

## RESEARCH REPORT

**A-type CpG ODN with higher binding affinity to LvToll1 could probably activate downstream IFN system-like antiviral response in shrimp *Litopenaeus vannamei***J Xu<sup>1,3</sup>, D Zhao<sup>4</sup>, M Sun<sup>1,3</sup>, L Wang<sup>2</sup>, Z Jia<sup>1,3</sup>, R Liu<sup>1</sup>, L Wang<sup>1,3</sup>, L Song<sup>2</sup><sup>1</sup>Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China<sup>2</sup>Key Laboratory of Mariculture & Stock enhancement in North China's Sea, Ministry of Agriculture, Dalian Ocean University, Dalian 116023, China<sup>3</sup>University of Chinese Academy of Sciences, Beijing 100049, China<sup>4</sup>Dalian Polytechnic University, Dalian 116034, China

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**Abstract**

CpG oligodeoxynucleotides (CpG ODNs) is a widely used immune adjuvant, which could activate various immune responses including antiviral response through interaction with Toll-like receptor 9 (TLR9) in mammals. In the present study, four types of CpG ODN (CpG-A, CpG-B CpG-C, and CpG-P) were synthesized and injected to the shrimp *Litopenaeus vannamei* in order to evaluate their immune enhancement effect in shrimp. The copy numbers of white spot syndrome virus in the shrimps treated with different types of CpG ODNs were of  $3.10 \times 10^5$  (CpG-A),  $8.32 \times 10^5$  (CpG-B),  $9.84 \times 10^5$  (CpG-C), and  $8.12 \times 10^5$  (CpG-P) copies  $\text{ng}^{-1}$  DNA respectively, which were significantly lower ( $p < 0.01$ ) than that in PBS group ( $1.70 \times 10^6$  copies  $\text{ng}^{-1}$  DNA). Surface plasmon resonance (SPR) assay revealed that the four types of CpG ODN displayed different binding affinity to LvToll1, LvToll2 and LvToll3, and the highest binding affinity was observed between CpG-A and LvToll1. Correspondingly, the mRNA transcripts of LvTolls were up-regulated significantly in CpG-A stimulated shrimps, which was significantly higher than that in CpG-B, CpG-C and CpG-P groups ( $p < 0.01$ ). The phagocytic rate and ROS level of shrimp hemocytes in CpG-A and CpG-B groups increased significantly compared with that in other groups, which were 1.63-fold, 9.98-fold ( $p < 0.01$ ) in CpG-A and 1.60-fold, 4.92-fold ( $p < 0.01$ ) in CpG-B higher than those in PBS group, respectively. Moreover, after CpG-A stimulation, the probable IFN level in shrimp plasma increased to 2.60-fold ( $p < 0.01$ ) of that in PBS group, and the mRNA expressions of IFN system-like antiviral genes (LvIRF, LvVago4 and LvSTAT) were also significantly up-regulated in CpG-A group, displaying a stronger response than that in CpG-B, CpG-C and CpG-P groups. The results indicated that CpG-A could promote the cellular and humoral immunity in shrimp, and induce relatively higher antiviral immune response among the four CpG ODNs. It provided useful information to understand the stimulatory effects of CpG ODNs in shrimp, promoting the application of CpG ODNs in aquaculture.

**Key Words:** *Litopenaeus vannamei*; CpG ODNs; CpG-A; LvTolls; SPR; IFN**Introduction**

Cytosine phosphate guanine oligodeoxynucleotides (CpG ODNs) were originally defined for bacterial DNA with unmethylated CpG dinucleotides in certain flanking sequences (CpG motifs), which had diverse stimulatory effects on the

innate and adaptive immune system (Krieg, 2002). There are four types of CpG ODNs with distinct structural and biological properties, including A-type, B-type, C-type and P-type (Bode *et al.*, 2011). A-type CpG ODN (CpG-A) has a phosphodiester core flanked by phosphorothioate terminal nucleotides. It contains a single CpG motif flanked by palindromic sequences and poly G tails at the 3' and 5' ends. B-type CpG ODN (CpG-B) and C-type CpG ODN (CpG-C) are both composed entirely of phosphorothioate nucleotides, while they resemble CpG-A in containing palindromic CpG motifs and thus can form stem loop structures or dimmers.

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P-type CpG ODN (CpG-P) contains double palindromes that can form hairpins at the GC-rich 3' ends (Shirota and Klinman, 2014). These CpG ODNs mainly participate in activating intracellular signaling pathways and triggering the proliferation, maturation and differentiation of immune cells, consequently increasing the secretion of cytokines and chemokines with disease-resistant effects (Klinman, 2004; Krieg, 2006).

CpG motifs are considered as a kind of pathogen-associated molecular patterns (PAMPs) due to their abundance in microbial genomes and rarity in animal genomes (Krieg, 2006). The immunogenicities of CpG ODNs have been examined in numerous preclinical studies, demonstrating that CpG ODNs could enhance both humoral (Tafaghodi *et al.*, 2006; Borges *et al.*, 2008) and cellular (Th1 cells and cytotoxic lymphocyte) (Cho *et al.*, 2000; Shirota *et al.*, 2000; Shirota and Klinman, 2011) immunity against pathogens in mammals. Toll-like receptor 9 (TLR9) in mammals is proved to be the receptor of CpG ODNs (Hemmi *et al.*, 2000; Bauer *et al.*, 2001; Takeshita *et al.*, 2001). CpG ODNs could be recognized by TLR9 to promote the maturation of antigen presenting cells (APCs) and secretion of cytokines like type I interferon (IFN), interleukin (IL) or tumor necrosis factor (TNF) (Colonna *et al.*, 2004). The immune responses triggered by different types of CpG ODN have been widely reported in vertebrates and the mechanisms are diverse. For example, CpG-A and CpG-P could strongly induce type I IFN production, while CpG-B and CpG-C could trigger the production of TNF- $\alpha$  and IL-6, respectively (Shirota and Klinman, 2014). Further studies have demonstrated that CpG-A could induce the IFN regulatory factor-7 (IRF-7) dependent antiviral immunity by activation of the TLR9 in plasmacytoid dendritic cells in mice (Honda *et al.*, 2005). Because of the effects in stimulation of immunity, many completed clinical trials have suggested a promising application of CpG ODN as adjuvants (Shirota and Klinman, 2014).

Recently, CpG ODNs have also been reported to trigger humoral and cellular immune responses in invertebrates. In the bivalve mollusc, bacterial DNA with unmethylated CpG motif could promote the antibacterial activity, lysozyme activity and prophenoloxidase production of hemolymph (Hong *et al.*, 2006). In crustacean, CpG-rich fragment containing tandem ODNs enhanced the immuno-protection efficiency and induced autophagy of Chinese mitten crab, *Eriocheir sinensis* (Sun *et al.*, 2013, 2015). CpG ODNs could also promote the generation of new hemocytes, increase

the phagocytic ability and the reactive oxygen species (ROS) level in the hemocytes of *Litopenaeus vannamei* (Sun *et al.*, 2013). Although CpG ODNs have been reported to induce immune response in crustacean, the mechanism of immune enhancement triggered by different type of CpG ODN is still not well understood. It has been reported that CpG ODNs could activate the IFN-related antiviral pathway in mammals (Levy *et al.*, 2011) and teleosts (Su *et al.*, 2016), and poly (I:C)-induced antiviral immunity has also been reported in Pacific white shrimp *L. vannamei*, which is similar to the IFN system in mammals (Wang *et al.*, 2013; Li *et al.*, 2015). In shrimp *L. vannamei*, the *LvVago4* gene is considered to be a cytokine functionally similar to IFN, which could restricts virus infection through activating the JAK-STAT pathway; the IRF-like gene *LvIRF* showed similar protein nature to mammalian IRFs and could be activated during virus infection. Therefore, the Vago system which is centered on the IRF-Vago-JAK/STAT axis plays a critical role in nucleic acid-induced antiviral immunity in shrimps, and exhibits similarity to the mammalian IFN system, suggesting an IFN system-like antiviral mechanism in shrimp (Li *et al.*, 2015).

