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An IL-6 gene in humphead snapper (*Lutjanus sanguineus*): Identification, expression analysis and its adjuvant effects on *Vibrio harveyi* OmpW DNA vaccine

Pujiang Huang[a,b,c,d,1], Jia Cai[a,b,c,e,f,g,1], Dapeng Yu[a,b,c,e], Jufen Tang[a,b,c,e,f], Yishan Lu[a,b,c,e,f], Zaohe Wu[b,c], Yucong Huang[a,b,c,e,f,*], Jichang Jian[a,b,c,e,f,**]

[a] College of Fishery, Guangdong Ocean University, Zhanjiang, PR China
[b] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang, PR China
[c] Guangdong Key Laboratory of Control for Diseases of Aquatic Economic Animals, Zhanjiang, PR China
[d] Shenzhen Fisheries Service and Aquatic Product Technology Extension Center, Shenzhen, PR China
[e] Shenzhen Research Institute of Guangdong Ocean University, Shenzhen, PR China
[f] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, PR China
[g] Guangxi Key Lab for Marine Biotechnology, Guangxi Institute of Oceanography, Guangxi Academy of Sciences, Beihai, PR China

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ABSTRACT

Interleukin 6 (IL-6) is a pleiotropic cytokine that plays important role in mediating the innate and adaptive immune responses against pathogen infection. In this study, an IL-6 homolog (Ls-IL6) was identified and characterized from humphead snapper, *Lutjanus sanguineus*. The full-length cDNA of Ls-IL6 was 1066 bp, containing an open reading frame (ORF) of 639 bp encoding 212 amino acids, 5′ untranslated region(UTR) of 63 bp and 3' UTR of 605 bp. The predicted Ls-IL6 protein had typical motif of IL-6 family and shared high identities to teleost IL-6s. Ls-IL6 extensively expressed in various tissues, and the highest expression of Ls-IL6 was detected in head kidney, spleen and thymus. *In vivo*, the transcript levels of Ls-IL6 were significantly up-regulated in response to *Vibrio harveyi* infection. Moreover, the DNA plasmid containing the OmpW of *V. harveyi* together with the gene encoding Ls-IL6 were successfully constructed and administered to fish, the protective efficacy of Ls-IL6 was investigated. Compared with the pcDNA-OmpW group, the level of specific antibodies against *V. harveyi* increased in pcDNA-IL6-OmpW injected group. After *V. harveyi* infection, the pcDNA-IL6-OmpW vaccinated fish showed higher relative percent survival (76%) than the relative survival of fish immunized with pcDNA-OmpW (60%). These results indicated that Ls-IL6 was involved in immune response against *V. harveyi* infection and could be applied as a promising adjuvant for DNA vaccines against *V. harveyi*.

1. Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that plays key roles in inflammation and immune regulation, tissue regeneration and hematopoiesis [1–4]. Functionally, they enhance inflammatory response by improving the productions of acute-phase proteins (APPs) such as serum amyloid A (SAA), haptoglobin, C-reactive protein (CRP), α-1 antitrypsin, and fibrinogen [5]. Also, IL-6 is closely linked to acquired immune response via modulating B cell differentiation into antibody-forming plasma cell, $CD8^+$T cells into cytotoxic T cells and T-follicular helper-cell differentiation [6–8]. Additionally, the involvement of IL-6 in pathogenic invasion has been well-described [9]. Thus, IL-6-contained plasmid DNA or IL-6 recombinant protein have been applied as an efficient adjuvant in mammalian vaccine against various pathogen [10–12].

To date, although IL-6 has been characterized in several fish species [13–19], the study focused on adjuvant role of fish IL-6 is limited.

* Corresponding author. Fisheries College, Guangdong Ocean University, Fisheries College, Guangdong Ocean University, No. 1 of Haida Road, Mazhang District, Zhanjiang, Guangdong Province, 524088, PR China.
** Corresponding author. Fisheries College, Guangdong Ocean University, Fisheries College, Guangdong Ocean University, No. 1 of Haida Road, Mazhang District, Zhanjiang, Guangdong Province, 524088, PR China.
E-mail addresses: hyczjou@163.com (Y. Huang), jianjc@gdou.edu.cn (J. Jian).
[1] These authors contributed to the work equally and should be regarded as co-first authors.

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Table 1
Sequences of primers used in this study.

Primers	Sequences (5′-3′)	Applications
LF1	AAGYAYGTGGAGAARGAG	Ls-IL6 fragment
LR1	GAYGAGGAAGYYGCGGAG	
P1	TCAAAGAAAAGATGAGGCACCAAG	3′ RACE
P2	ACAGGCCCAACGCTTTCGAC	
SP1	TCTGTCGAAAGCGTTGGGC	5′RACE
SP2	CTGCTCCTCCTGGCTGCTGGT	
SP3	AGTTGGCTGCTGTATTTCCTG	
DF	AGCAGCTCATCATGCCTTCTAC	
DR	TTGGGAATAGAAACTGTTGG	Ls-IL6 genomic DNA
β-actinS	GTCATGTGGATCAGCAAGCAGGA	Real-Time PCR
β-actinA	CGCCCGAGTGTGTATGAGAAATG	
IL6S	GTCTTCCTGCTCTGTGCGGT	
IL6A	GGATTATCACCCTCCTCAAACG	
IL6 F	CGCGGATCCATGGTCCCGCTGGATGAC	pET-LsIL6 construction
IL6 R	CCGCTCGAGTCAAACTTCCTCCGGGCTCCT	
W-P1	CGCGGATCCATGGTCCCGCTGGATGAC	Ls-IL6-OmpW fusion
W-P2	GCTGCCGCCACCGCCGCTTCCGCCACCGCCGCTTCCACCGCCACCAACTTCCTCCGGGCTCCTCAG	
W-P3	GGTGGCGGTGGAAGCGGCGGTGGCGGAAGCGGCGGTGGCGGCAGCCACAGTGAAGGTGACTTCATT	
W-P4	CCGCTCGAGTTAGAACTTGTAACCACCGC	
WF	CGCGGATCCATGAAAAAAACAATCTGCAGT	OmpW amplification
WR	CCGCTCGAGTTAGAACTTGTAACCACCG	

Humphead snapper (*Lutjanus sanguineus*) is an important marine fish that widely cultured in the south China. However, the snapper culture has been suffering from the outbreaks of disease caused by *Vibrio* spp. in recent years [20]. In the present study, an IL-6 (Ls-IL6) homolog was identified from humphead snapper (*L. sanguineus*). The tissue distribution and expression profiles of Ls-IL6 upon *Vibrio harveyi* infection was investigated *in vivo*. Given the *Vibrio* spp. outer membrane protein OmpW had been identified as an efficient DNA vaccine against *Vibrio* spp. infection in snapper [21], the immune adjuvant effect of Ls-IL6 was also evaluated.

