Aquaculture 446 (2015) 236–241

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

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

Tomoya Kono [a], Gouranga Biswas [b,1], Jean Fall [c], Tohru Mekata [d], Jun-ichi Hikima [a], Toshiaki Itami [a], Masahiro Sakai [a,*]

[a] Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan
[b] Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan
[c] Institute of Fisheries and Aquaculture, University Cheikh, Austra Diop, UCAD II, Dakar BP5005, Senegal
[d] National Research Institute of Aquaculture, Tsuiura, Kamiura, Saiki City, Oita 879-2602, Japan

ARTICLE INFO

Article history:
Received 16 March 2015
Received in revised form 14 April 2015
Accepted 28 April 2015
Available online 5 May 2015

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

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 the heart and lymphoid organ of combined rVP28 + poly I:C or imiquimod immunized shrimps at 1, 3 and 7 days after WSSV challenge. Shrimps which 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.

Statement of relevance: Major contributors to economic losses in shrimp aquaculture are viral diseases, of which white spot syndrome virus (WSSV) is the most important one due to its rapid spread and economic impact. In this paper, we present an immunization method towards prevention of WSSV disease in kuruma shrimp using adjuvants such as poly I:C or imiquimod along with a sub-unit protein (rVP28).

1. Introduction

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

Adjuvants are helper substances that are added to vaccine formulations in order to improve immune response to the incorporated antigen (Bowden et al., 2003). In the conventional vaccines, adjuvants are used to elicit an early, high and long-lasting immune response. Numerous compounds are under evaluation as immunological adjuvants and peptide-carriers to improve the immune response. The double-stranded synthetic RNA, poly I:C has been widely used as a viral mimic to examine immune responses in vertebrates (Huang et al., 2006). In shrimps, antiviral immunity is induced by double-stranded RNA (dsRNA) that confers protection against WSSV infection (Robalino et al., 2004). Although the exact mechanism of action is unknown, imiquimod, a nucleoside analog of imidazoquinoline is an agonist for

* Corresponding author. Tel./fax: +81 985 587219.
E-mail address: m.sakai@cc.miyazaki-u.ac.jp (M. Sakai).
[1] Present address: Kakdwip Research Centre of Central Institute of Brackishwater Aquaculture (ICAR), Kakdwip, South 24 Parganas, West Bengal, PIN-743347, India.

http://dx.doi.org/10.1016/j.aquaculture.2015.04.033
0044-8486/

toll-like receptors (TLRs) 7 and 8 (Schön and Schön, 2007). In mammalian models, imiquimod acts on induction of cytokines, such as interferon alpha (IFN-α), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) (Gupta and Khandelwal, 2004). The desire for new and improved adjuvants stems not only from the need to make existing inactivated vaccines more potent, but also to gain features such as antigen-spreading ability, stimulation of T-cell immunity, and longer-lasting protective immunity. Therefore, the benefits of 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-rVP28 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 rVP28 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) 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. The 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 the 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 the heart and 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), the internal control gene β-actin, and their respective primers with GenBank accession numbers are presented in Table 1. PCR products were separated by 1.5% agarose

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	AB333779
Mj Toll Rv	TTTGATGAGAGCACGACAATC	21	
Mj TNF Fw	AAGAAAACCCCCAGGAAGAA	20	AB385697
Mj TNF Rv	AACCAGTGTGCACTCCAGA	19	

[a] Fw = Forward.
[b] Rv = Revers.

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 ± 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 μg) + poly I:C and rVP28 (2.5 μg) + imiquimod, respectively (Fig. 3). There was a significantly lower survival of 35 and 33.3% in shrimp groups which received only rVP28 (2.5 μg) 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 the 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 μg), rVP28 (2.5 μg) + poly I:C or rVP28 (2.5 μg) + imiquimod (Figs. 4B and 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 μg), rVP28 (2.5 μg) + poly I:C or rVP28 (2.5 μg) + imiquimod when compared to the control group (Fig. 6A,B). However, expression of these genes in the heart of shrimps which received rVP28 (2.5 μg), or rVP28 (2.5 μg) + imiquimod was elevated when compared to the control group (Fig. 6A,B).