The shrimp *L. vannamei* is a prosperous commodity in the world, but its farming has been seriously disturbed by virus, especially white spot syndrome virus (WSSV). Even the immune defense mechanism of shrimp has been well documented (Li and Xiang, 2013), the effective approaches as well as novel therapeutic agents are in urgent to be developed for better controlling WSSV in shrimp aquaculture. Our previous studies have revealed that tandem CpG ODNs could induce antiviral immunity in shrimp (Zhang *et al.*, 2010). In the present study, four types of CpG ODN (CpG-A, CpG-B CpG-C, and CpG-P) were employed to stimulate the shrimp *L. vannamei* with the objectives (1) to detect the copy number of WSSV and the expression levels of *LvTolls* in the hepatopancreas of shrimps after the treatment of four types of CpG ODNs, (2) to evaluate the binding activities of *LvTolls* to various CpG ODNs by Surface plasmon resonance (SPR), (3) to investigate the cellular immune responses by detection of phagocytic rate, ROS level in shrimps hemocytes, and humoral antiviral immunity via analysis of the IFN system-like related genes and the probable IFN level in plasma after the treatments with different CpG ODNs, and (4) to evaluate the immune enhancement effect of different CpG ODNs and provide theoretical guidance for the application of different CpG ODNs as immunopotentiator in aquaculture.

**Table 1** Names and sequences of the CpG ODNs used in this study

Name	Sequence (5'---3')
CpG 2216 (CpG-A)	GGGGGACGATCGTCGGGGGG
CpG 2006 (CpG-B)	TCGTCGTTTTGTCGTTTTGTCGTT
CpG 2395 (CpG-C)	TCGTCGTTTTCGGCGCGCGCCG
CpG 23617(CpG-P)	TCGTCGACGATCGGCGCGCGCCG
CpG-negative 2317 (CpG-N)	TGCTGCTTTTGTGCTTTTGTGCTT

## Materials and Methods

### *Shrimps and CpG ODNs*

Shrimps, *Litopenaeus vannamei*, about 10 cm in length, were collected from a commercial farm in Qingdao, China, and cultured in tanks at  $22 \pm 1$  °C for 14 days before processing. Four types of CpG ODNs, including 2216 (A type), 2006 (B type), 2395 (C type) and 23617 (P type) (Table 1), which had been demonstrated to be effective in mammalian and aquatic animals (Chen *et al.*, 2007; Martinson *et al.*, 2007; Samulowitz *et al.*, 2010; Zhang *et al.*, 2010), were employed in the present study. The DNA sequence of the four types of CpG ODNs and the non-CpG ODN (CpG-N) 2317 were synthesized by Sangon Biotech Co. (China) with the purity above 99.9 %.

### *Virus preparation*

WSSV was extracted and purified from gill tissue of WSSV infected shrimps via the method of differential centrifugation described by Xu *et al.* (2007) with some modification. Briefly, *L. vannamei* shrimps with white spots on the cuticle were selected. One hundred milligram of the diseased shrimp gill were homogenized in 45 mL TNE buffer (400 mmol L<sup>-1</sup> NaCl, 50 mmol L<sup>-1</sup> Tris-HCl, 5 mmol L<sup>-1</sup> EDTA, pH 8.5) with 1 mmol L<sup>-1</sup> PMSF (Phenylmethanesulfonyl fluoride), and then centrifuged at 3,500g, 4 °C for 5 min. The supernatant was filtered through 0.45 µm membrane, and centrifuged at 30,000g for 30 min. The pellet was resuspended with TM buffer (10 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 50 mmol L<sup>-1</sup> Tris-HCl, pH 7.5) and centrifuged three times. Finally, the pellet was dissolved with 1 mL TM buffer containing 0.3 % NaN<sub>3</sub>. The copy number of WSSV stock solution was quantified by the Real-time PCR, then diluted with PBS (phosphate buffer saline, 0.1 mol L<sup>-1</sup>, pH 7.4) to a final concentration of 10<sup>7</sup> copies µL<sup>-1</sup> for subsequent experiments (stored at -80°C).

### *CpG ODNs treatment, WSSV infection and sample collection*

A total of 432 shrimps were randomly divided into two groups (group I and group II). In group I, the 162 shrimps were randomly separated to six sub-groups including CpG-A group, CpG-B group, CpG-C group, CpG-P group, CpG-N group and PBS group to survey the WSSV copy numbers after virus infection, so the shrimp number in each treatment was 27. The CpG ODNs were diluted in sterilized PBS at a final concentration of 10 µg mL<sup>-1</sup>, and 40 µL of CpG ODNs was injected into each shrimp from the second abdominal muscle using a syringe. The dosage of CpG ODNs was selected according to the published papers (Zhang *et al.*, 2010; Sun *et al.*, 2013, 2015). In the CpG-N and PBS groups, the shrimps received the injection of same volume non-CpG DNA (10 µg mL<sup>-1</sup>) or sterilized PBS. The shrimps were returned to the tanks and received an injection of 100 µL WSSV stock (10<sup>4</sup> copies µL<sup>-1</sup>) at 12 h after the CpG treatment. Nine shrimps were randomly sampled at first, third and fifth day post WSSV challenge in

each group, and the gill of every 3 shrimps in the same group was mixed as a single sample for the further DNA extraction to measure the virus copy numbers. In group II 270 shrimps were randomly separated into six sub-groups, and the CpG ODNs treatment was performed as described in the group I, and there were 45 shrimps in each treatment. Nine shrimps in each group were sampled at 0, 3, 6, 12, 24 h post injection. Tissues (hemocytes, plasma and hepatopancreas) from three shrimps in the same group at the same time point were mixed together as a single sample and there were three replicates for each group. The hemolymph (about 0.8 mL each shrimp) was collected from the pericardial cavity through the intersegmental membrane between the cephalothorax and the first abdominal segment using a syringe (2.5 mL) with 0.8 mL pre-cooled (4 °C) anticoagulant solution (115 mmol L<sup>-1</sup> glucose, 27 mmol L<sup>-1</sup> sodium citrate, 336 mmol L<sup>-1</sup> NaCl, 9 mmol L<sup>-1</sup> EDTA·Na<sub>2</sub>·2H<sub>2</sub>O, pH 7.4). The collected hemolymph was immediately centrifuged at 1,000g, 4 °C for 10 min to harvest hemocytes for the determination of phagocytosis and ROS. The supernatants (plasma) were stored at -80 °C for subsequent enzyme activity examination. The hepatopancreas was collected and added into 1.5 mL microcentrifuge tube with 600 µL Trizol reagent (TaKaRa, Japan), stored at -80 °C for subsequent RNA extraction.

### *Quantitative real-time PCR analysis of WSSV copy numbers*

The qRT-PCR technique was employed to determine the WSSV viral numbers according to the previous report (Zhang *et al.*, 2010) with some modification. Briefly, the recombinant plasmid of a 154 bp WSSV DNA fragment was used as the standard DNA for SYBR Green fluorescent qRT-PCR in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA). The WSSV copy numbers were estimated by the target amplicon in the plasmid, and the target amplicons were used to make 10-fold serial dilutions from 1×10<sup>10</sup> to 1×10<sup>2</sup> copies µL<sup>-1</sup>. These dilutions were employed as absolute standards to make a curve for quantification. Total genomic DNA of gill tissue in infected shrimps was extracted following the instructions of TIANamp Marine Animals DNA Kit (TIANGEN, China), and the DNA concentration was determined by using Nanodrop 2000 (Thermo Scientific, USA). The WSSV DNA fragment in total genomic DNA were examined by qPCR and the copy numbers of WSSV were calculated with the standard curve.

### *Analysis of phagocytic rate of shrimp hemocytes*

The phagocytic activity of hemocytes was measured according to the previous description with some modification (Wu *et al.*, 2008). Briefly, hemocytes from different groups were resuspended with cell resuspension (modified L-15 medium), and then incubated with the Latex Beads (Sigma, USA) under low speed of rotation at room temperature for 1 h. After three times of washing with cell

resuspension solution, the phagocytic rate of hemocytes was analyzed by FACS Arial II flow cytometer (FCM) (Becton, Dickinson and Company).