2. Materials and methods

2.1. Fish and sampling

Healthy humphead snapper (body weight 500–600 g) were obtained from local commercial market (Zhanjiang, China). The fish were maintained in aerated sea water tank at 28 °C and fed for two weeks prior to experimental manipulation. Before the experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney as well as spleen. No bacteria were detected from any of the examined tissues of the sampled fish.

Two hundred healthy humphead snapper were divided randomly into two groups (one hundred fish per group), and injected intraperitoneally with 200 μL of PBS (control), live *V. harveyi* (the value of LD_{50} is 7.2×10^5 cfu g^{-1}) with the concentration of 5.0×10^7 cells mL^{-1}, respectively. At 6 h post injection, samples from liver, head kidney, spleen, thymus, gut, muscle and gill were taken from five individuals following anaesthetization with tricaine methanesulfonate (Sigma). The samples were frozen in liquid nitrogen immediately after dissection, and stored at −80 °C for further analysis. All organ samples were used for cDNA cloning and gene expression.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the sampled tissues of humphead snapper using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The quality of total RNA was detected by electrophoresis on 1% agarose gel. The first-strand cDNA synthesis was carried out according to protocols of M-MLV Reverse Transcriptase (Promega, USA) after DNaseI treatment (Promega, USA). The RNA isolated from spleen was used for templates synthesis of RACE PCR through SMARTer RACE cDNA Amplification Kit (Clontech, USA) after DNaseI treatment (Promega, USA).

2.3. Amplification of cDNA sequence and DNA sequence

To clone the partial sequence of snapper IL-6 (Ls-IL6), degenerate primers LF1 and LR1 (Table 1) were designed based on a multiple sequence alignment of the fish IL-6 genes reported in GenBank (Table 2). The amplified PCR products using LF1 and LR1 were purified, ligated into the pMD18-T vector (TaKaRa, Japan) and cloned. Then the positive clones were sequenced by SANGON BIOTECH (Shanghai, China). To amplify the full-length sequence of Ls-IL6, the 5'and 3′ ends of the Ls-IL6 cDNA were amplified following the manufacturer's protocol of SMARTer RACE cDNA amplification kit (Clontech, USA). The sequences of the primers used were listed in Table 1. The RACE PCR condition and assembly of Ls-IL6 cDNA were performed as mentioned in Ref. [22].

The DNA template was extracted from muscle tissue by genomic DNA extraction kit (TIANGEN BIOTECH, China) according to user's

Table 2
GenBank accession numbers of IL-6 used in this study.

Protein	Accession no.
Lutjanus Sanguineus	JX683126
Epinephelus coioides	AFE62919
Danio rerio	AFC76325
Ailuropoda melanoleuca	AEY70473
Felis catus	BAA02507
Equus caballus	AAB87703
Sus scrofa	AFK92986
Ovis aries	CAA44363.1
Macaca mulatta	AAA99978
Mus musculus	ABG81953
Gallus gallus	ADL14564
Paralichthys olivaceus	ABJ53333.1
Takifugu rubripes	CAD67609
Oncorhynchus mykiss	ABI48359
Homo sapiens	AAC41704
Hippoglossus hippoglossus	ADP55202
Sparus aurata	ABY76175
Beluga Whale	Q9XT80
Genomic DNA	**Accession no.**
Homo sapiens	Y00081
Rattus norvegicus	M26745
Mus musculus	M20572
Paralichthys olivaceus	DQ884914
Takifugu rubripes	AJ544722

manual. After obtaining the cDNA sequence of IL-6 gene, the genomic DNA sequence Ls-IL6 was amplified through primers DF and DR (Table 1).

2.4. Bioinformatics analysis

The potential open reading frame (ORF) was analyzed with ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight and theoretical isoelectric point from the deduced amino acid sequence were calculated referring to the online software ProtParam (http://www.expasy.ch/cgi-bin/protparam). The similarity analysis of nucleotide and amino acid sequence was performed using BLAST program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein analysis was performed using ExPASy online tools (http://www.expasy.org/structural bioinformatics). Multiple-sequence alignments were carried out using ClustalX2.0, and the phylogenetic tree was constructed using the MEGA 6.0 software.

2.5. Real-time PCR assay

Total RNA extraction and cDNA synthesis were performed as described in 2.2. qRT-PCR was carried out in a Bio-Rad iQ5 Real-time PCR System (Bio-Rad) using the Trans Start™ Green qPCR Super- Mix (TransGen) as described previously [13]. Melting curve analysis of amplification products was performed at the end of each qRT-PCR to confirm that only one qRT-PCR product was amplified and detected. The expression level of Ls-IL6 was analyzed using comparative threshold cycle method ($2^{-\triangle\triangle Ct}$) with β-actin as described in Ref. [23].

2.6. Expression and purification of recombinant Ls-IL6

To obtain recombinant Ls-IL6 protein (rLs-IL6), Ls-IL6 CDS was subcloned into pET-32a (+) vector to obtain plasmid pET-LsIL6. The recombinant plasmid pET-LsIL6 was transformed into *E. coli* BL21 cells, and the fusion protein was expressed under induction conditions of exposure to isopropyl-*b*-*d*-thiogalactoside (IPTG 1 mmol) at 37 °C for 4 h. Then, eluted the target protein from purification column by imidazole elution buffer, and the eluted protein desalination was performed by ultrafiltration (Millipore). All the proteins were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and Coomassie blue staining solution was used for protein staining.

2.7. Antibody preparation

To obtain antibody against Ls-IL6, the purified rLs-IL6 was quantified by UV method and the protein was mixed with equal volume of Freund's adjuvant (Sigma) to immunize mouse by hypodermal injection at a dose of 100 μg per mouse at 7-day intervals. Freund's complete adjuvant (FCA) was used in the first injection and Freund's incomplete adjuvant (FIA) in the subsequent three injections. The mouse anti-LsIL6 serum was collected at 3 days after the last immunization. Simultaneously, the negative serum was produced as control by immunizing mouse with PBS using the same procedures. The separated antiserum obtained by centrifugation was stored at −80 °C until used.