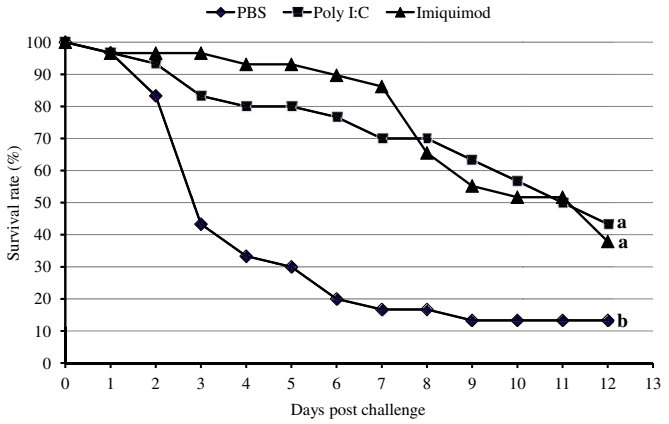

Fig. 2. Survival (%) of shrimps injected with 100 μL of poly I:C and imiquimod (100 μg shrimp^{-1}) and PBS is plotted against the time after WSSV challenge. Small letters besides the final survival at 12 days post challenge indicate significant difference ($P < 0.05$) among different injected groups.

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 7 days post immunization. Similarly, Rajeshkumar et al. (2009) showed that an oral delivery of chitosan nanoparticles that 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

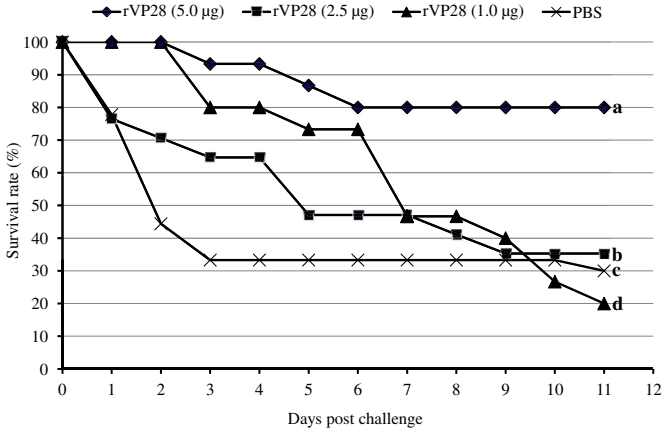

Fig. 1. Survival (%) of shrimps injected with different doses of rVP28 (5.0, 2.5, 1.0 μg shrimp^{-1}) and PBS is plotted against the time after WSSV challenge. Small letters besides the final survival at 11 days post challenge indicate significant difference ($P < 0.05$) among different injected groups.

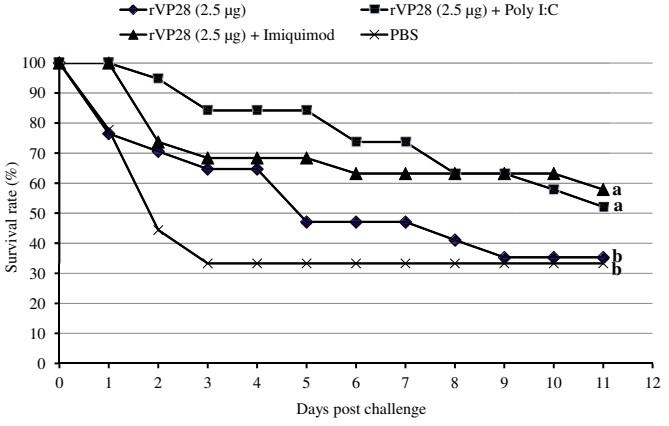

Fig. 3. Survival (%) of shrimps injected with rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) and PBS is plotted against the time after WSSV challenge. Small letters besides the final survival at 11 days post challenge indicate significant difference ($P < 0.05$)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