#### Measurement of intracellular ROS level

The Reactive Oxygen Species Assay Kit (Beyotime, China), based on the peroxide-sensitive fluorescent probe DCFH-DA, was used to detect the intracellular ROS level. The hemocytes (about 100 cell mL<sup>-1</sup>) from shrimps were harvested and incubated in the cell resuspension solution at room temperature for 20 min. After three times of washing with cell resuspension solution, the intracellular ROS level in hemocytes was analyzed by FACS Arial II flow cytometer (Becton, Dickinson and Company).

#### Analysis of CpG ODNs binding activities of LvToll-ECDs by SPR

The recombinant extracellular domain (ECD) proteins of three verified toll-like receptors (LvToll1 (ABK58729.1), LvToll2 (AEK86516.1) and LvToll3 (AEK86517.1) in *L. vannamei* were employed for the SPR assay. Preparation of LvToll-ECDs was conducted according to the method from Sun's description (Sun *et al.*, 2014). CpG ODNs binding analysis of LvToll-ECDs (LvToll1-ECD, LvToll2-ECD, LvToll3-ECD) was performed at 20 °C on a BIAcore T200 SPR instrument (GE Healthcare, USA). The anti-His tag antibody (GE Healthcare, USA) was previously covalently immobilized onto the CM5 sensor chip surface with the included immobilization buffer and Amine Coupling Kit. The recombinant LvToll-ECDs proteins with 6xHis tag were diluted in HBS-EP (GE Healthcare, USA), which was the running buffer containing 10 mmol L<sup>-1</sup> HEPES (pH 7.5), 150 mmol L<sup>-1</sup> NaCl, 3 mmol L<sup>-1</sup> EDTA and 0.005 % (v/v) surfactant P20. Then they were

injected to the loading tube and captured by the immobilized anti-His tag antibody to 240 (for rLvToll-ECDs) and 200 (for rTrx) response units (RU), respectively. The CpG ODNs (100 μmol L<sup>-1</sup>) were injected to the loading tube and passed over adjacent target. Flow cells were controlled at a flow rate of 30 μL min<sup>-1</sup> for 2 min. After 5 min dissociation, recombinant proteins and bound analyses were removed by a 60 s wash with 10 mmol L<sup>-1</sup> glycine-HCl (pH 1.5) at a flow rate of 30 μL min<sup>-1</sup>. After subtracting the control values, the data were analyzed with a 1:1 Langmuir binding model using the BIAcore T200 evaluation software.

#### RNA extraction, cDNA synthesis and qRT-PCR analysis of immune related genes

The RNA extraction and cDNA synthesis were conducted as the previous report (Sun *et al.*, 2015) with some modification. Total RNA was extracted from hepatopancreas using Trizol reagent (Invitrogen, USA). First-strand cDNA synthesis was carried out based on Promega M-MLV RT Usage information with random hexamers primer (Sangon, China). The mRNA expression levels of immune related genes were detected by SYBR Green fluorescent qRT-PCR. Gene specific primers for LvTolls, LvIRF, LvVago4 and LvSTAT (primer P5-P16) (Table 2) were used to amplify the corresponding products. The shrimp 18S rRNA fragment, amplified with primers P3 and P4 (Table 2), was chosen as reference for internal standardization. After the PCR program, data were analyzed by using the SDS 2.0 software (Applied Biosystems). The relative expressions of related genes were calculated by the 2<sup>-ΔΔCT</sup> method. All the data were given in terms of relative mRNA expressed as mean ± SD (N = 4) (Zhou *et al.*, 2012).

**Table 2** Summary of Primers in this study

Primer	Sequence (5'---3')
P1 (WSSV-154-RT-F)	CCAGTTCAGAATCGGACGTT
P2 (WSSV-154-RT-R)	AAAGACGCCTACCCTGTTGA
P3(18SrRNA-RT-F)	TATACGCTAGTGGAGCTGGAA
P4(18SrRNA-RT-R)	GGGAGGTAGTGACGAAAAAT
P5(LvToll1-RT-F)	TCGACCATCCCTTTTACACC
P6(LvToll1-RT-R)	TTGCCTGGAAGGTCTGATTC
P7(LvToll2-RT-F)	CATGCCTGCAGGACTGTTTA
P8(LvToll2-RT-R)	GGCCTGAGGGTAAGGTCTTC
P9(LvToll3-RT-F)	TCGTACAACCAGCTGACGAG
P10(LvToll3-RT-R)	ATACTTCAGGTGGGCCACAG
P11(LvIRF-RT-F)	CTTTCGCTACTGGGCTCTTGC
P12(LvIRF-RT-R)	GGTCGTAGTGCTTCGGTTTCTC
P13(LvVago4-RT-F)	GCGAGAGGGAAAAGGAAAACAGG
P14(LvVago4-RT-R)	CCAGCACTTCCCAGGGTGGTCTG
P15(LvSTAT-RT-F)	AGCCCCTGTCTGAGCGAAA
P16(LvSTAT-RT-R)	GGTGTCTCTTGTAACTTCATCA

#### Measurement of probable IFN level in plasma

The probable IFN level in plasma was determined by using the commercial fish IFN ELISA kit (Jiangsu Kete biological technology, China) following the manufacturer's instructions. Briefly, the 96 wells plates coated by the purified fish IFN antibody were incubated with 50  $\mu$ L plasma (diluted 5-fold with sample diluent) from different groups at 37 °C for 30 min. After five times of washing, the plate was incubated with 50  $\mu$ L Horseradish Peroxidase (HRP)-fish IFN antibody at 37 °C for 30 min. After the final washing of five times, chromogenic agent and stop buffer solution were added, and the absorbance was detected at 450 nm by a precision microplate reader (BioTek, USA).

#### Data analysis

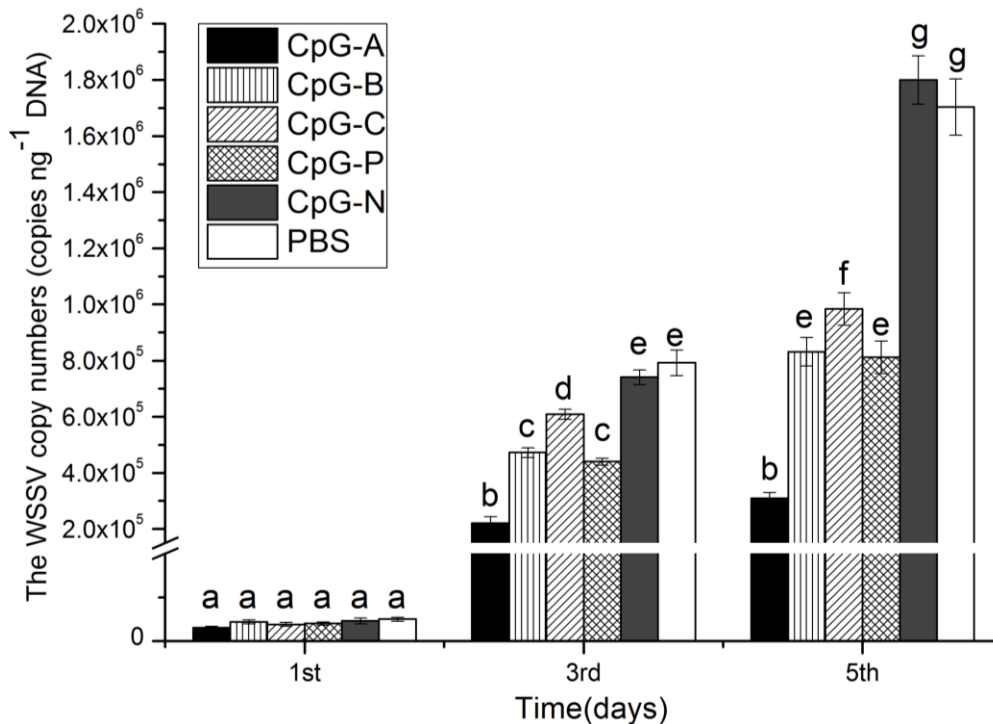
For all experimental results, the data from three samples were recorded and analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests using SPSS 16.0 program. Differences were considered to be statistically significant at a *p* value of 0.05 or less.

