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Full Length Research Paper

Expression of innate immune-related genes of Kuruma shrimp, *Marsupenaeus japonicus,* after challenge with *Vibrio nigripulchritudo*

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The expression of five innate immune-related genes crustin (MjCrus), lysozyme (MjLyz), penaeidin (MjPEN), Toll-like Receptors (MjToll), and Tumor Necrosis Factor (MjTNF) in the lymphoid organ (LO) and intestine of kuruma shrimp (*Marsupenaeus japonicus*) was investigated after challenge with *Vibrio nigripulchritudo*. Bacteria (1 x 10^5 CFU/ml) were injected into the second abdominal segment of the shrimp. The LO and intestine were isolated at 3, 12, 24, and 48 h post-injection, and total RNA was extracted. The expression of MjCrus, MjLyz, MjPEN MjToll, and MjTNF was determined by semi-quantitative RT-PCR. In the LO, expression levels of MjCrus, MjLyz, MjPEN, MjToll, and MjTNF were significantly higher in infected shrimp than in uninfected animals. The intestine of shrimp infected with *V. nigripulchritudo* exhibited higher expression of MjCrus, MjLyz, MjPEN, and MjTNF than did that of uninfected animals. However, intestinal MjToll expression levels were similar in both groups. These results suggested that MjCrus, MjLyz, MjPEN, MjToll, and MjTNF may play an important role in the immune defense of kuruma shrimp exposed to *V. nigripulchritudo*.

Key words: Kuruma shrimp *Marsupenaeus japonicus*, innate immune-related gene, infection, *Vibrio nigripulchritudo*.

INTRODUCTION

The production of important species of cultivated penaeid shrimp has increased exponentially since the early 1970s (Soonthornchai et al., 2010). In Japan, kuruma shrimp, *Marsupenaeus japonicus*, is one of the most valuable species of cultured aquatic animals. However, the occurrence of serious and problematic disease outbreaks is increasing rapidly (Tanticharoen et al., 2008). Vibriosis is the most common bacterial disease, causing mass mortality of cultured shrimp worldwide (Lavilla-Pitogo et al., 1998). The Vibrio species isolated from diseased shrimp include *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*, and *V. vulnificus* (Adams, 1991; Lavilla-Pitogo et al., 1998; Leangphibul et al., 1985; Lightner, 1988; Ruangpan and Kitao, 1991). The infection caused by *V. nigripulchritudo*, which was first isolated in New Caledonia in 1995, has been reported as the etiological agent causing high mortality in *Litopeanaeus stylirostris* (Goarant et al., 2004). On affected farms, *V. nigripulchritudo* were isolated from pond water, pond sediment, and renewal water (Goarant et al., 2004). Mass mortality of cultured kuruma shrimp infected with *V. nigripulchritudo* occurred at a shrimp farm in Kagoshima, Japan. High mortality was observed during July and August of 2005, the cumulative mortality was about 80%. The moribund shrimps displayed sluggish behavior and spiral swimming pattern, but they did not exhibit any gross external pathological abnormalities (Sakai et al., 2007).

Shrimps are vulnerable to a wide array of bacterial and viral pathogens. While shrimp do not possess an adaptive immune system, they possess an innate

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immune system that effectively protects against pathogens (Lee and Söderhäll, 2002). A better understanding of the innate immune system of shrimp will undoubtedly help us to develop strategies for disease control and sustainable shrimp farming. The lymphoid organ (LO) and intestine of penaeid shrimps are thought to have immune function. For example, Burgents et al. (2005) proposed that the LO has bacteriostatic effects, and Van de Braak et al. (2002) suggested that it may be the major phagocytic organ in shrimp. The intestine is a favorable site for invasion of pathogens carried in the water, food, and sediment (Jayabalan et al., 1982). It was previously demonstrated that an influx of hemocytes enters the intestine of *Penaeus monodon* following exposure to *V. harveyi*. Moreover, the hemocytes associated with the basal lamina of *S. ingentis* were reported to fight pathogens entering the body via the midgut (Chen et al., 1992).

