificial challenge with WSSV was carried out by immersion method described previously by Kono et al. (2009) after 7 days post-injection. Challenged shrimps from each group were distributed in 3 replicate tanks (n = 20) and maintained in seawater flow-through system. Shrimp survival from each tank was recorded until mortality stopped.

In the second experimental trial, the protective effect of poly I:C and imiquimod against WSSV in kuruma shrimp was investigated. Shrimps were i.m. injected with 100 µg of poly I:C (Sigma-Aldrich) or imiquimod [1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4 amine] (LKT Laboratories, Inc., St. Paul, MN, USA) dissolved in 100 µL PBS. A control group was injected with 100 µL PBS. WSSV artificial challenge was carried out by immersion at 7 days post-injection and challenged shrimps from each group were distributed in 3 replicate tanks (n = 20) facilitated with seawater flow-through system. Survival from each group was monitored until mortality stopped.

In the third experimental trial, the efficiency of using rVP28 + adjuvant (poly I:C or imiquimod) in protecting kuruma shrimp against WSSV after injection was determined. Shrimps divided into four groups (n = 90) were i.m. injected with 100 µg of total protein containing 2.5 µg rVP28 only, 2.5 µg rVP28 + 100 µg poly I:C or imiquimod dissolved in 100 µL PBS. A control group had an i.m. injection of 100 µL PBS. WSSV artificial challenge was carried out by immersion at 7 days post-injection and challenged shrimps from each group were distributed in 3 replicate tanks (n = 30). Survival from each group was recorded daily for 11 days. For expression analysis of innate immune related genes, nine shrimps were used from each group having three individuals from each replicate. Heart and lymphoid organ (LO) from shrimps were collected at 1, 3 and 7 days after WSSV infection for RNA extraction and cDNA synthesis.

2.4. Total RNA isolation and cDNA synthesis

Total RNA was extracted from heart and LO of kuruma shrimp using ISOGEN (Nippon Gene, Osaka, Japan) in accordance with the manufacturer's instructions. The amount of nucleic acid in each total RNA sample was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). The purity of each total RNA sample was assessed by measuring the ratio of O.D. 260 nm/O.D. 280 nm. cDNA was synthesized from 1.0 µg of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) following the manufacturer's instructions, and this cDNA was used as a template for polymerase chain reaction (PCR).

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

Expression of innate immune-related genes in heart and lymphoid organ (LO) of WSSV challenged shrimps injected with combination of rVP28 and poly I:C or imiquimod from the third trial was determined. PCR was conducted with the primer combinations according to the protocol of Kono et al. (2014). The immune-related genes (Mj Rab7, Mj penaeidin, Mj crustin, Mj lysozyme, Mj TNF and Mj Toll) and the internal control gene β -actin, their respective primers with GenBank accession numbers are presented in Table 1. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under a transilluminator. The immune-related gene/ β -actin ratio was determined by densitometry; specifically, the photo-stimulated luminescence values were measured using Science Lab99 Image Gauge software (Fujifilm, Tokyo, Japan) (Biswas et al., 2012; Kono et al., 2010).

2.6. Statistical analysis

Differences between the final survival rates of WSSV challenged shrimps and quantified relative expressions of a particular gene in immunized and control group shrimps at each time interval were evaluated with ANOVA followed by Tukey's test using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL). All data are expressed as mean $\pm$ standard deviation (S.D.).

3. Results

3.1. Survival of immunized shrimps after WSSV challenge

The highest survival of 80% was obtained in the group immunized with 5 µg shrimp⁻¹ of rVP28, whereas the groups injected with 2.5 and 1 µg shrimp⁻¹ had 35 and

20% survival, respectively (Fig. 1). The negative control group (PBS injected) had a survival of 30%.

The second trial investigated the protective effects of poly I:C and imiquimod against WSSV in kuruma shrimp. The highest survival (43.3%) was obtained in the group immunized with poly I:C, while imiquimod injected group had 37.9% survival at 12 days post challenge (Fig. 2). However, a significantly lower survival of 13.3% was observed in PBS injected control group ($P<0.05$).

In the third trial, at 11 days post challenge survival was 52.1 and 57.9% after experimental challenge with WSSV in kuruma shrimps immunized with rVP28 (2.5 μ g) + poly I:C and rVP28 (2.5 μ g) + imiquimod, respectively (Fig. 3). There was a significantly lower survival of 35 and 33.3% in shrimp groups received only rVP28 (2.5 μ g) and PBS, respectively.