### Results

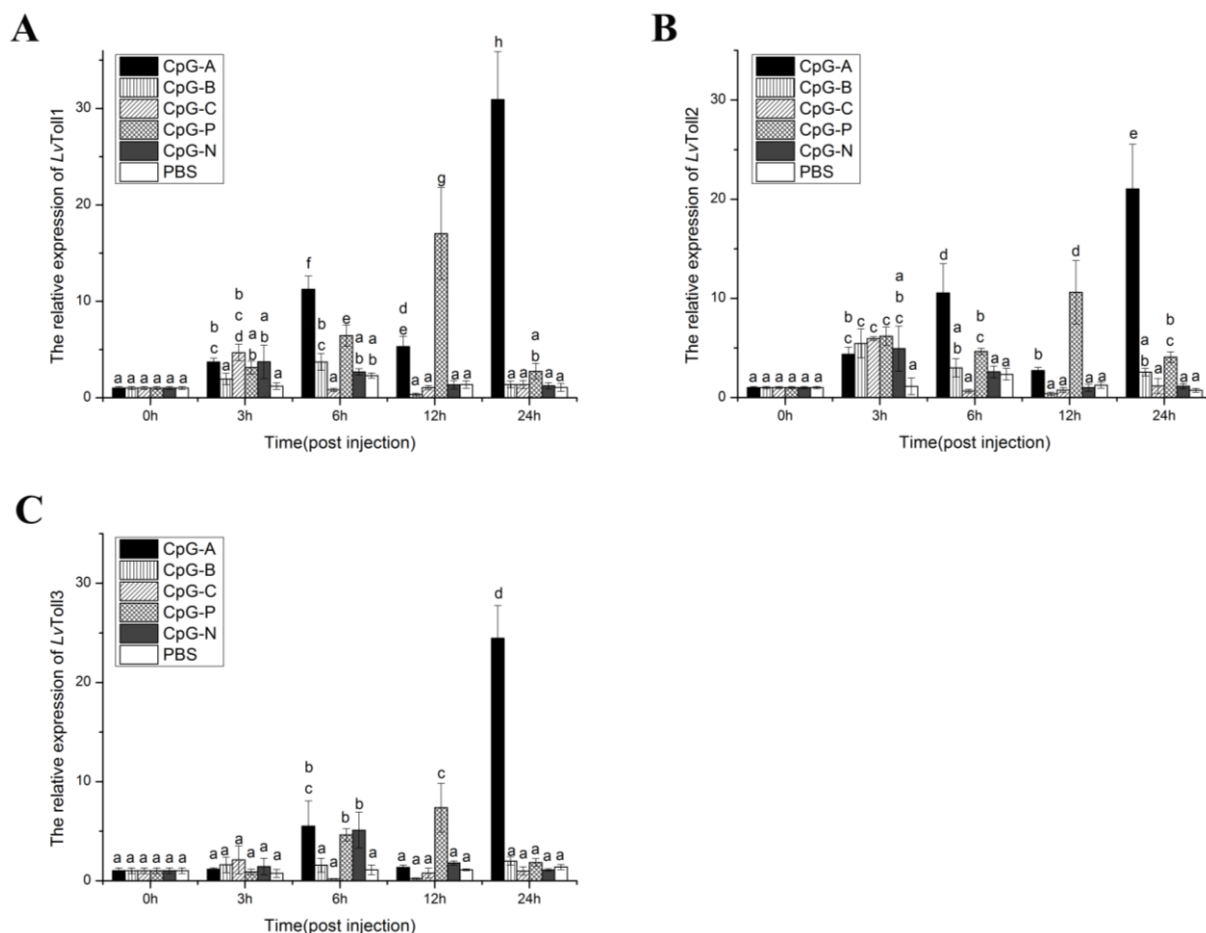
#### The alternations of WSSV copy numbers after CpG ODN treatments

The WSSV copy numbers in the gill tissue of

shrimps pretreated with different types of CpG ODNs were significantly lower than those in CpG-N and PBS-pretreatment shrimps on the third and fifth day post WSSV challenge. On the first day after WSSV challenge, no significant difference of the WSSV copy numbers was observed between CpG-pretreatment groups and controls (Fig. 1). On the third day, the WSSV numbers in the shrimps pretreated with CpG-A, CpG-B, CpG-C and CpG-P were  $2.20 \times 10^5$ ,  $4.72 \times 10^5$ ,  $6.09 \times 10^5$  and  $4.40 \times 10^5$  copies  $\text{ng}^{-1}$  DNA, whereas the numbers in CpG-N and PBS-pretreatment shrimps were  $7.41 \times 10^5$  and  $7.92 \times 10^5$  copies  $\text{ng}^{-1}$  DNA. On the fifth day post WSSV challenge, the WSSV copy number of shrimps in CpG-A, CpG-B, CpG-C and CpG-P groups were  $3.10 \times 10^5$ ,  $8.32 \times 10^5$ ,  $9.84 \times 10^5$  and  $8.12 \times 10^5$  copies  $\text{ng}^{-1}$  DNA respectively, which were significantly lower, approximately 18.19 %, 48.82 %, 57.75 % and 47.65 % (*p* < 0.01) respectively, than that in PBS group. Moreover, the WSSV copy numbers in CpG-A group was the lowest among the four types of CpG ODN groups, especially on the fifth day post WSSV challenge, the numbers in CpG-B, CpG-C and CpG-P groups was 2.68-fold, 3.17-fold and 2.62-fold (*p* < 0.01) higher than that of CpG-A group respectively. No significant difference was observed between the control groups (treated with CpG-N and PBS) throughout the experiment.



**Fig. 1** The WSSV copy numbers in gill tissue in CpG-A, CpG-B, CpG-C, CpG-P, CpG-N and PBS-pretreatment shrimps after WSSV infection on the first, third and fifth day. Data presented as mean  $\pm$  S.D. (N = 4). The significant differences between the treated groups and control groups were subjected to one-way analysis of variance (one-way ANOVA).



**Fig. 2** Temporal expression of (A) *LvToll1*, (B) *LvToll2* and (C) *LvToll3* mRNA detected by real-time PCR in shrimp hepatopancreas after *in vivo* CpG-A, CpG-B, CpG-C, CpG-P, CpG-N and PBS injection at 3, 6, 12 and 24 h. Comparison of the level of mRNA (relative to 18S rRNA gene) was normalized to 0 h. Data presented as mean  $\pm$  S.D. (N = 4). The significant differences between the treated groups and control groups were subjected to one-way analysis of variance (one-way ANOVA).