To our knowledge, there is no information about how antimicrobial peptides (MjCrus, MjLyz, MjPEN), MjToll, and MjTNF respond when shrimp are confronted with *V. nigripulchritudo*. In order to design efficient strategies for disease control and insure the long-term survival of shrimp aquaculture, we investigated the expression of these genes in the LO and in the intestine of shrimp infected with *V. nigripulchritudo*.

MATERIALS AND METHODS

Bacterial strain

V. nigripulchritudo E15 strain used in this study was kindly provided by Kyushu Medical Co., Ltd, Kitakyushu, Fukuoka, Japan. The bacterium was grown in Marine Broth 2216 E (Difco, Detroit, Michigan, USA) at 27°C with continuous shaking overnight. Bacterial cells were harvested from stationary phase cultures and re-suspended in sterile saline solution. Cell counts were estimated from the optical density (O.D.) values at 600 nm, and the corresponding colony-forming units (CFU) were obtained from a serial dilution of bacterial culture grown on marine agar plates.

Bacterial infection

A preliminary pathogenicity test of *V. nigripulchritudo* E15 for kuruma shrimp was conducted. The artificial infection was done by intramuscular injection of 0.1 ml of four different bacterial suspensions containing 1 x 10^7, 1 x 10^6, 1 x 10^5, or 1 x 10^4 CFU/shrimp into the second abdominal segment of the shrimp. PBS was injected into a control group. In the high dose (107 CFU/ml) group, all shrimps died 1 day after challenge. However, at a dose of 105 CFU/ml half of the shrimps died at the time of the monitoring (7 days after injection). In the control group no mortality was observed (our unpublished data). Therefore, we decided to use 1 x 105 CFU/ml for the gene expression study.

Healthy kuruma shrimp (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 with running artificial seawater at 20°C and fed commercial diet (Higashimaru, Japan) once a day. The health status of experimental animals was further ascertained by culturing hemolymph and hepatopancreas from few sampled animals on Marine Broth 2216 E (Difco, Detroit, Michigan, USA) for the presence of any bacterial pathogens. To know whether shrimp LO and Intestine express some immune-related genes, ten healthy shrimp which were not given any infection (time 0) served as the control. The artificial infection was conducted by injecting the shrimp (n = 30) with 0.1 ml of bacterial suspension (1 x 105 CFU/ml). At 3, 12, 24, and 48 h post-injection, intestine and LO were isolated for total RNA extraction.

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

Total RNA was extracted from the LO and intestine 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 mg 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). Amplification of the EF-α1 gene was used as an internal control. All PCR reactions were performed according to the protocol of Kono et al. (2004): 1 µl cDNA was mixed with 5 µl buffer, 5 µl dNTPs (10 µM each dNTP), 0.5 µl Taq polymerase (5 units/µl), 5 µl each of gene-specific primer (5 µM), and 28.5 µl distilled water. The immune-related genes and the control, their respective primers, and the optimum conditions for each amplification reaction are shown in Table 1. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under a transilluminator. The expected size of each PCR product is listed in Table 1. The immune-related genes/EF-1α ratio was determined by densitometry; specifically, the photostimulated luminescence values were measured using Science Lab99 Image Gauge software (Fujifilm, Tokyo, Japan).

Transcriptional expression of shrimp immune genes after *V. nigripulchritudo* infection

Time-course reverse transcriptase polymerase chain reaction (RT-PCR) assays were used to investigate the transcriptional regulation of five shrimp immune-related genes during *V. nigripulchritudo* infection. For this assay, the expression levels of individual genes were first normalized using EF-α1 as an internal control and then expressed relative to the basal expression in the 0 h post-injection. sample, which was collected just before challenge.

Statistical analysis

The data obtained from the RT-PCR analysis were subjected to one way analysis of variance (ANOVA) using SPSS software 14; the ANOVA was followed by Tukey's test. Differences were considered significant at $P<0.05$.

RESULTS

Crustin

In the LO of challenged shrimp, the expression of MjCrus was significantly higher than that in unchallenged shrimp

Table 1. Primer sequences used in this study with their optimum conditions.