2.8. Immunohistochemical (IHC) analysis

The complete tissues (thymus, head kidney and spleen) of humphead snapper were collected and fixed in freshly prepared 4% paraformaldehyde in 100 mM PBS (pH 7.4) containing 10 Mm EDTA at room temperature for 1 h. The samples were dehydrated, embedded in paraffin wax using Embedder (Leica) and then sectioned into 6 μm using a rotary microtome (Leica). The sections were treated with xylene for deparaffinization, rehydrated and unmasked following the standard IHC methods. Endogenous peroxidase activity was blocked by immersing the sections for 10 min in absolute methanol containing 0.3% hydrogen peroxide. Subsequently, the sections were treated at 4 °C with the primary antibody (mouse anti-LsIL6 sera) at a dilution of 1:100 (sterile PBS, pH 7.4) for 1 h in a humidity chamber. Sections were later washed in PBS and incubated with goat antimouse horseradish peroxidase (HRP) conjugate (Sigma) at a dilution of 1:100 for 30 min at room temperature. The antibody-bound proteins were then visualized by DAB-H_2O_2. Finally, the slide was observed by Leica DM6000B microscope (Leica). Negative controls for all samples were done using negative serum instead of primary antibody.

2.9. Construction of the plasmids of pcDNA-OmpW, pcDNA-IL6-OmpW

The sequence encoding OmpW were screened from draft genome sequence of *V. harveyi* strain kept in our laboratory (data not published). The primers used for OmpW amplification were designed according to open reading frames (ORF). To detect the immune adjuvant effects of Ls-IL6, the fusion genes IL6-(G4S)3-OmpW (designated as IL6-OmpW) were amplified by SOE (splicing by overlap extension) PCR. For Ls-IL6 contained a signal peptide, the signal peptide sequences were removed from OmpW ORFs and the rest sequences were applied for primers design. Then fragments of OmpW, IL6-OmpW were subcloned into pcDNA3.1 (+) vector. Subsequently, the transformants were screened and sequenced to confirm the target gene. The correct constructed recombinant plasmids were named as pcDNA-OmpW and pcDNA-IL6-OmpW. For the purpose of vaccination, the recombinant plasmids above were extracted with Endo-Free Plasmid Maxi Kit (OMEGA, USA).

2.10. Vaccination and challenge

The plasmids pcDNA-OmpW and pcDNA-IL6-OmpW were diluted to 400 μg/mL with PBS, respectively. Healthy humphead snapper (body weight 40–50 g) were divided randomly into three groups (200 fish/group) and injected intramuscularly with 50 μL of PBS (control), pcDNA-OmpW and pcDNA-IL6-OmpW, respectively. At 35 days post-vaccination, the humphead snapper were randomly selected from each experimental group (one group with three replicate tanks, 30 fish/replicate) for challenge. The selected fish was intraperitoneal injected with a dose of 100 μL per fish containing 1×10^8 CFU mL^{-1} live *V. harveyi* according to the preliminary experiment. Cumulative mortality and clinical symptom were recorded daily until the mortality progress ceased at 15 days post-infection. Dead fish were autopsied to determine the cause of death. The presence of *V. harveyi* in the tissues was determined by bacterial culture on TSA and sequencing. Relative percent survival (RPS) was calculated as: RPS % = 1-(%mortality of vaccinated group/% mortality of control group) × 100% [24].

2.11. Detection of the serum antibodies against V. harveyi by enzyme-linked immunosorbent assay (ELISA)

Specification of serum antibody detection against *V. harveyi* was determined by ELISA. Each well of flat-bottom microplates was covered with 100 μL diluted *V. harveyi* (1×10^8 CFU mL^{-1}) overnight at 4 °C. After washing with phosphate buffered saline tween (PBST) and blocking with 5% BSA in PBS for 1 h at 37 °C, the serum (1:20 diluted in PBS) collected from different experimental groups was added 100 μL per well in triplicate and then incubated for 2 h at 37 °C. The secondary and third antibody were rabbit-anti-snapper serum (1:1000 diluted in PBS, kept in our laboratory) and goat-anti-rabbit Ig-alkaline phosphatase conjugate (1:1000 diluted in PBS, BOSTER Biological Technology co. ltd, USA), which were added 100 μL per well and incubated at 37 °C for 2 h, respectively. 100 μL of TMB substrate solution is added to each well and will be transformed into a blue precipitate, the amount of which is linearly proportional to the amount of enzyme in the well. After 15 min Incubation at room temperature, 50 μl of Stop Solution was added into each well and absorbance was measured at 450 nm.