*The mRNA expressions of LvToll1, LvToll2 and LvToll3 in hepatopancreas of shrimps after CpG ODNs treatment*

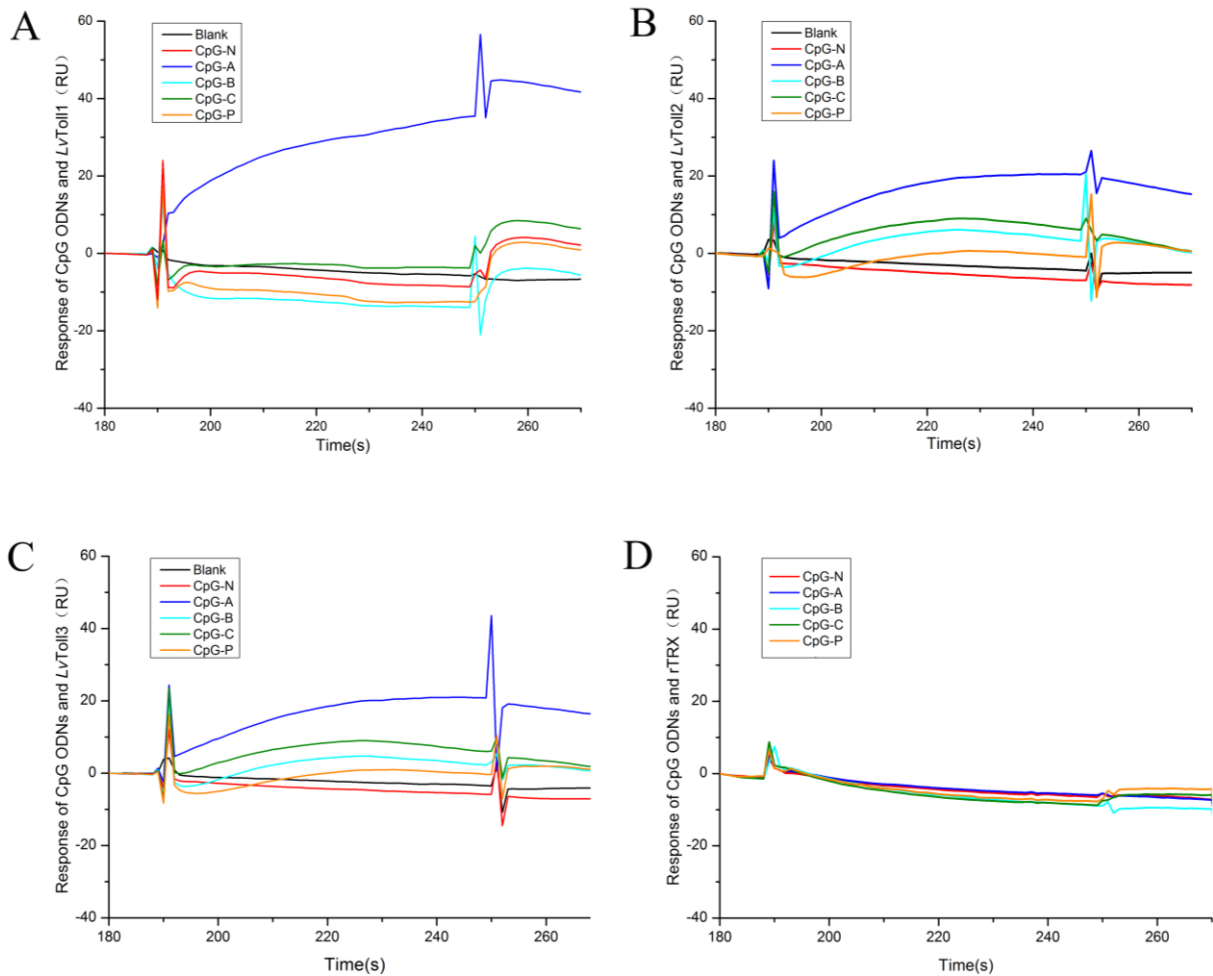
The mRNA expression levels of three verified toll-like receptors (*LvToll1*, *LvToll2* and *LvToll3*) in hepatopancreas from shrimps were detected at 0, 3, 6, 12 and 24 h post the treatments with CpG ODNs. The expression levels of *LvTolls* in CpG-A and CpG-P groups were significantly up-regulated compared with those in CpG-N and PBS groups, while those in the CpG-B, CpG-C groups did not change dramatically.

After CpG-A stimulation, the mRNA expression levels of *LvToll1*, *LvToll2* and *LvToll3* were all up-regulated at 3 h and peaked at 24 h (Fig. 2), which were 28.62-fold ( $p < 0.01$ ), 22.82-fold ( $p < 0.01$ ) and 17.59-fold ( $p < 0.01$ ) of that in PBS group, respectively (Fig. 2). After the stimulation of CpG-P, the mRNA transcripts of *LvToll1*, *LvToll2* and *LvToll3* increased and reached the maximum level at 12 h, which were 12.52-fold ( $p < 0.01$ ), 8.50-fold ( $p < 0.01$ ) and 6.64-fold ( $p < 0.01$ ) of those in PBS group,

respectively (Fig. 2). No significant difference was observed between the control groups (treated with CpG-N and PBS) throughout the experiment. In addition, the maximum mRNA expression levels of *LvToll1*, *LvToll2* and *LvToll3* in CpG-A group were 1.82-fold, 1.98-fold and 3.32-fold ( $p < 0.01$ ) of that in CpG-P group, respectively, which were also significantly higher than that in CpG-B and CpG-C groups.

*The binding affinity of different CpG ODNs to LvToll-ECDs*

In order to determine the binding affinity of CpG ODNs to *LvTolls*, three *LvToll*-ECDs (*LvToll1*-ECD, *LvToll2*-ECD, and *LvToll3*-ECD) and rTrx (the control protein) with His tag were captured by the chip-immobilized anti-His antibodies. CpG ODNs, including CpG-A, CpG-B, CpG-C and CpG-P, were screened by passing through the chip-immobilized proteins to examine the molecular interaction with CpG-N as control. CpG-A displayed a rapid association with *LvToll1*-ECD on SPR with



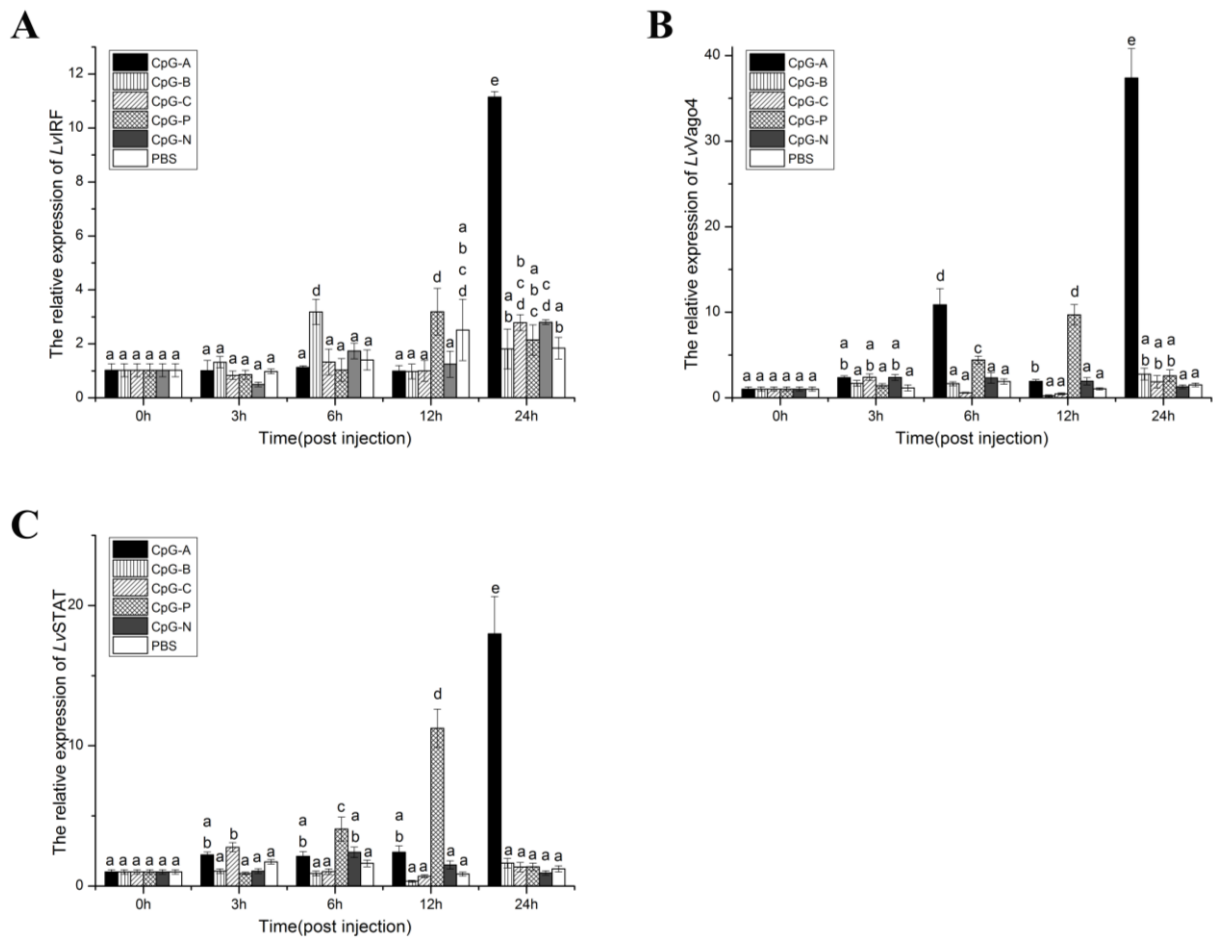
**Fig. 3** Surface plasmon resonance measurement of the binding between different CpG ODNs and LvToll-ECDs. The sensorgrams of different CpG-ODNs binding to (A) LvToll1-ECD, (B) LvToll2-ECD, (C) LvToll3-ECD and (D) rTrx. All data shown were reproducible and representative of at least three independent experiments.