Primers	Sequence (5'-3')	Cycle No	An. Temp (°)	Amplicon size (bp)	Accession No.
MjLyz-F	TCCTAATCTAGTCTGCAGGGA	35	58	516	AB080238
MjLyz-R	CTAGAATGGGTAGATGGA				
MjPEN-F	GCTGCACCCACTATAGTCTTT	30	60	339	AU175636
MjPEN-R	CTACCATGGTGATGAAACAAA				
MjCrus-F	CATGGTGGTGGCTTAGGAAA	35	62	300	AB12174
MjCrus-R	GTAGTCGTTGGAGCAGGTTA				
MjToll-F	TCTTTCTGGTGTTTTAGCTACTGTAA	30	60	300	AB333779
MjToll-R	TTTGATGAGAGCACGACAATG				
MjTNF-F	AAGAAAACCCCCAGGAAGAA	30	60	324	M NM_165735
MjTNF-R	AACCAGTGTGCACTCCATGA				
MjEF1-α-F	GTCTTCCCCTTCAGGACGTA	25	55	373	AB458256
MjEF1-α-R	GAACTTGCAGGCAATGTGAG				

at 12 h post-injection (Figure 1a). However, in the intestine, MjCrus expression was significantly up-regulated at 3 h after *V. nigripulchritudo* challenge compared to the unchallenged group. In the intestine, significantly higher expression of MjCrus transcripts was also observed at 24 and 48 h post-injection. compared to the unchallenged group (Figure 1b).

Lysozyme

In the LO, significantly higher MjLyz expression was observed at 3 and 48 h post-injection (Figure 2a) relative to the controls. In the intestine, MjLyz expression was significantly higher at 3 h post-injection. compared with unchallenged group (Figure 2b).

Penaeidin

The expression of MjPEN in the LO was significantly higher in challenged than in unchallenged shrimps at 12 and 48 h post-injection (Figure 3a). In the intestine, MjPEN expression was significantly higher at 3 h post-injection. relative to controls and was induced to its highest expression at 12 h post-injection. (Figure 3b).

Toll-like receptor

In the LO of challenged shrimp, MjToll expression was significantly higher than in unchallenged animals from 3 h to 24 h post-injection and peaked at 12 h post-injection (Figure 4a). In the intestine, the expression of MjToll was not affected by *V. nigripulchritudo* infection (Figure 4b).

Tumor necrosis factor

The expression level of MjTNF in the LO was significantly higher in challenged animal from at 12 and 48 h post-injection (Figure 5a). In the intestine, the expression of MjTNF was significantly higher in challenged animals from 3 to 48 h post-injection. (Figure 5b).

DISCUSSION

Penaeidins are constitutively synthesized and stored in the shrimp hemocytes, localized in granulocyte cytoplasmic granules, and released in response to appropriate stimuli such as infection (Destoumieux et al., 2000). Analysis of expression levels in *L. vannamei* revealed that PEN3 expression decreased from 6 -12 h after microbial challenge and then increased to normal levels after 48 h post-injection. (Munoz et al., 2002). In contrast, our results showed that the expression of MjPEN was up-regulated in the LO 12 and 48 h after *V. nigripulchritudo* inoculation. Moreover, expression of MjPEN in the intestine of *V. nigripulchritudo* challenged shrimp was significantly higher than in unchallenged animals at 3 h post-injection. and was induced to its highest expression at 12 h post-injection. These observations confirmed the up-regulation of penaeidins after bacterial challenge, as seen with PEN2 and PEN4 after *V. harveyi* injection (Wang et al., 2010)