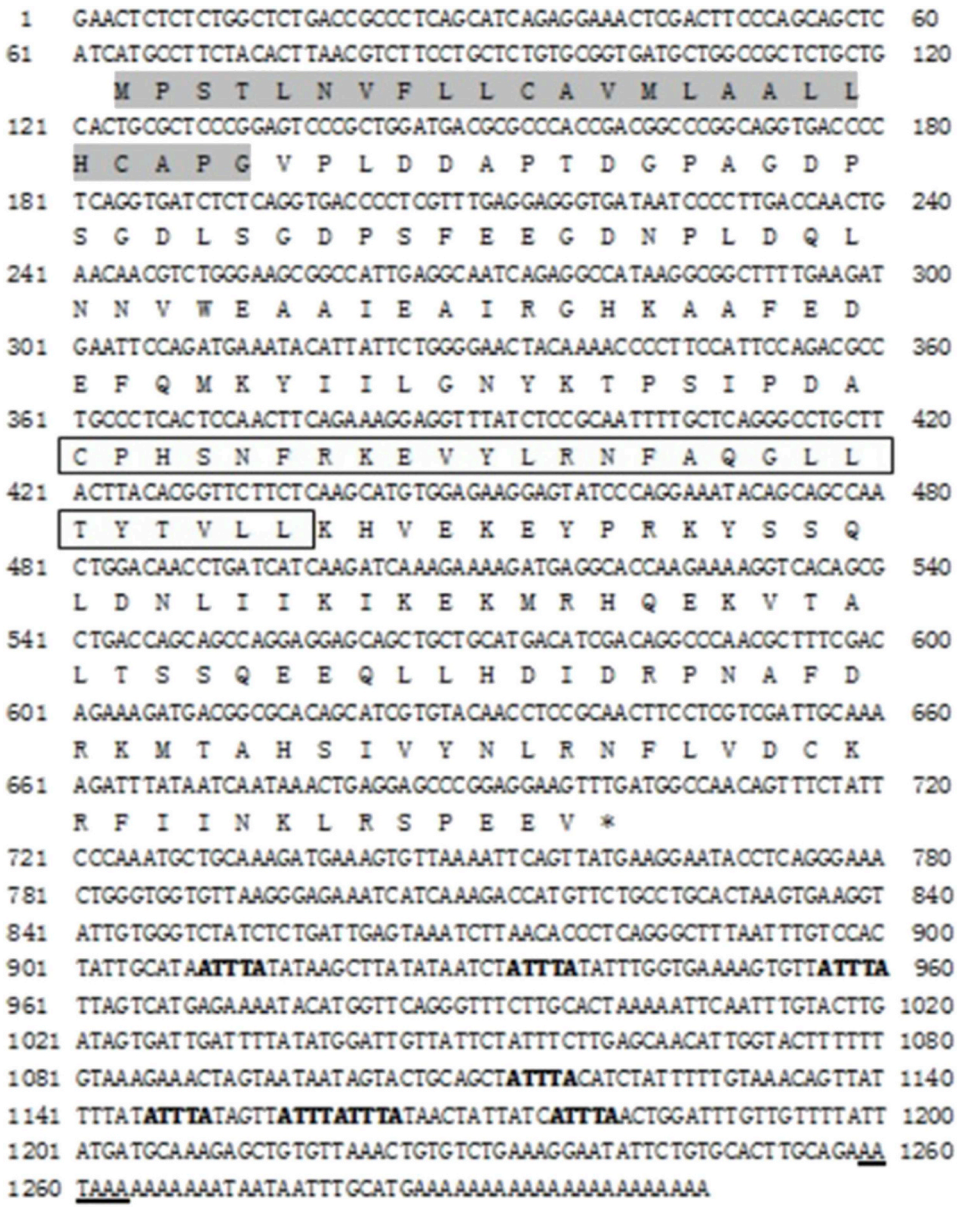

Fig. 1. Full-length nucleotide sequences and deduced amino-acid sequences of Ls-IL6. The signal peptide and the signature motif of IL-6 family were marked with grey and box, respectively. The unstable signals were indicated with bold. The polyadenylation signal was underlined.

2.12. Statistical analysis

All of the statistical analyses were carried out with SPSS 19.0 software (SPSS Inc., IBM, Armonk, NY, USA). The differences among different group were determined using a one-way analysis of variance (ANOVA). In all cases, the results were expression as the means ± SD (standard deviation), and the significance level was defined as $p < 0.05$.

3. Results

3.1. Characterization of Ls-IL6 sequence

The Ls-IL6 cDNA (GenBank accession no. JX683126) was comprised of a 63 bp 5-UTR, a 639 bp ORF and a 605 bp 3′-UTR including seven instability signals (ATTTA) (Fig. 1). The predicted Ls-IL6 protein was 212 aa in length, contained a signal peptide and typical motif of IL-6 family (Fig. 2). The predicted molecular mass and theoretical pI of the mature protein was 24 KDa and 5.65, respectively. The phylogenetic analysis shows that the fish IL-6 was clustered into a separate group apart from other species and Ls-IL6 was closest to *T. rubripes* IL-6 (Fig. 3).

When we cloned and analyzed the genomic sequence of Ls-IL6, we found that the Ls-IL6 had five exons and four introns. Strikingly, Ls-IL6 gene had a conserved exon-intron organization that found in human and fish, even in terms of both positions and phases of the introns. However, except the first exon, the other Exons and introns displayed variance in length among compared IL-6 (Fig. 4).

3.2. Tissue distribution of Ls-IL6

To detect the tissue distribution of Ls-IL6, several tissues including liver, spleen, thymus, head kidney, gill, muscle and gut were collected. The transcript levels of Ls-IL6 were detected by qRT-PCR. As shown in Fig. 5A, Ls-IL6 mRNA is distributed in all examined tissues, with higher expression in the head kidney, spleen and thymus, lowest level in liver. After *V. harveyi* infection, Ls-IL6 transcriptions were induced significantly in spleen, thymus and head kidney (Fig. 5A).

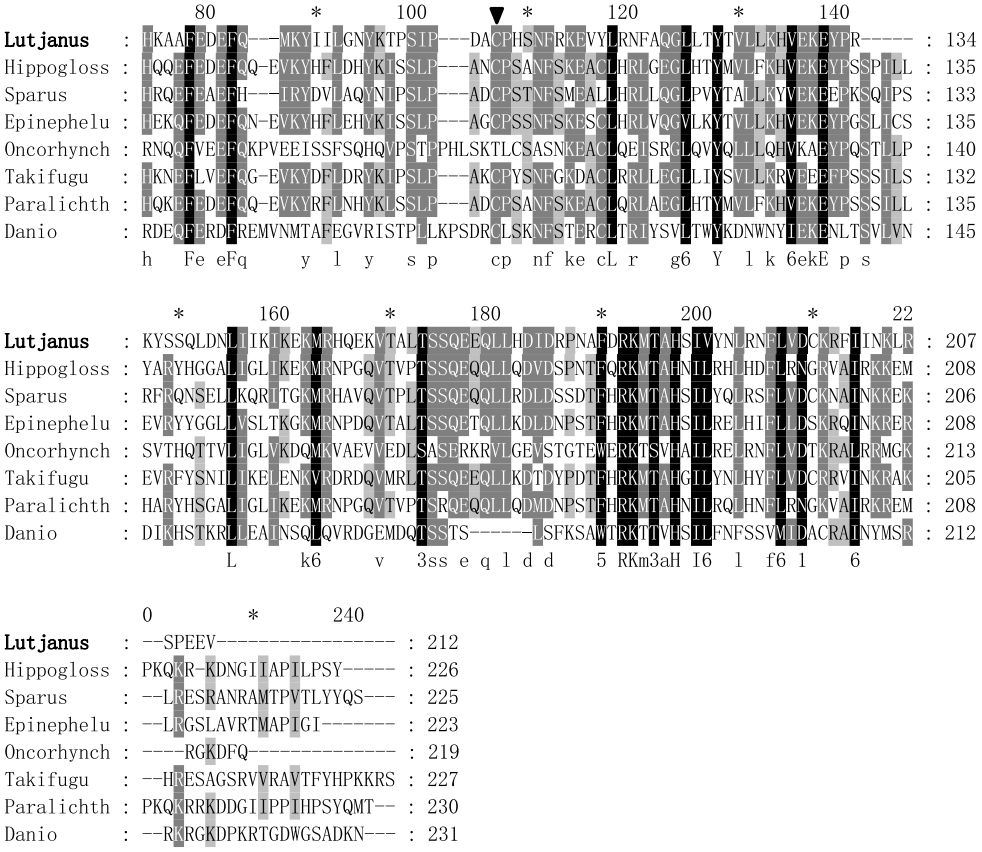

Fig. 2. Multiple alignments of Ls-IL6 amino acid sequence with other known IL-6s. The conserved cystein was indicated with arrow. GenBank accession numbers are listed in Table 2.