the binding signal of 35 RU, while other CpG ODNs showed no binding affinity to LvToll1-ECD (Fig. 3A). All the four types of CpG-ODNs exhibited low binding affinity to LvToll2-ECD with the signals of 20, 7, 10 and 3 RU, respectively (Fig. 3B). Similarly, the signals of LvToll3-ECD for CpG-A, CpG-B, CpG-C and CpG-P were 20, 5, 7 and 3 RU respectively (Fig. 3C). No binding signal was observed in CpG-N and blank groups throughout the experiment. And all the four types CpG ODNs did not display any binding signal with rTrx (Fig. 3D). CpG-A exhibited the strongest binding activity among the three LvToll-ECD and it displayed a binding signal of 35 RU with LvToll1-ECD, which was significantly higher than that of CpG-A with LvToll2-ECD (20 RU) and LvToll3-ECD (20 RU).

*The mRNA expression of LvIRF, LvVago4 and LvSTAT genes in hepatopancreas after CpG ODNs treatment*

LvIRF (KM277954), LvVago4 (HQ541161.1) and LvSTAT (AGT28261.1) were selected to investigate the induction of CpG ODNs on the

activation of the IFN system-like response in shrimp. After stimulations of CpG-A and CpG-P, the mRNA expression of the three genes increased significantly compared with that in CpG-N group and PBS group. The mRNA expression levels of LvIRF, LvVago4 and LvSTAT were significantly up-regulated and reached peak at 24 h after injection of CpG-A, which were 6.08-fold, 24.92-fold and 14.73-fold ( $p < 0.01$ ) of those in PBS group, respectively (Fig. 4). At 12 h post CpG-P injection, the mRNA expression levels of LvVago4 and LvSTAT genes were also significantly up-regulated, which were 9.24-fold and 13.08-fold ( $p < 0.01$ ) compared with those in PBS group (Figs 4B, C). In addition, CpG-B could induce the up-regulation of LvIRF at 6 h with 2.27-fold ( $p < 0.01$ ) of that in PBS group and CpG-C could induce the up-regulation of LvSTAT at 3 h with 1.60-fold ( $p < 0.01$ ) of that in PBS group, which were significantly lower than that in CpG-A and CpG-P group (Figs 4A, C). No significant difference in the expression level of LvIRF, LvVago4 and LvSTAT was observed between the control groups (treated with CpG-N and PBS) throughout the experiment.



**Fig. 4** Temporal expression of (A) *LvIRF*, (B) *LvVago4* and (C) *LvSTAT* mRNA detected by qRT-PCR in shrimp hepatopancreas after *in vivo* CpG-A, CpG-B, CpG-C, CpG-P, CpG-N and PBS injection at 3, 6, 12 and 24 h. Comparison of the level of mRNA (relative to 18S rRNA gene) was normalized to 0 h. Data presented as mean  $\pm$  S.D. (N = 4). The significant differences between the treated groups and control groups were subjected to one-way analysis of variance (one-way ANOVA).

#### *The probable IFN protein level in plasma after CpG ODNs treatment*

The probable IFN protein level in plasma of shrimps after CpG ODN treatments was determined using the fish IFN ELISA kit. CpG-A, CpG-B and CpG-P could induce the increase of probable IFN level in plasma. The concentration of probable IFN in CpG-A group was significantly up-regulated at 3 h and reached the peak (2.60-fold,  $p < 0.01$ ) at 24 h (Fig. 5). Compared with the probable IFN protein level in PBS group (Fig. 5), CpG-B group increased at 6 h (1.39-fold,  $p < 0.01$ ) and CpG-P group also increased at 24 h (1.91-fold,  $p < 0.01$ ) respectively, while they were both significantly lower than that in CpG-A group. In addition, there was a significant decrease in CpG-C group at 6h and an increase in CpG-N group at 3h in comparison to the PBS control (Fig. 5).

#### *The phagocytic rate of shrimp hemocytes after CpG ODNs treatment*

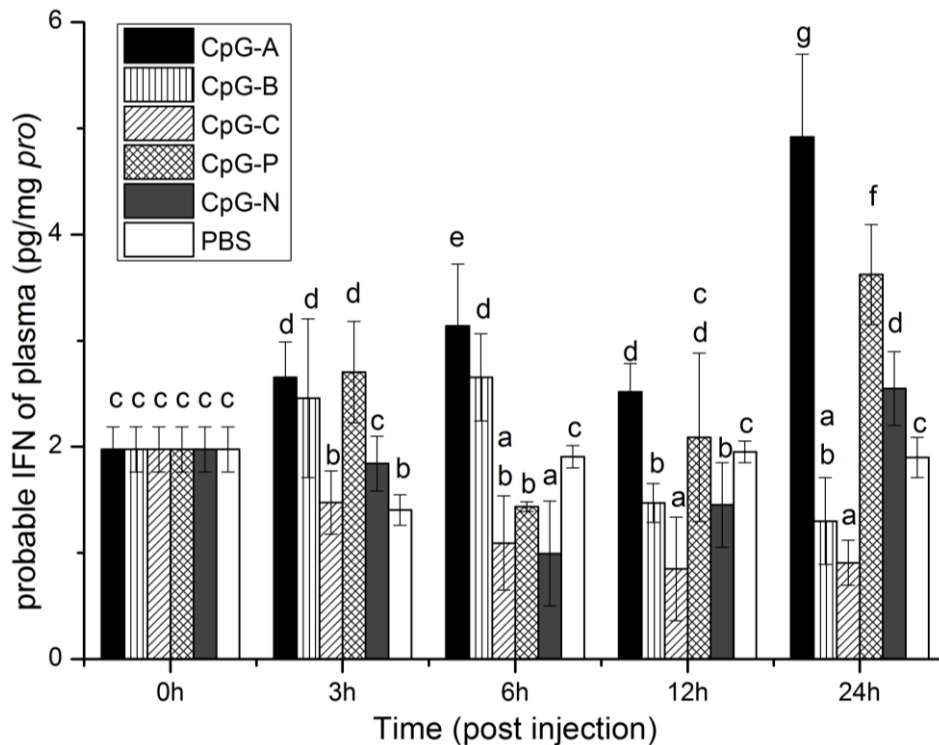
The phagocytic rate of shrimp hemocytes was recorded by using the relative fluorescence intensity. After injections of CpG-A, CpG-B, CpG-C and

CpG-P, the phagocytic rate of hemocytes increased in comparison with that from the shrimps treated with CpG-N and PBS. In CpG-A group, the phagocytic rate of hemocytes was up-regulated at 3 h and reached the peak at 6 h (1.63-fold of that in PBS group,  $p < 0.01$ ) (Fig. 6). In CpG-B group, the phagocytic rate increased to the maximum at 6 h (1.60-fold of that in PBS group,  $p < 0.01$ ) (Fig. 6). In CpG-P and CpG-C groups, the peak level of the phagocytic rate was detected at 6 h and 12 h, which were 1.40-fold and 1.15-fold ( $p < 0.01$ ) of that in PBS group, respectively (Fig. 6). The maximum levels of phagocytic rate in CpG-A group and CpG-B group were significantly higher than that in CpG-C and CpG-P groups. There was no obvious difference between the control (CpG-N and PBS) groups throughout the experiment.