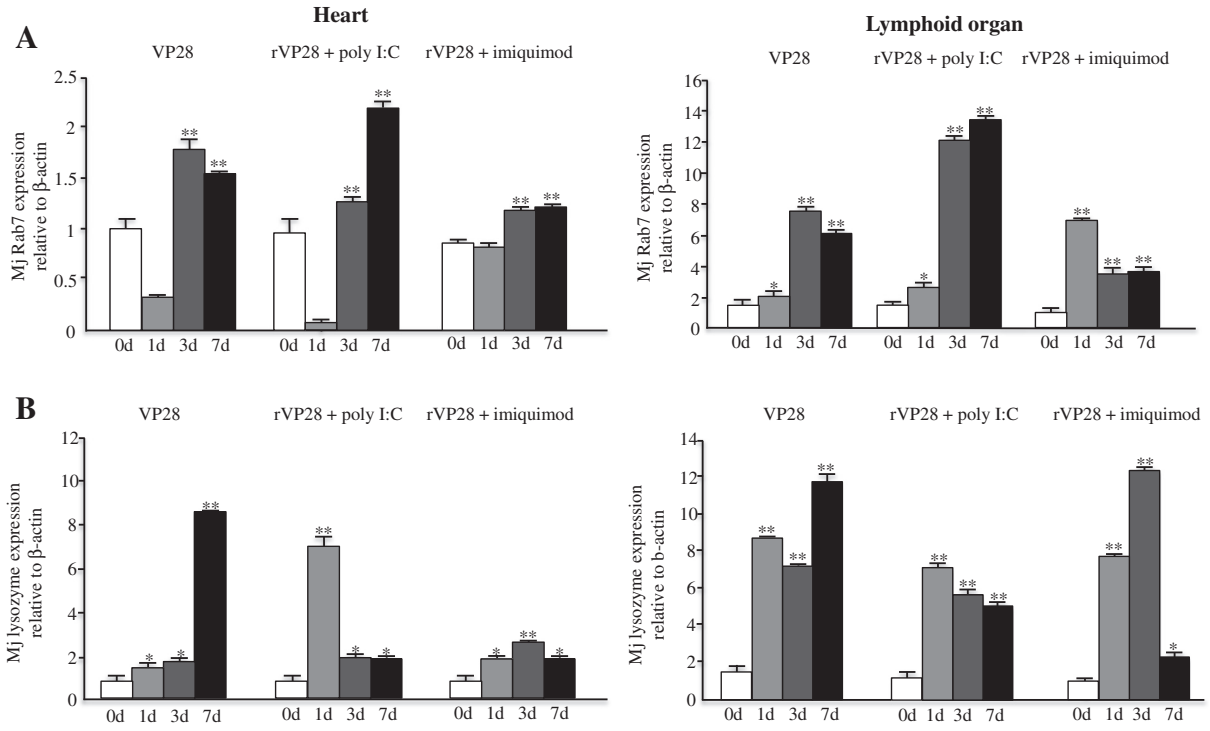

Fig. 4. Effects of injection immunization of rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) on Mj Rab7 (A) and Mj lysozyme (B) gene expressions of kuruma shrimp (*M. japonicus*: Mj) in the heart and lymphoid organ at 1, 3 and 7 days post WSSV challenge. Data indicate 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 (**$P < 0.01$; *$P < 0.05$) in immunized groups at different time points compared to the control (0d).