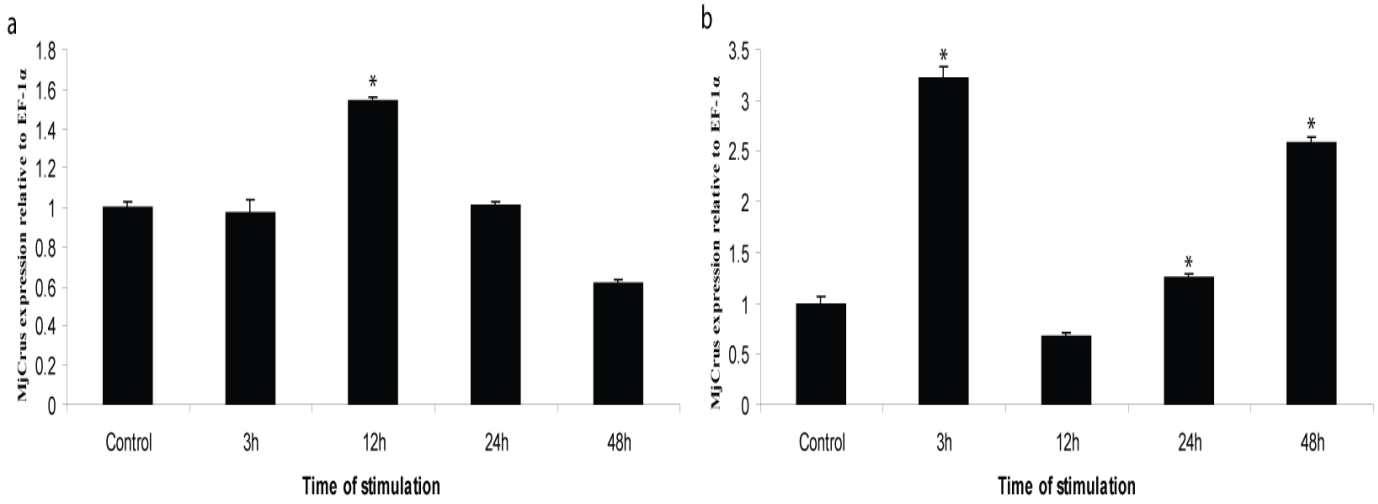

Figure 1. Expression of shrimp crustin gene in lymphoid organ (a) and intestine (b) at 3, 12, 24 and 48 h post *V. nigripulchritudo* infection. Data are presented as mean ± SD of triplicate samples. Relative expressions were normalized with elongation factor-1α (EF-1α) and the basal expression level at 0 h post-injection. Asterisks indicate significant up-regulation of the target gene in the *V. nigripulchritudo* treatment group compared to the unchallenged group (control) at the same time point (P<0.05).