#### *The ROS level in hemocytes after injection of CpG ODNs*

The level of ROS generation in hemocytes after CpG ODNs injection was recorded by using the relative fluorescence intensity. CpG-A, CpG-B and CpG-C could increase the ROS generation in





**Fig. 5** Determination of probable IFN in plasma after injection of CpG ODNs using the commercially fish IFN ELISA kit. Data presented as mean  $\pm$  S.D. (N = 3). The significant differences between the treated groups and control groups were subjected to one-way analysis of variance (one-way ANOVA).

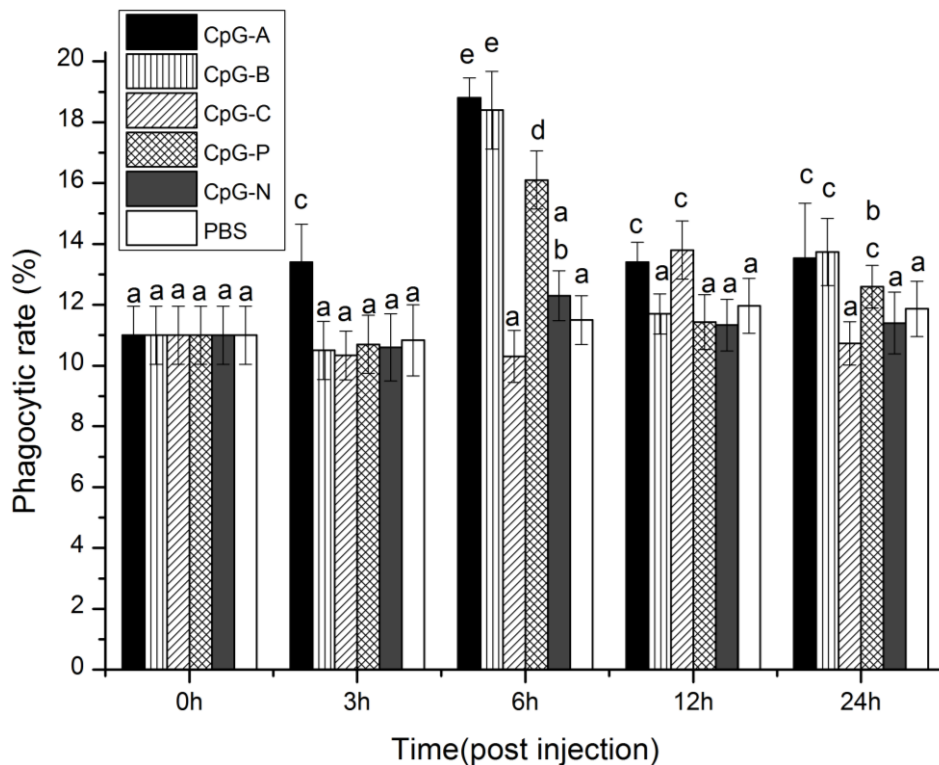
hemocytes compared with CpG-N and PBS. After injection of CpG-A and CpG-B, the ROS levels increased significantly at 3 h and reached the peak at 6 h, which were 9.98-fold ( $p < 0.01$ ) for CpG-A and 4.92-fold ( $p < 0.01$ ) for CpG-B of those in PBS group, then they recovered to the original level at 24 h (Fig. 7). In the CpG-C group, the ROS levels also increased at 6 h, which were 2.22-fold ( $p < 0.01$ ) compared with those in PBS group (Fig. 7). No significant difference was detected among the CpG-P, CpG-N and PBS groups during the whole experimental period with exception of 6 h post injection, at which the ROS levels in CpG-P and CpG-N groups were 1.88-fold and 1.92-fold ( $p < 0.01$ ) of that in PBS group, respectively (Fig. 7).

## Discussion

CpG ODNs with unmethylated CpG dinucleotides (CpG motifs) are one type of important immunomodulators which could induce various immune responses. Four types of CpG ODNs with diverse characteristics exhibit different cellular and humoral immune effects in mammals (Shirota and Klinman, 2014). For example, A and P-type ODNs can trigger plasmacytoid dendritic cells (pDCs) to mature and secrete IFN- $\alpha$ , whereas B-type ODNs mainly stimulate NF $\kappa$ B-mediated signaling pathway resulting in strong B cell activation (Krug *et al.*, 2001; Vollmer and Krieg, 2009). In our previous studies, tandem ODNs sequence has been observed to bind

LvTolls, activate antiviral associated factors and enhance the survival rates of *L. vannamei* (Zhang *et al.*, 2010; Sun *et al.*, 2013; Yi *et al.*, 2014), but the exactly effects of a certain type of CpG ODNs is still not clear. In the present study, four types of CpG ODNs (CpG-A, CpG-B, CpG-C, and CpG-P) were employed to evaluate their immune enhancement effects.

It is widely demonstrated that CpG ODNs have antiviral effects in multiple vertebrate species. In woodchuck, the combination therapy with CpG ODNs could induce early antiviral response and enhance the inhibition of viral replication in the hepadnaviral infection (Meng *et al.*, 2016). Similarly, the antiviral DNA vaccine containing multi-copy of CpG fragment showed increased immunogenicity to viral hemorrhagic septicemia virus (VHSV) in fish (Martinez-Alonso *et al.*, 2011). Recently, CpG ODN has been applied as an immunopotentiator against pathogens in crustacean aquaculture and the previous studies have revealed that CpG ODNs could increase the survival rate after WSSV injection (Zhang *et al.*, 2010; Yi *et al.*, 2014). It is generally believed that higher survival rate against virus should be associated with lower viral loads (Jang *et al.*, 2009). In the present study, all the four kinds of CpG ODNs were observed to be able to inhibit WSSV copies in shrimps on the third and fifth day after treatment. CpG-A displayed the strongest inhibition on WSSV, while CpG-B, CpG-C and CpG-P were less efficient. The results indicated that



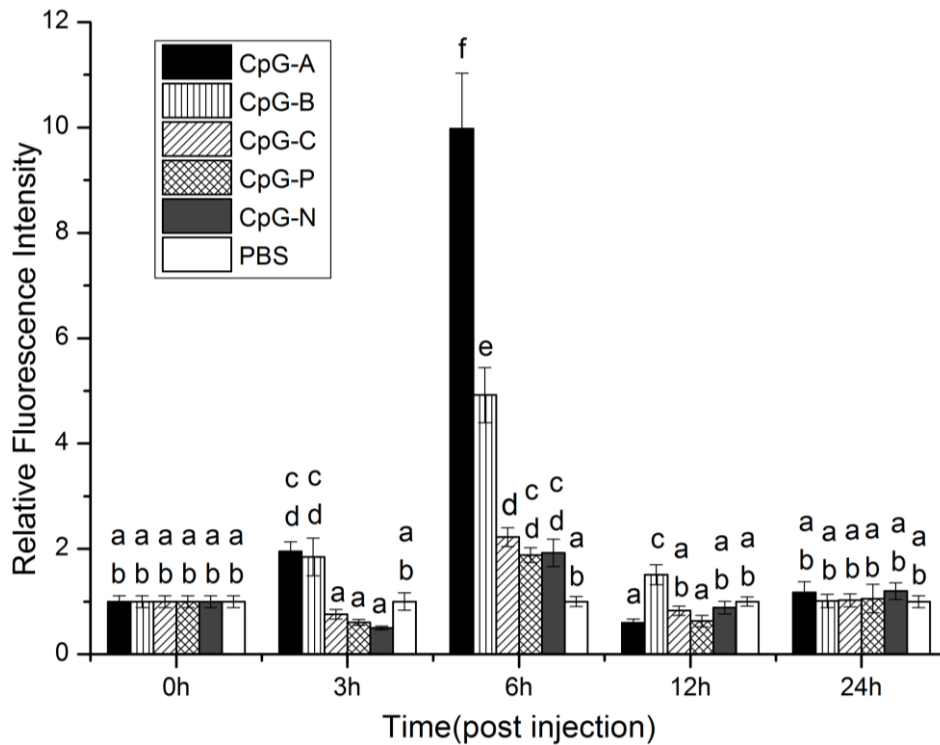
**Fig. 6** Hemocyte phagocytic rate of CpG-A, CpG-B, CpG-C, CpG-P, CpG-N and PBS treated shrimps at 3, 6, 12, 24 h post injection. Data presented as mean  $\pm$  S.D. (N = 3). The significant differences between the treated groups and control groups were subjected to one-way analysis of variance (one-way ANOVA).