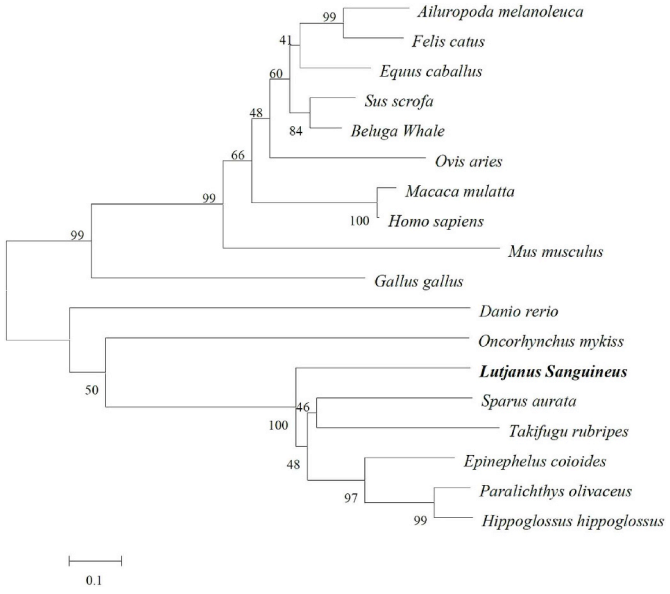

Fig. 3. Phylogenetic tree of Ls-IL6s constructed using the neighbor-joining method. The numbers in each branch indicate percentage bootstrap values for 1000 replicates.

3.3. Expression profiles of Ls-IL6 following V. harveyi in spleen, thymus and head kidney

qRT-PCR was applied to explore the expression patterns of Ls-IL6 after *V. harveyi* infection. The result showed that the expression level of Ls-IL6 increased markedly in various detected tissues and reached to peak at 6 h (thymus) and 9 h (head kidney) and 12 h (spleen). (Fig. 5B).

3.4. Preparation for antibody of Ls-IL6

To obtain recombinant protein of Ls-IL6 (rLs-IL6), pET-IL6 was transformed in *E. coli* BL21. The rLs-IL6 gene was efficiently expressed after IPTG induction (Fig. 6, lane 2). The expressed rLs-IL6 was purified and used for antibody preparation (Fig. 6, lane 3). Western blotting of the antisera and rLs-IL6 showed a typical protein recognition (Fig. 6, lane 4).

3.5. Distribution of Ls-IL6-producing cells

To clarify Ls-IL6 protein-producing cells in the major immune organs of humphead snapper, IHC analysis was performed. The results indicated that Ls-IL6-producing cells were detected in head kidney, spleen and thymus with positive signals, which also implying that antisera of rLs-IL6 can recognize the native IL6 proteins (Fig. 7d and e,f).