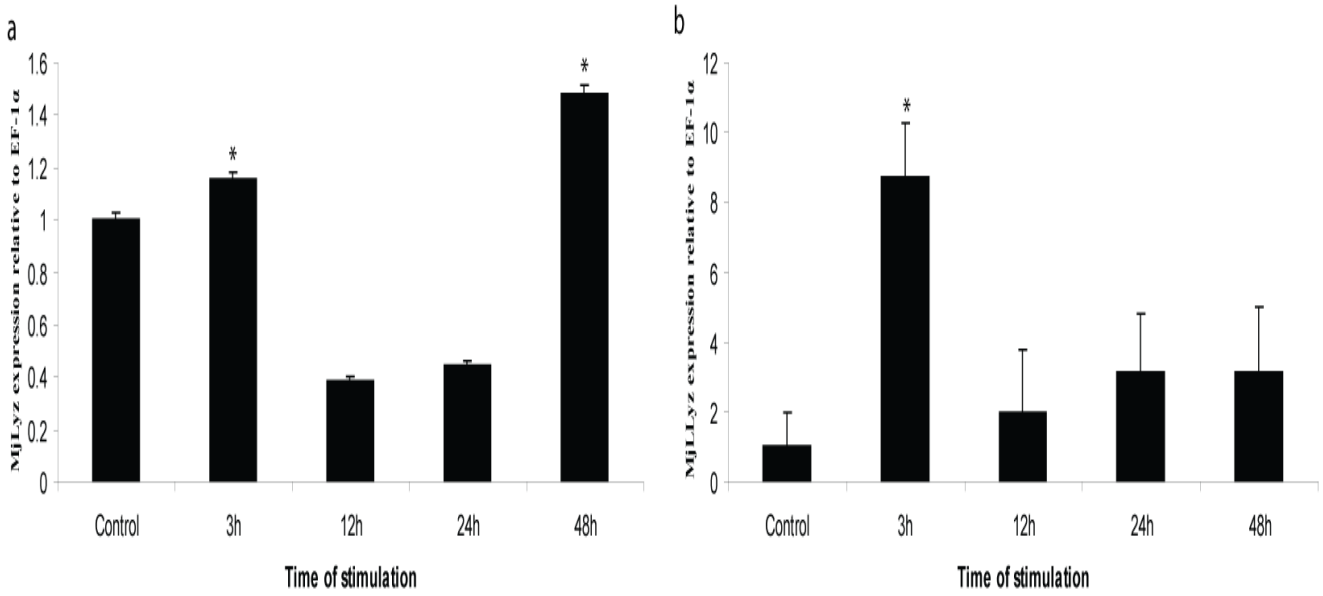

Figure 2. Expression of shrimp lysozyme gene in lymphoid organ (a) and intestine (b) at 3, 12, 24 and 48 h post *V. nigripulchritudo* infection. Data are presented as mean ± SD of triplicate samples. Relative expressions were normalized with elongation factor-1α (EF-1α) and the basal expression level at 0 h post-injection. Asterisks indicate significant up-regulation of the target gene in the V. nigripulchritudo treatment group compared to the unchallenged group (control) at the same time point (P<0.05).

and PEN5, a novel class, in *F. chinensis* (Kang et al., 2007). Hu et al. (2006) found that the expression level of PEN5 was induced within 3 h after *V. alginolyticus* or *Aerococcus viridans* injection and that a higher protein level of PEN5 was maintained up to 24 h after injection. Furthermore, the expression of MjPEN gene was significantly higher relative to controls in the LO and intestine of kuruma shrimp after injection of a DNA vaccine encoding viral envelope protein (VP28) of penaeid rod-shaped DNA virus (PRDV) (Kono et al., 2009). These results suggest that MjPEN is concerned with systemic innate immunity.

Crustins are antibacterial proteins containing a four-disulphide core (4DSC) or a whey acidic protein (WAP)

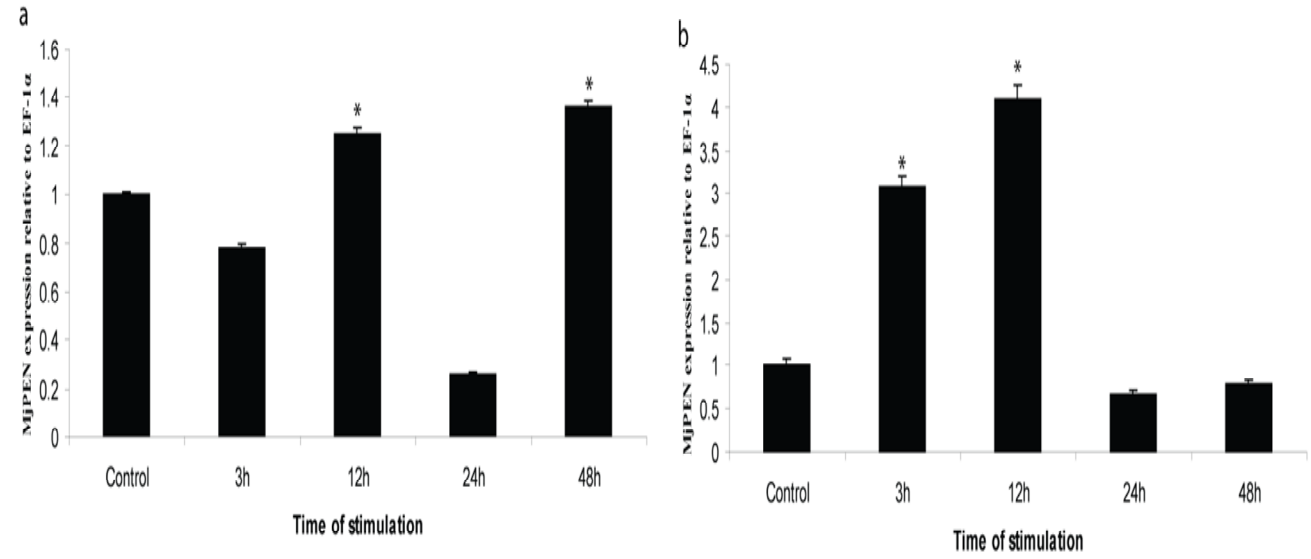

Figure 3. Expression of shrimp penaeidin gene in lymphoid organ (a) and intestine (b) at 3, 12, 24 and 48 h post *V. nigripulchritudo* infection. Data are presented as mean ± SD of triplicate samples. Relative expressions were normalized with elongation factor-1α (EF-1α) and the basal expression level at 0 h post-injection. Asterisks indicate significant up-regulation of the target gene in the V. nigripulchritudo treatment group compared to the unchallenged group (control) at the same time point (P<0.05).