CpG-A should play an important role in shrimp immune response against the invading of WSSV. The biological properties of different types of CpG ODN are diverse in mammals, and it is suspected that the difference of anti-WSSV effects should be related to their involvements in the activation of shrimp immune system.

CpG motifs are considered as one of PAMPs due to their abundance in microbial genomes and rarity in animal genomes (Bauer and Wagner, 2002). As a classical type of PAMPs, CpG ODNs could be recognized by some conserved TLRs in various species (Keestra *et al.*, 2010; Kumagai *et al.*, 2008). So far, three Tolls (*LvToll1*, *LvToll2* and *LvToll3*) have been identified from *L. vannamei* (Wang *et al.*, 2012; Yang *et al.*, 2007). In the present study, the mRNA expression levels of *LvTolls* in hepatopancreas of shrimps after CpG ODNs stimulation and the binding affinity between *LvToll*-ECDs and CpG ODNs were investigated. After treated with CpG-A and CpG-P, the expression levels of *LvTolls* were significantly up-regulated, while the treatments of CpG-B and CpG-C could not change the expression of *LvTolls* significantly. Meanwhile, CpG-A exhibited the strongest activity to induce the expression of *LvTolls*, and the expression of *LvToll1* was highest after CpG-A treatment. It was speculated that the antiviral effects of CpG ODNs were associated with their promotion of *LvToll* expressions. Correspondingly, CpG-A exhibited the strongest binding activity towards *LvToll1*-ECD in

SPR assay, which was significantly higher than that towards *LvToll2*-ECD and *LvToll3*-ECD, indicating that *LvToll1* should be the main receptor of CpG-A in shrimp. Furthermore, ECD of *LvToll1* was found to share high identity with that of *HsTLR9* (56%), the receptor of CpG ODNs in human (Bauer *et al.*, 2001; Sun *et al.*, 2014), providing convincing evidence to our conclusion that *LvToll1* was the receptor of CpG-A.

The activation of the TLR signaling pathway by CpG ODNs could induce a series of cellular immune responses. For instance, the recognition of CpG DNA by TLR could activate the expression of genes involved in phagocytosis in mice cells (Doyle *et al.*, 2004). As a powerful cellular response, phagocytosis plays a pivotal role in clearance of influenza virus and Hepatitis C virus (HCV) in mammals (Fujimoto *et al.*, 2000; Huber *et al.*, 2001; Bang and Saito, 2015). Similarly, phagocytosis also plays important roles in antiviral immunity in crustaceans (Wang and Zhang, 2008; Liu *et al.*, 2009). The accumulated evidences have proved that CpG ODNs could induce phagocytic activity in various aquatic animals (Tassakka and Sakai, 2002; Zhao *et al.*, 2016). In the present study, the phagocytic rate was significantly enhanced by CpG-A and CpG-B treatment, providing direct evidence for the resistance to WSSV infection of shrimps immunized by CpG ODNs. It has been suggested that the enhanced phagocytosis might contribute to the partial protective functions of CpG



**Fig. 7** Intracellular ROS level of CpG-A, CpG-B, CpG-C, CpG-P, CpG-N and PBS treated shrimps at 3, 6, 12, 24 h post injection. Data presented as mean  $\pm$  S.D. (N = 3). The significant differences between the treated groups and control groups were subjected to one-way analysis of variance (one-way ANOVA).

ODNs in decreasing the virus load of WSSV (Zhang *et al.*, 2010). Apart from phagocytosis, CpG ODNs could also increase the ROS generation in immune cells. For instance, CpG DNA promoted the ROS burst in murine B cells, which was linked to the activation of NF $\kappa$ B and then accelerated the leukocyte gene transcription and cytokine secretion (Yi *et al.*, 1998). As a product of cellular metabolism, ROS is involved in the regulation of viral infection (Skulachev, 1998; Waris *et al.*, 2005). In human, ROS could disrupt the activity of HCV replication complexes in hepatoma cells to inhibit its replication (Choi *et al.*, 2004). Moreover, ROS was demonstrated to be essential in the defense against influenza A virus infection through IFN-related immune responses (Kim *et al.*, 2015). In the present study, the intracellular ROS level in hemocytes of CpG-A and CpG-B stimulated shrimps increased significantly, which displayed a similar trend with the phagocytic rate. The boosted ROS level was previously reported to contribute to the decreased WSSV copies in CpG ODNs pre-treated shrimps (Sun *et al.*, 2013). The results suggested that the inhibition CpG-A and CpG-B on WSSV might partly result from the increase of cellular immunity including the phagocytic rate and intracellular ROS generation.

Apart from activation of cellular immunity, the recognition of CpG DNA or single-stranded RNA by pattern recognition receptors (PRRs) also promotes

humoral immunity including the secretion of proinflammatory cytokines, chemokines and type I IFNs, which are the hallmarks of host innate immune system in defending against viral infections in mammals (Sadler and Williams, 2008; Takeuchi and Akira, 2009). In vertebrates, CpG DNA could be recognized by TLR9 and then activated the downstream IFN regulation factor (IRF) to promote the secretion of type I IFN (Kumar *et al.*, 2009; Takeuchi and Akira, 2009), and consequently activated JAK/STAT pathway via the interaction with the IFN receptor (Sadler and Williams, 2008). Li *et al.* (2015) reported that shrimp might possess an IRF-Vago-JAK/STAT regulatory axis, which was similar to the IRF-IFN-JAK/STAT antiviral axis of vertebrates, indicating that shrimp might possess an IFN system-like antiviral mechanism. The Vago gene from arthropods encodes a viral-activated secreted peptide that restricts virus infection through activating the JAK-STAT pathway and it is considered as a cytokine functionally similar to IFN; as Vago has been suggested to be an IFN-like protein and IRFs to be conserved from invertebrates to mammals, it could be seen that the shrimp Vago system exhibits similarity to the mammalian IFN system (Li *et al.*, 2015). In the present study, the temporal expression of IFN system-like related genes (*LvIRF*, *LvVago4* and *LvSTAT*) were detected after the treatments with different types of CpG ODN. The mRNA expression of *LvIRF*, *LvVago4* and

LvSTAT in hepatopancreas of CpG-A and CpG-P treated shrimps were dramatically up-regulated compared with that in the control groups and other CpG ODNs groups. Moreover, the level of probable IFN in plasma was also increased significantly in CpG-A and CpG-P groups, indicating that CpG-A and CpG-P might promote the secretion of the probable IFN in shrimp. Meanwhile, all the above responses were significantly higher in CpG-A group than that in CpG-P group. In vertebrates, type I IFNs activate intracellular signaling pathways and regulate the expression of a set of genes, which are involved in eliminating viral components from infected cells and conferring resistance to viral infection on uninfected cells. Therefore, the high antiviral effect of CpG-A was possible related to the downstream IFN system-like response. These results indicated that CpG-A might induce the IRF-Vago-JAK/STAT pathway in shrimp, and the CpG-mediated antiviral pathway might be evolutionarily conserved in crustacean.

In conclusion, CpG-A can stimulate relatively higher antiviral effects in shrimp *L. vannamei* with effective inhibition to WSSV. The antiviral effects of CpG-A are directly related to its preferable binding affinity to LvToll1 as well as the following increased cellular and humoral immunity including the phagocytotic activity, the ROS level and the IFN system-like antiviral response. These results provided theoretical basis for the application of CpG-A as an immunologic adjuvant in shrimp aquaculture.

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