3.2. Expression of immune-related genes in rVP28+adjuvant injected shrimps

In immunized shrimps, the expression of Rab7 was significantly increased in heart and LO at almost all the time points post treatment when compared to the control group (Fig. 4A). Expression of antimicrobial peptide (AMP) genes (Mj lysozyme, Mj penaeidin and Mj crustin) were significantly elevated in shrimps immunized with rVP28 (2.5 μ g), rVP28 (2.5 μ g) + poly I:C or rVP28 (2.5 μ g) + imiquimod (Fig. 4B; Fig. 5A,B). Mj Toll and Mj TNF genes were significantly higher expressed at all the time points in LO of shrimps injected with rVP28 (2.5 μ g), rVP28 (2.5 μ g) + poly I:C or rVP28 (2.5 μ g) + imiquimod when compared to the control group (Fig. 6A,B). However, expression of these genes in heart of shrimps received rVP28 (2.5 μ g), or rVP28 (2.5 μ g) + imiquimod was elevated when compared to the control group (Fig. 6A,B).

4. Discussion

WSSV is one of the most dreaded viral diseases for cultured shrimps in the world. In our study, the protective effects of rVP28 against WSSV in kuruma shrimp significantly increased when administered in combination of poly I:C or imiquimod after 7 days post immunization. Similarly, Rajeshkumar et al. (2009) showed that an oral delivery of chitosan nanoparticles encapsulated DNA construct (pVP28) to *P. monodon* provided a significant protection against experimental challenge with WSSV.

The present study also demonstrated the protective immunity against WSSV by immunization with the poly I:C or imiquimod at 7 days post injection in kuruma shrimps. Recently, it was reported that fish immunization using poly I:C offers protection against nodavirus (Nishizawa et al., 2009). The efficacy of poly I:C immunization has been experimentally confirmed for infectious hematopoietic necrosis in rainbow trout, *Oncorhynchus mykiss* (Kim et al., 2009) and viral nervous necrosis (VNN) in sevenband grouper, *Epinephelus septemfasciatus* (Nishizawa et al., 2009). In mammals, several previous studies have demonstrated the antiviral effect of imiquimod against Rift Valley fever virus and Banzi virus (Harrison et al., 1994; Perry and Lamb, 1999; Hengge et al., 2000). Moreover, imiquimod could enhance the effectiveness of Herpes Simplex Virus (HSV) immunization, especially in reducing recurrent HSV disease in guinea pig (Bernstein et al., 1993). In fish, it has been observed that an imiquimod derivative S-27609 induces antiviral activities through the TLR7-MyD88-dependent signaling pathway (Kileng et al., 2008).

Results of the present study showed that survival enhanced from 35% to 80% as dose of rVP28 increased from 2.5 to 5 µg. These results are consistent with a previous study that showed survival rate as dose-dependent, increasing from 60.7 to 80.3% as the dose of rVP28 increased from 1 to 50 mg shrimp⁻¹. At a dose of 50 mg shrimp⁻¹, the recombinant protein provided protection as early as 1 day after feeding (72.5% survival) (Caipang et al., 2008).

In this study, WSSV were used for challenging the shrimps injected with rVP28 (2.5 µg), rVP28 (2.5 µg) + poly I:C or rVP28 (2.5 µg) + imiquimod. From the results, immune-related genes were indeed significantly up-regulated in heart and LO of WSSV-infected shrimps at 1, 3 or 7 days post infection. A significantly higher level of Rab7 gene expression in heart and LO was observed at all time points when compared to the control group. Previous reports exhibited an elevation in protection against WSSV infection by the injection of recombinant Rab7 protein, which plays an important role for the attachment of WSSV at the early infection stage (Sritunyalucksana et al., 2006). In another study, expression of Mj Rab7 gene involved in WSSV infection was significantly increased in the intestine compared with that of control shrimps at all the time periods after vaccination (Kono et al., 2010). Similarly, in our previous study, oral delivery of rVP28 caused an up-regulation of Rab7 gene in heart and LO of kuruma shrimps (Kono et al., 2014). The AMPs with their multiple and complementary properties have an

essential role in defense against a wide variety of pathogens in penaeid shrimps (Destoumieux et al., 1997; O'Leary and Gross, 2006; Cuthbertson et al., 2008). Our results indicated that expression of AMP genes (Mj lysozyme, Mj penaeidin and Mj crustin) in shrimp immunized with rVP28 (2.5 µg), rVP28 (2.5 µg) + poly I:C or rVP28 (2.5 µg) + imiquimod was significantly higher in heart and LO than that of the negative control group. Previously, Kono et al. (2010) reported that expression of these AMP genes was significantly increased in intestine and lymphoid organ of kuruma shrimps after immunization with WSSV DNA vaccine. However, our previous study of oral delivery of rVP28 to kuruma shrimps exhibited an up-regulation of Mj lysozyme and Mj penaeidin genes in heart and LO (Kono et al., 2014). We found that the expression of Mj Toll and Mj TNF was significantly increased at all time points in LO of shrimps immunized with rVP28 (2.5 µg), rVP28 (2.5 µg) + poly I:C or rVP28 (2.5 µg) + imiquimod when compared to the control group. Our study disagreed with previous WSSV-infection experiment revealing that *F. chinensis* Fc Toll expression level had diminished considerably soon after viral challenge (Yang et al., 2008).