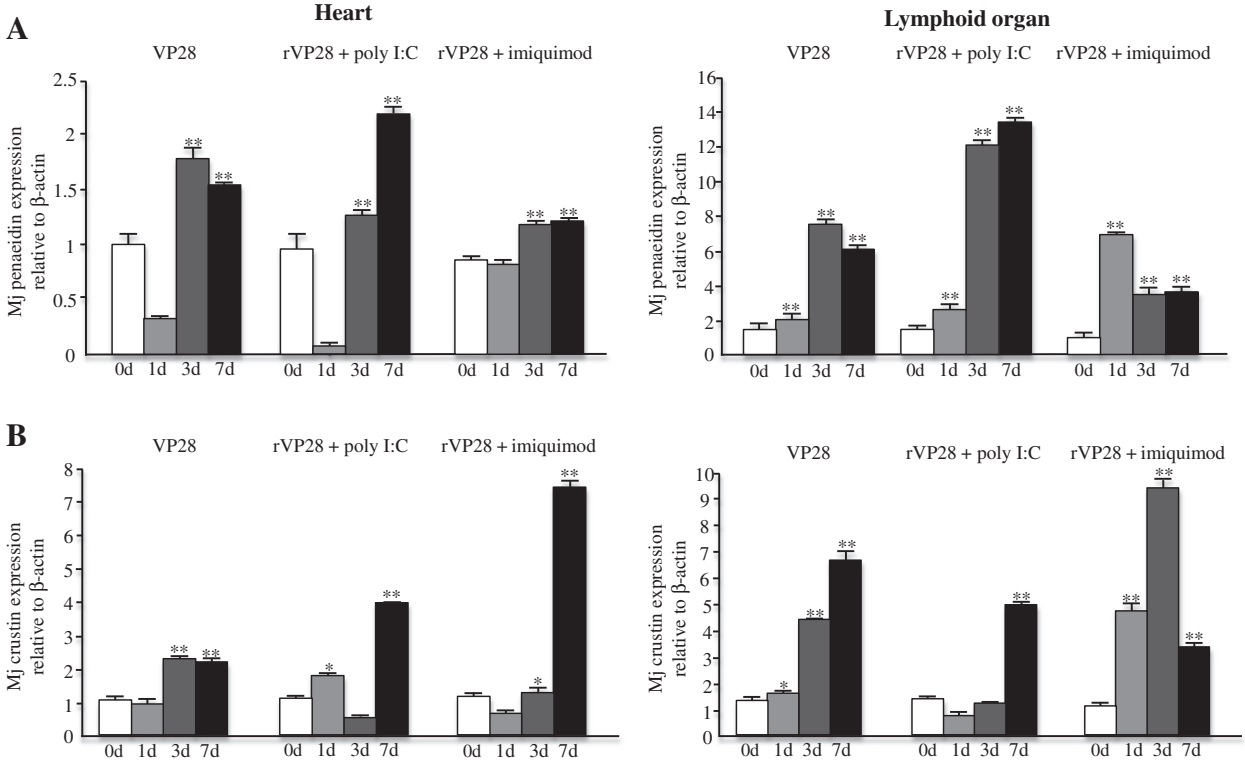

Fig. 5. Effects of injection immunization of rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) on Mj penaeidin (A) and Mj crustin (B) gene expressions of kuruma shrimp (*M. japonicus*: Mj) in the heart and lymphoid organ at 1, 3 and 7 days post WSSV challenge. Data 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 (**$P < 0.01$; *$P < 0.05$) in immunized groups at different time points compared to the control (0d).