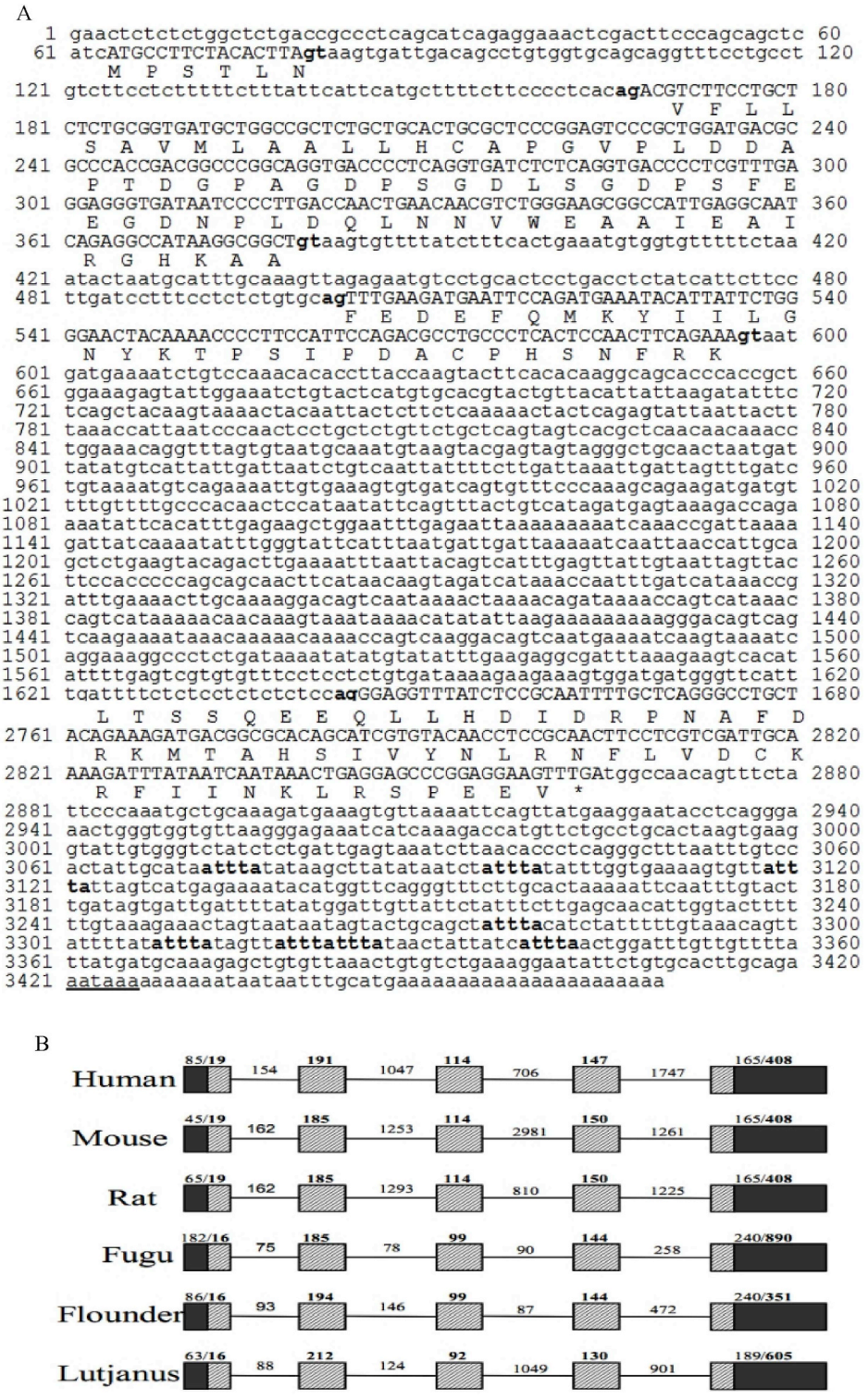

Fig. 4. Comparison of the gene organization and intron/exon sizes between Red snapper IL-6 and known IL-6 genes. The number of base pairs in the introns and exons of the IL-6 genes is shown where numbers in bold indicate the regions containing the coding sequence. Shadow boxes indicate coding regions and open boxes indicate untranslated regions of exons for each gene. The accession numbers of the IL-6 genes are listed in Table 2.

3.6. Tissue distribution and in vivo expression of pcDNA-OmpW, pcDNA-IL6-OmpW

Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) analyses were performed to detect the presence of plasmids pcDNA-OmpW, pcDNA-IL6-OmpW, respectively. The results showed that the OmpW, IL6-OmpW were detected in muscle, spleen, head kidney, and liver of vaccinated fish at 7d (Fig. 8).

3.7. Response of specific serum antibodies

The specific serum antibodies of vaccinated flounder in each experimental group were detected by enzyme-linked immunosorbent assay (ELISA) from day 1–35 post-vaccination (Fig. 9). Compared to the

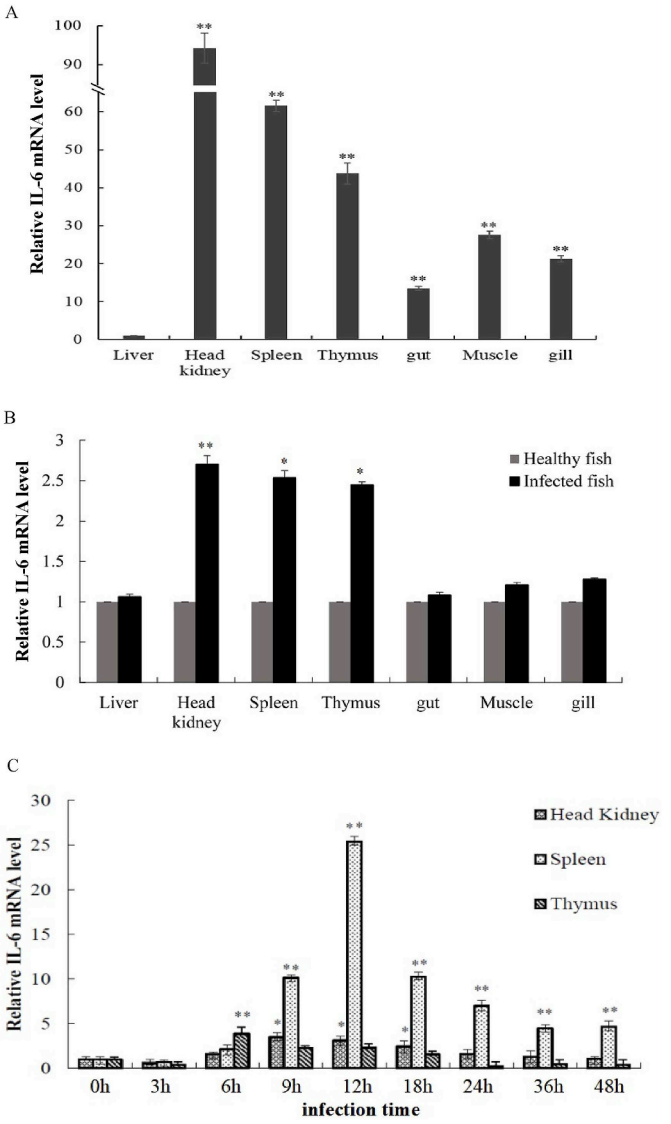

Fig. 5. (A)The mRNA expression level of Ls-IL6 in different tissues of healthy fish. Data was presented as a ratio to Ls-IL6 mRNA expression in liver. (B)The mRNA expression level of Ls-IL6 in different tissues of fish after *V. harveyi* infected. The expression of Ls-IL6 in infected fish were calculated as the folds changed based on the expression level in healthy fish. (C) The mRNA expression level of Ls-IL6 in head kidney, spleen and thymus at different time points after challenge with *V. harveyi*. The relative expression of Ls-IL6 was calculated in *V. harveyi*-challenged fish as the folds relative to that in PBS-injected fish at the same time point. Vertical bars represented the means ± SD (n = 3), levels of significance were set as *($p < 0.05$) or **($p < 0.01$).

PBS control group, the levels of specific serum antibodies in the other vaccination groups showed the same dynamics trend. The levels gradually increased and reached their peak levels at day 21 post-vaccination, then descended slowly ($p < 0.05$) (see Fig. 10).

3.8. Protection against V. harveyi infection

The cumulative mortality rates of vaccinated fish after challenging with live *V. harveyi* at 15 days post-vaccination are shown in Fig. 9. In the blank control group, the challenged fish began to die Day 6, then increased rapidly and the cumulative mortality finally reached 100% at Day 10. The RPS of the OmpW, IL6-OmpW group reached 60% and 76%, respectively. The bacteria tests of the lesions separated from the infected flounder also demonstrated that *V. harveyi* was the only type of pathogen that caused the death of snapper.