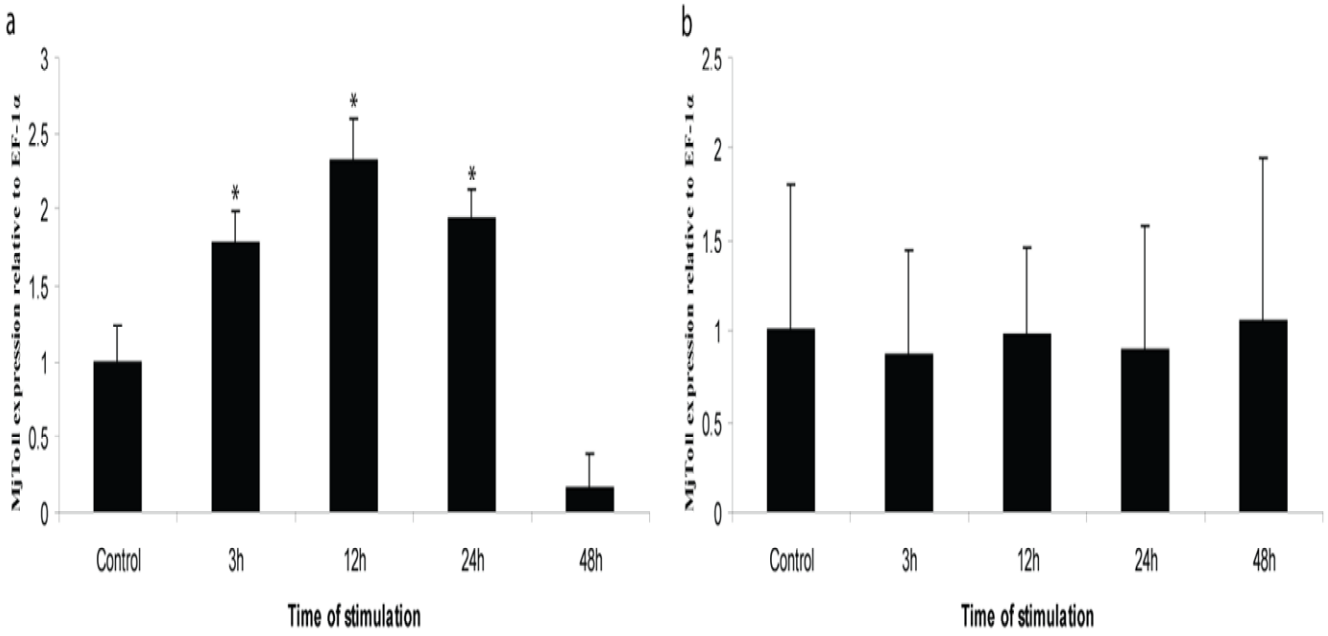

Figure 4. Expression of shrimp Toll receptor gene in lymphoid organ (a) and intestine (b) at 3, 12, 24 and 48 h post *V. nigripulchritudo* infection. Data are presented as mean ± SD of triplicate samples. Relative expressions were normalized with elongation factor-1α (EF-1α) and the basal expression level at 0 h post-injection. Asterisks indicate significant up-regulation of the target gene in the V. nigripulchritudo treatment group compared to the unchallenged group (control) at the same time point (P<0.05).

domain that are unique to crustacean species (Rattanachai et al., 2004). In *P. monodon*, the transcription level of crustin-like antimicrobial peptide (Crus-like Pm) was shown to be substantially elevated after *V. harveyi* injection (Amparyup et al., 2008). In addition, using dsRNA-mediated RNA interference, Shockey et al. (2009) demonstrated that crustin is involved in protective responses induced by *V. penaeicida* infection. Furthermore, Plcrustin1 from *P. leniusculus* was up-regulated after challenge with