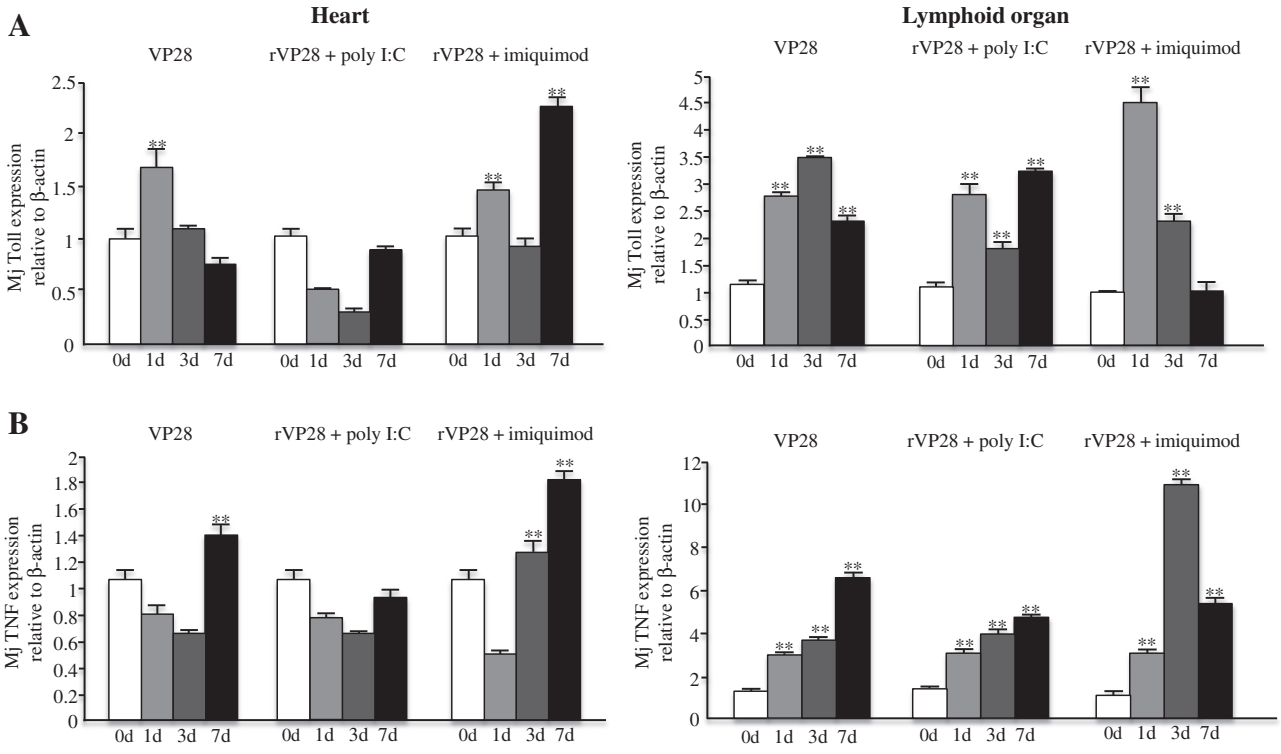

Fig. 6. Effects of injection immunization of rVP28 (2.5 μg), rVP28 (2.5 μg) + poly I:C (100 μg), rVP28 (2.5 μg) + imiquimod (100 μg) on Mj Toll (A) and Mj TNF (B) gene expressions of kuruma shrimp (*M. japonicus*: Mj) in the heart and lymphoid organ at 1, 3 and 7 days post WSSV challenge. Data indicate 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 control (0d).

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; Hengge et al., 2000; Perry and Lamb, 1999). 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 the heart and LO of WSSV-infected shrimps at 1, 3 or 7 days post infection. A significantly higher level of Rab7 gene expression in the 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 the 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 (Cuthbertson et al., 2008; Destoumieux et al., 1997; O'Leary and Gross, 2006). 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 the 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 the 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 the 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.

Acknowledgments

This research was partially supported by an A-STEP program, Feasibility study stage, Exploratory research (contract no. AS242Z01684N) of Japan Science and Technology Agency (JST). GB received a scholarship from the MEXT (Monbukagakusho), Japan during this study.

References

Bernstein, D.I., Miller, R.L., Harrison, C.J., 1993. Effects of therapy with an immunomodulator (imiquimod, R-837) alone and with acyclovir on genital HSV-2 infection in guinea-pigs when begun after lesion development. Antivir. Res. 20, 45–55.

Biswas, G., Korenaga, H., Nagamine, R., Kono, T., Shimokawa, H., Itami, T., Sakai, M., 2012. Immune stimulant effects of a nucleotide-rich baker's yeast extract in the kuruma shrimp, *Marsupenaeus japonicus*. Aquaculture 366–367, 40–45.

Bowden, T.J., Adamson, K., MacLachlan, P., Pert, C.C., Bricknell, I.R., 2003. Long-term study of antibody response and injection-site effects of oil adjuvants in Atlantic halibut (*Hippoglossus hippoglossus* L). Fish Shellfish Immunol. 14, 363–369.

Caipang, C.M., Verjan, N., Ooi, E.L., Kondo, H., Hirono, I., Aoki, T., Kiyono, H., Yuki, Y., 2008. Enhanced survival of shrimp, *Penaeus* (*Marsupenaeus*) *japonicus* from white spot syndrome disease after oral administration of recombinant VP28 expressed in *Brevibacillus brevis*. Fish Shellfish Immunol. 25, 315–320.

Cuthbertson, B.J., Deterding, L.J., Williams, J.G., Tomer, K.B., Etienne, K., Blackshear, P.J., Büllesbach, E.E., Gross, P.S., 2008. Diversity in penaeidin antimicrobial peptide form and function. Dev. Comp. Immunol. 32, 167–181.

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., Bachére, E., 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. 272, 28398–28406.

FAO, 2014. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations, Rome, Italy.

Gupta, D., Khandelwal, R.L., 2004. Modulation of insulin effects on phosphorylation of protein kinase B and glycogen synthesis by tumor necrosis factor-α in HepG2 cells. Biochim. Biophys. Acta 1671, 51–58.

Harrison, C.J., Miller, R.L., Bernstein, D.I., 1994. Post-therapy suppression of genital herpes simplex virus (HSV) recurrences and enhancement of HSV-specific T-cell memory by imiquimod in guinea pigs. Antimicrob. Agents Chemother. 38, 2059–2064.

Hengge, U.R., Goos, M., Arndt, R., 2000. Topical treatment of warts and mollusca with imiquimod. Ann. Intern. Med. 132, 95.