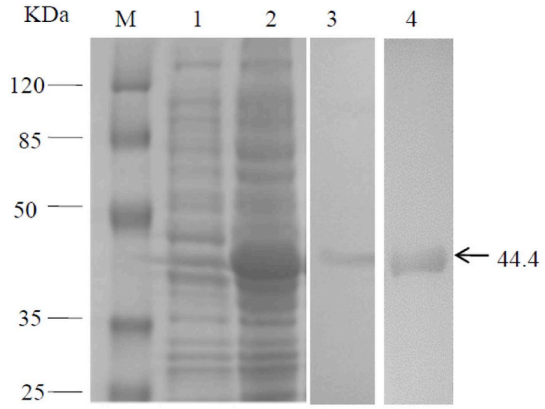

Fig. 6. SDS-PAGE (Lane 1–3) and Western blot analysis (Lane 4). Lane M, protein molecular marker; lane 1, pET-LsIL6 without IPTG induction; lane 2, pET-LsIL6 with IPTG induction; lane 3, purified Ls-IL6 protein; lane 6, purified Ls-IL6 protein (using mouse anti-LsIL6 serum as the primary antibody).

4. Discussion

In this study, a fish IL-6 homolog (Ls-IL6) from humphead snapper (*Lutjanus sanguineus*) was identified and characterized. Ls-IL6 possessed a typical motif C-X(9)-C-X(6)-GL-X(2)-Y-X(3)-L of IL-6 family, a signal peptide and a conserved cystine, which were also found in other known fish IL-6. Like reported IL-6 genes [13,18,19], the genomic exon-intron organization of the Ls-IL6 gene is same as IL-6 genes from rat, mouse and human, with five exons and four introns.

Tissue distribution showed that Ls-IL6 was mainly expressed in head kidney, spleen and thymus, which was similar with the findings in Nile tilapia [17], halibut [25], snout bream [26] and fugu [13]. It was possible that Ls-IL6 could play vital roles in lymphocytes differentiate, mature and interact [27–31]. In the test of bacterial infection, Ls-IL6 was significantly up-regulated in thymus, head kidney and spleen, which was in accordance with IL6s of Nile tilapia [17], rainbow trout [32], flounder [19] and seabream [18]. These results indicated that Ls-IL6 might be involved in host defense against bacterial infection.

Cytokines are low-molecular-weight proteins that contribute to mediate the communication for immune system and play an essential role in host defense against pathogens [33]. The adjuvant effects of cytokines in the form of recombinant protein, DNA plasmid (separate gene or fusion-gene) have already been recorded in various mammalian models [10–12,34–36]. Previous research revealed that the recombinant protein and DNA plasmid of flounder IL-6 also can be used as adjuvants to enhance the immune response and evoke immune protections against bacterial infection [37]. In this work, the plasmid containing Ls-IL6 and OmpW of *V. harveyi* (pcDNA-IL6-OmpW) vaccinated fish induced higher immune protection compared with pcDNA-OmpW vaccinated fish, which was consistent with the finding of flounder IL-6 [37]. Given IL-6 could promote B cell differentiation and antibody production [21,38], induce the differentiation of thymocytes and splenic cytotoxic T-lymphocytes as well [8,39], the future research should clarify the mechanisms of fusion gene IL6-OmpW on immune response against bacterial infection.

In summary, an IL-6 cDNA (Ls-IL6) was cloned from humphead snapper, *Lutjanus sanguineus*. Ls-IL6 possessed characteristic motif of IL-6 family shared high identities to teleost IL-6s. qRT-PCR analysis revealed that Ls-IL6 was expressed mainly in immune organs and induced by *Vibrio harveyi* infection. This data showed the involvement of Ls-IL6 in immune response against bacterial infection. Furthermore, the DNA vaccine co-expressing Ls-IL6 and OmpW increased the level of protective immunity after *V. harveyi* challenged, indicating that Ls-IL6 could be applied as a potent immune adjuvant for the vaccine development in control and prevention of fish disease.

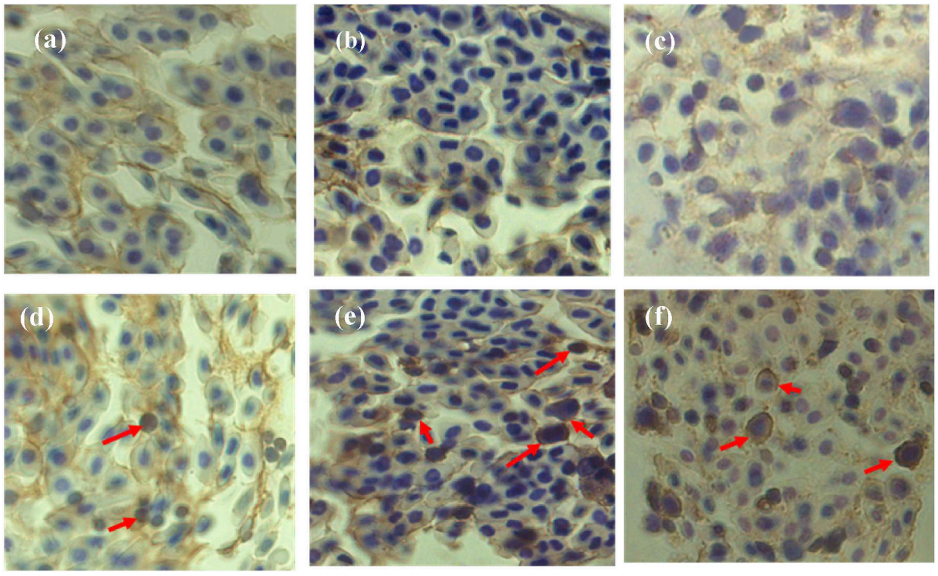

Fig. 7. Distribution of Ls-IL6 in the head kidney (a, d) spleen (b, e) and thymus (c, f) tissue of *L. sanguineus* by immunohistochemistry. (a), (b) and (c) are control groups that use negative serum as the primary antibody. (d), (e) and (f) are experimental groups that use mouse anti-LsIL6 serum as the primary antibody. Black arrows represent positive signals. Arrows represent positive signals.