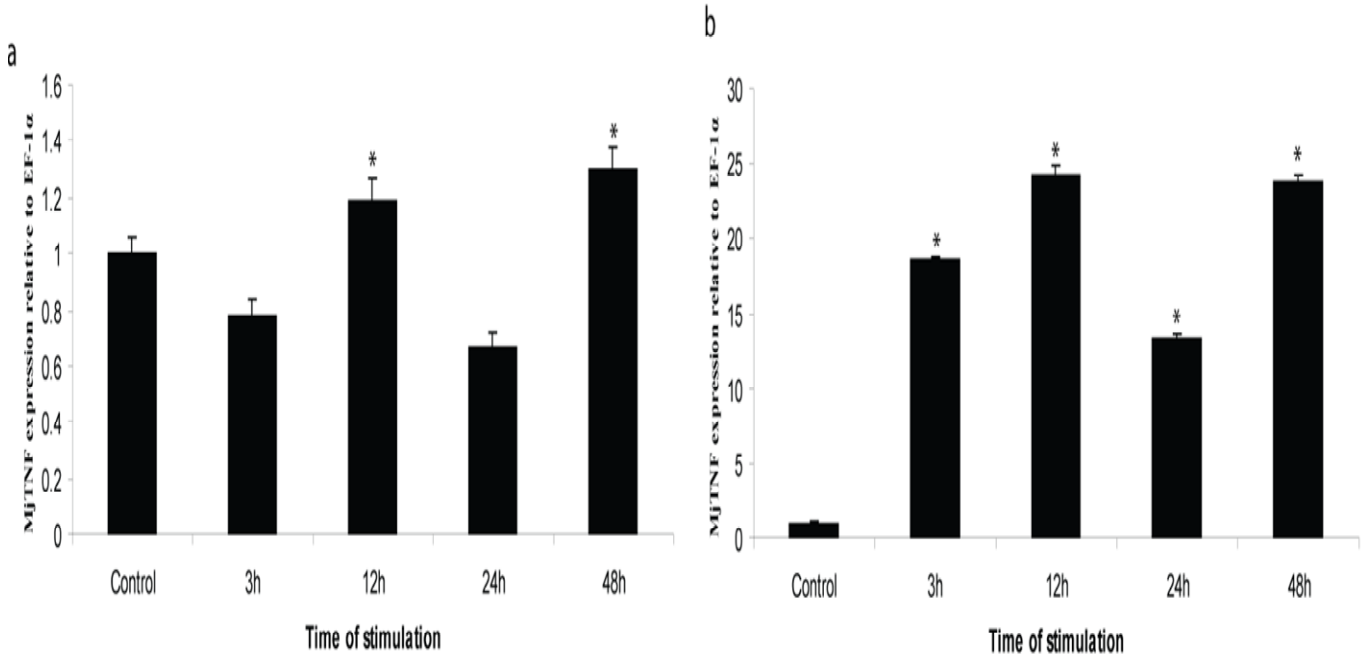

Figure 5. Expression of shrimp Tumor necrosis factor gene in lymphoid organ (a) and intestine (b) at 3, 12, 24 and 48 h post *V. nigripulchritudo* infection. Data are presented as mean ± SD of triplicate samples. Relative expressions were normalized with elongation factor-1α (EF-1α) and the basal expression level at 0 h post-injection. Asterisks indicate significant up-regulation of the target gene in the V. nigripulchritudo treatment group compared to the unchallenged group (control) at the same time point (P<0.05).

Escherichia coli or *Acinetobacter* sp. (Jiravanichpaisal et al., 2007). To assess the efficiency of DNA vaccine in kuruma shrimp, the expression of MjCrus in vaccinated shrimp was examined. The results suggested that MjCrus was significantly higher in the LO and intestine of kuruma shrimp after DNA vaccination (Kono et al., 2009). Similarly, in the current study, the *in vivo* expression of MjCrus was up-regulated 3 and 12 h after the *V. nigripulchritudo* challenge in the intestine and the LO, respectively. In contrast, Vargas-Albores et al. (2004) reported that injection of *V. alginolyticus* could cause a significant decrease of crustin I in *L. vannamei*. Moreover, injection of *L. anguillarum* caused a down-regulation of the expression of crustin-like peptides in *Homarus gammarus* within 3 - 6 h (Hauton et al., 2006). These results, together with the transcript expression pattern, indicate that the proteins in the crustin family have diverse functions (Vatanavicharn et al., 2009).