Huang, C.C., Duffy, K.E., San Mateo, L.R., Amegadzie, B.Y., Sarisky, R.T., Mbow, M.L., 2006. A pathway analysis of poly (I:C)-induced global gene expression change in human peripheral blood mononuclear cells. Physiol. Genomics 26, 125–133.

Johnson, K.N., van Hulten, M.C.W., Barnes, A.C., 2008. "Vaccination" of shrimp against viral pathogens: phenomenology and underlying mechanisms. Vaccine 26, 4885–4892.

Kileng, O., Albuquerque, A., Robertsen, B., 2008. Induction of interferon system genes in Atlantic salmon by the imidazoquinoline S-27609, a ligand for toll-like receptor 7. Fish Shellfish Immunol. 24, 514–522.

Kim, C.S., Kosuke, Z., Nam, Y.K., Kim, S.K., Kim, K.H., 2007. Protection of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. Fish Shellfish Immunol. 23, 242–246.

Kim, H.J., Oseko, N., Nishizawa, T., Yoshimizu, M., 2009. Protection of rainbow trout from infectious hematopoietic necrosis (IHN) by injection of infectious pancreatic necrosis virus (IPNV) or poly(I:C). Dis. Aquat. Org. 83, 105–113.

Kimbrell, D.A., Beutler, B., 2001. The evolution and genetics of innate immunity. Nat. Rev. Genet. 2, 256–267.

Kono, T., Sonoda, K., Kitao, Y., Mekata, T., Itami, T., Sakai, M., 2009. The expression analysis of innate immune-related genes in kuruma shrimp *Penaeus japonicus* after DNA vaccination against penaeid rod-shaped DNA virus. Fish Pathol. 44, 94–97.

Kono, T., Fall, J., Korenaga, H., Takayama, H., Iizasa, T., Mekata, T., Itami, T., Sakai, M., 2010. Immunomodulation by DNA vaccination against white spot syndrome virus (WSSV). Asian Fish. Sci. 23, 435–446.

Kono, T., Fall, J., Korenaga, H., Sudhakaran, R., Biswas, G., Mekata, T., Itami, T., Sakai, M., 2014. Recombinant VP28 produced by cell-free technique confers protection in kumruam shrimp (*Marsupenaeus japonicus*) against white spot syndrome virus. Turk. J. Fish. Aquat. Sci. 14, 547–555.

Lotz, J.M., 1997. Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. World J. Microbiol. Biotechnol. 13, 405–413.

Nishizawa, T., Takami, I., Kokawa, Y., Yoshimizu, M., 2009. Fish immunization using a synthetic double-stranded RNA Poly(I:C), an interferon inducer, offers protection against RGNNV, a fish nodavirus. Dis. Aquat. Org. 83, 115–122.

O'Leary, N.A., Gross, P.S., 2006. Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*. Gene 371, 75–83.

Otta, S.K., Shubha, G., Joseph, B., Chakraborty, A., Karunasagar, I., Karunasagar, I., 1999. Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. Dis. Aquat. Org. 38, 67–70.

Perry, C.M., Lamb, H.M., 1999. Topical imiquimod: a review of its use in genital warts. Drugs 58, 375–390.

Rajeshkumar, S., Venkatesan, C., Sarathi, M., Sarathbabu, V., Thomas, J., Anver Basha, K., Saahul Hameed, A.S., 2009. Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). Fish Shellfish Immunol. 26, 429–437.

Robalino, J., Browdy, C.L., Prior, S., Metz, A., Parnell, P., Gross, P., Warr, G., 2004. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J. Virol. 78, 10442–10448.

Rosenberry, B., 2001. World Shrimp Farming 2001. Shrimp News International, San Diego.

Schön, M.P., Schön, M., 2007. Imiquimod: mode of action. Br. J. Dermatol. 157 (Suppl. 2), 8–13.

Sritunyalucksana, K., Wannapapho, W., Lo, C.F., Flegel, T.W., 2006. PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. J. Virol. 80, 10734–10742.

Supamattaya, K., Hoffmann, R.W., Boonyaratpalin, S., Kanchanaphum, P., 1998. Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus pelagicus*, mud crab *Scylla serrata* and krill *Acetes* sp. Dis. Aquat. Org. 32, 79–85.

Yang, C., Zhang, J., Li, F., Ma, H., Zhang, Q., Jose Priya, T.A., Zhang, X., Xiang, J., 2008. A toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. Fish Shellfish Immunol. 24, 564–574.