In conclusion, the results of the present study suggest that injection administration of poly I:C or imiquimod + sub-unit protein (rVP28) provides significant protection and induces immune response in kuruma shrimps against WSSV. Moreover, poly I:C and imiquimod have potentials to be used as adjuvants or immunostimulants in shrimp immunization. Further studies may focus on optimizing the combination of rVP28 and poly I:C or imiquimod, investigating the effect of their oral delivery and also combination of DNA vaccine construct with poly I:C or imiquimod to protect shrimp against WSSV.

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Figure Legends

Fig. 1. Survival (%) of shrimps injected with different doses of rVP28 (5.0, 2.5, 1.0 μg shrimp⁻¹) and PBS is plotted against the time after WSSV challenge. Small letters besides the final survival at 11 days post challenge indicate significant difference ($P<0.05$) among different injected groups.

Fig. 2. Survival (%) of shrimps injected with 100 μL of poly I:C and imiquimod (100 μg shrimp⁻¹) and PBS is plotted against the time after WSSV challenge. Small letters besides the final survival at 12 days post challenge indicate significant difference ($P<0.05$) among different injected groups.

Fig. 3. Survival (%) of shrimps injected with rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) and PBS is plotted against the time after WSSV challenge. Small letters besides the final survival at 11 days post challenge indicate significant difference ($P<0.05$) among different injected groups.

Fig. 4. Effects of injection immunization of rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) on Mj Rab7 (A) and Mj lysozyme (B) gene expressions of kuruma shrimp (*M. japonicus*: Mj) in heart and lymphoid organ at 1, 3 and 7 days post WSSV challenge. Data indicate innate Mj Rab7 or Mj lysozyme gene PCR product after normalization against β -actin gene product. Data are presented as mean $\pm$ S.D. ($n = 9$ shrimps). Asterisks indicate significant difference (** $P<0.01$; * $P<0.05$) in immunized groups at different time points compared to the PBS injected group (Con).

Fig. 5. Effects of injection immunization of rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) on Mj penaeidin (A) and Mj crustin (B) gene expressions of kuruma shrimp (*M. japonicus*: Mj) in heart and lymphoid organ at 1, 3 and 7 days post WSSV challenge. Data indicate innate Mj Rab7 or Mj lysozyme gene PCR product after normalization against β -actin gene product. Data are presented as mean $\pm$ S.D. ($n = 9$ shrimps). Asterisks indicate significant difference (** $P<0.01$; * $P<0.05$) in immunized groups at different time

points compared to the PBS injected group (Con).

Fig. 6. Effects of injection immunization of rVP28 (2.5 µg), rVP28 (2.5 µg) + poly I:C (100 µg), rVP28 (2.5 µg) + imiquimod (100 µg) on Mj Toll (A) and Mj TNF (B) gene expressions of kuruma shrimp (*M. japonicus*: Mj) in heart and lymphoid organ at 1, 3 and 7 days post WSSV challenge. Data indicate innate Mj Rab7 or Mj lysozyme gene PCR product after normalization against β-actin gene product. Data are presented as mean ± S.D. (n = 9 shrimps). Asterisks indicate significant difference (** $P < 0.01$) in immunized groups at different time points compared to the PBS injected group (Con).

Table 1

Primers used for expression analysis in this study.

Name	Sequence (5'→3')	Length (mer)	Accession no.
Mj Rab7 Fw ^a	CTCGCAAGAAGATTCTCCTG	20	AB379643
Mj Rab7 Rv ^b	CTTCGTTGATACCGCCCTAT	20	
Mj lysozyme Fw	TCCTAATCTAGTCTGCAGGGA	21	AB080238
Mj lysozyme Rv	CTAGAATGGGTAGATGGA	18	
Mj penaeidin Fw	GCTGAACCCACTATAGTCTTT	21	AU175636
Mj penaeidin Rv	CTACCATGGTGATGAAACAAA	20	
Mj crustin Fw	CATGGTGGTGGCTTAGGAAA	19	AB121740
Mj crustin Rv	GTAGTCGTTGGAGCAGGTTA	20	
Mj Toll Fw	TCTTTCTGGTGTTTTAGCTACTGTAA	26	AB33379
Mj Toll Rv	TTTGATGAGAGCACGACAATC	21	
Mj TNF Fw	AAGAAAACCCCCAGGAAGAA	20	AB385697
Mj TNF Rv	AACCAGTGTGCACTCCAGA	19	

^aFw= Forward; ^bRv= Reverse

Figure(s)

Fig. 1

Figure(s)

Fig. 2

Figure(s)

Fig. 3

Figure(s)

Fig. 4

Figure(s)

Fig. 5

Figure(s)

Fig. 6