Lysozyme (muramidase, EC.3.2.1.17) is widely distributed among eukaryotes and prokaryotes. It is considered to be an integral component of the innate immune system, and protects against microbial infections (Ji et al., 2009). It catalyzes the hydrolysis of bacterial cell walls and acts as a nonspecific innate immunity molecule upon invasion of bacterial pathogens (Jollès and Jollès, 1984). The expression and antimicrobial activity of various lysozymes have been studied in penaeid shrimp (Shockey et al., 2009). The *in vivo* mRNA expression of lysozyme was significantly increased 36 h post-injection after *V. harveyi* stimulation (Wang et al., 2010). It was previously reported that expression of lysozyme transcripts increased after challenges with the Vibrio species *V. campbellii* and *V. alginolyticus*, in *L. vannamei* and *F. merguiensis* (Burgents et al., 2007; Mai and Hu, 2009). Recently, Kono et al. (2009) found that the expression of Mjlyz in the LO and intestine of kuruma shrimp increased significantly after DNA vaccination. These results are consistent with our observations that the MjLyz transcription was up-regulated 3 h after *V. nigripulchritudo* injection in both the LO and intestine. In the LO, the transcript level of MjLyz was the highest at 48 h post-injection. Taken together, these results suggest that lysozyme is an important component of the shrimp anti-gram-negative bacterial defense system.

Tolls and Toll-like receptors (TLRs) are recognized as major Pattern Recognition Receptors (PRRs), and they are involved in the signaling pathway for the innate immunity activation and are genetically conserved from insects to mammals (Mekata et al., 2008). Using real-time PCR assays, it was demonstrated that expression levels of *Fenneropenaeus chinensis* Toll gene (FcToll) were distinctly modulated after bacterial or viral stimulation, with significant up-regulation after 5 h post-*Vibrio anguillarum* challenge (Yang et al., 2008). *Litopenaeus vannamei* Toll gene (LvToll) was significantly up-regulated 24 h after challenge with *V. harveyi* (Wang et al., 2010). Our results showed that in the LO of kuruma shrimp MjToll expression was

up-regulated from 3 to 24 h after *V. nigripulchritudo* injection. In contrast, the expression level of MjToll in the intestine was not significantly affected by *V. nigripulchritudo* infection. Conversely, upon *in vitro* immuno-stimulation of shrimp LO tissue, MjToll was not modulated by lipopolysaccharide (LPS) or flagellin (Mekata et al., 2008). Taken together, these results suggest that MjToll might be involved in innate host defense, especially against the pathogen *V. nigripulchritudo*.

Tumor necrosis factors (TNFs) are potent inflammatory cytokines implicated in inflammation, apoptosis, cell proliferation, and a general stimulation of immune system of vertebrate. Recently, a tumor necrosis factor (TNF) gene has been isolated and characterized in kuruma shrimp, *M. japonicus*, providing the first conclusive evidence for the existence of the TNF ligand in shrimp (Mekata et al. 2010). They demonstrated that, in LO cells, a high expression level of MjTNF was observed *in vivo* 4 h after stimulation with *V. penaeicida*. Similarly, in the present study, the expression level of MjTNF in the LO was elevated 12 and 48 h after *V. nigripulchritudo* challenge. However, in the intestine, the expression level of MjTNF was elevated within 3 h after *V. nigripulchritudo* injection and an elevated level of MjTNF expression was maintained up to 48 h post-injection. The present study confirmed the existence of TNF genes in kuruma shrimp and suggested that MjTNF might play a role in the innate immune defense of shrimp.

In conclusion, our results suggested that the quantification of expression levels of MjCrus, MjLyz, MjPEN, MjToll, and MjTNF is useful for evaluating the immune status of *M. japonicus*. Our understanding of the innate immune response of shrimp against *V. nigripulchritudo* is still in its early stages. On the basis of our results, it will be of great interest to determine the expression profiles of these innate immune-related genes in *M. japonicus* in response to *in vivo* stimulation with immunostimulants such as lipopolysaccharide (LPS), β-Glucans or cytidine-phosphateguanosine (CpG) and its resistance against *V. nigripulchritudo*.

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