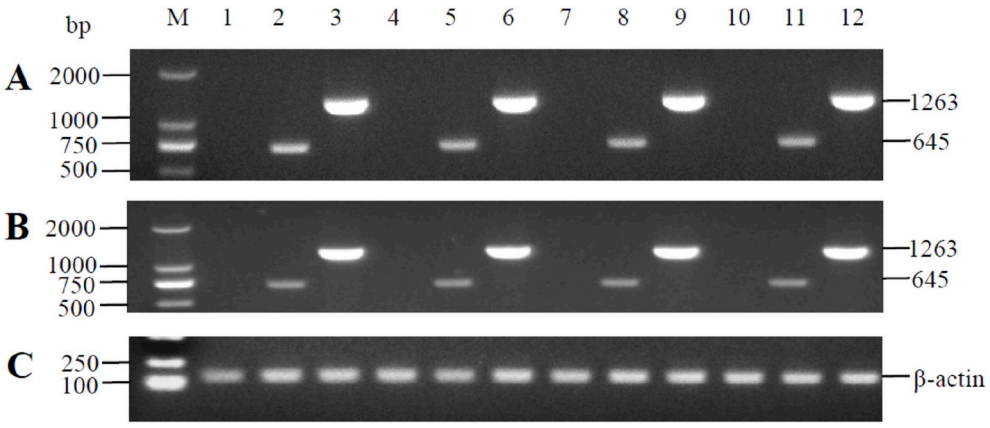

Fig. 8. Detection of plasmid pcDNA-OmpW (A) and pcDNA-LsIL6-OmpW transcription (B) by polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) in vaccinated fish. Flounder reverse transcription polymerase chain reaction (RT-PCR) in vaccinated fish. Snapper were vaccinated with phosphate buffered saline (PBS) (Lanes 1, 4, 7, and 10), pcDNA-OmpW (Lanes 2, 5, 8, and 11), and pcDNA-IL-6-Omp W(Lanes 3, 6, 9 and 12); DNA and RNA were extracted from muscle (Lanes 1, 2, and 3), spleen (Lanes 4, 5, and 6), head kidney (Lanes 7, 8, and 9), and liver (Lanes 10, 11, and 12) at 7 d post-vaccination. The β-actin (C) was used as an internal control. Lane M, DNA marker.

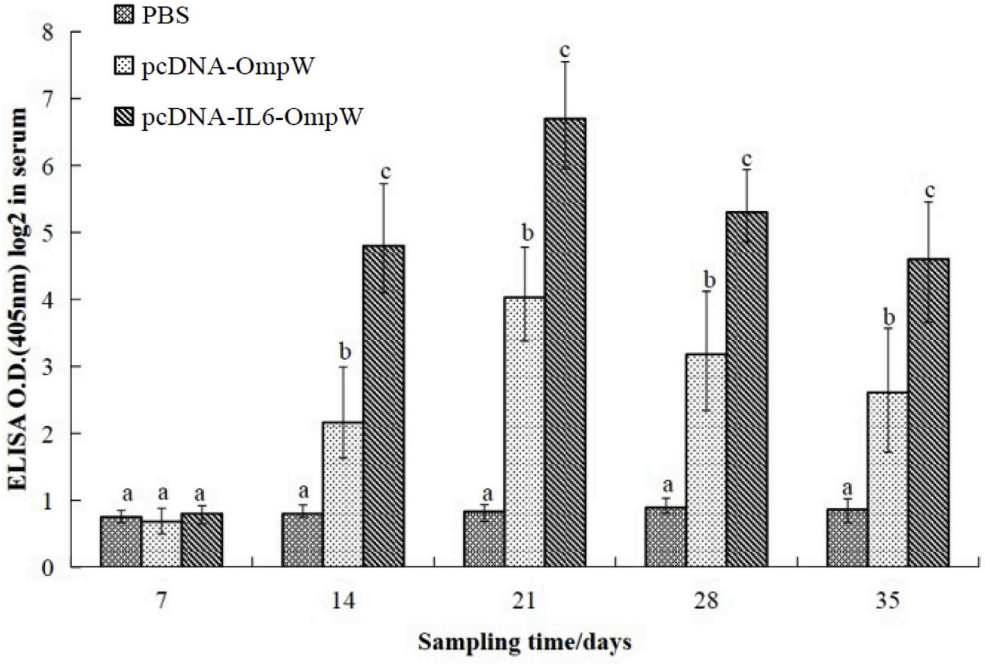

Fig. 9. Serum antibodies detection in vaccinated fish by enzyme-linked immunosorbent assay (ELISA). Serum was randomly collected from three fish at day 7, 14, 21, 28 and 35 post-vaccination. Results are expressed as the means ± SD (n = 3). Different letters on the bars represent the statistical significance ($p < 0.05$).

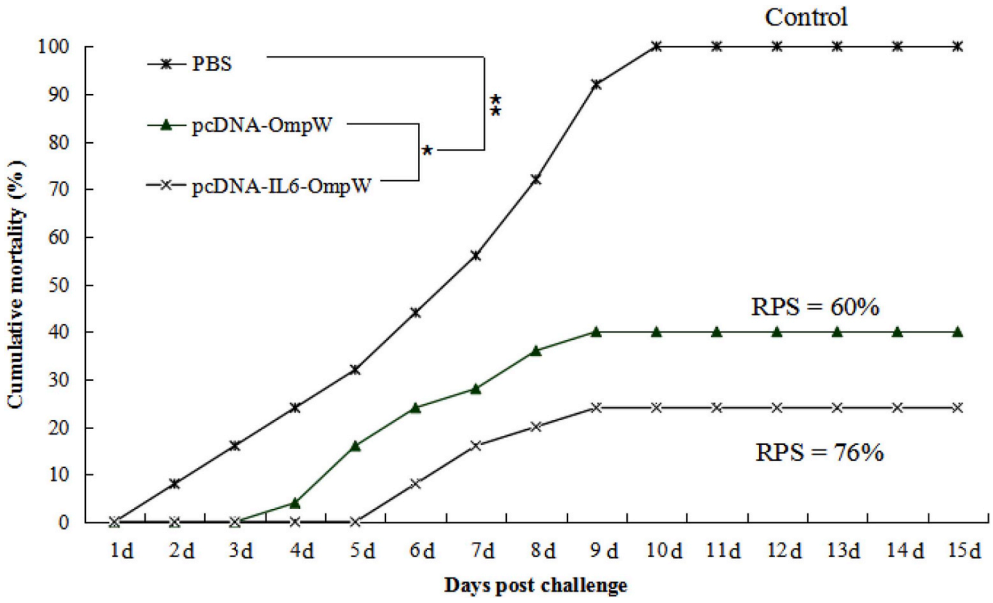

Fig. 10. Cumulative mortality rates of vaccinated snapper after challenge with live *V. harveyi* at day 35 post-vaccination. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. The different letters above the bars indicate the significant difference among different groups at the same time point (*$p < 0.05$ or **$p < 0.01$).

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