Fish and Shellfish Immunology 93 (2019) 1018–1027

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Generation and functional evaluation of a DNA vaccine co-expressing *Vibrio anguillarum* VAA protein and flounder interleukin-2

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ARTICLE INFO

Keywords:
Vibrio anguillarum
Paralichthys olivaceus
Bicistronic DNA vaccine
IL2
Immune response

ABSTRACT

In our previous study, a DNA plasmid encoding the VAA gene of *Vibrio anguillarum* was constructed and demonstrated to confer moderated protection against *V. anguillarum* challenge. Here, a bicistronic DNA vaccine (pVAA-IRES-IL2), co-expressing the VAA gene of *V. anguillarum* and Interleukin-2 (IL2) gene of flounder, was constructed to increase the protective efficacy of VAA DNA vaccine. The potential of pVAA-IRES-IL2 to express both VAA and IL2 in transfected HINAE cell lines was confirmed by immunofluorescence assay. Further, the variation of $sIgM^+$, $CD4\text{-}1^+$, $CD4\text{-}2^+$ lymphocytes and production of VAA-specific antibodies in flounder, which was intramuscularly immunized with three DNA plasmids (pIRES, pVAA-IRES, pVAA-IRES-IL2), were investigated, respectively. The bacterial burden and relative percentage survival (RPS) of flounder exposed to *V. anguillarum* infection were both analyzed to evaluate the efficacy of bicistronic DNA plasmid. Our results revealed that the percentages of $sIgM^+$, $CD4\text{-}1^+$, $CD4\text{-}2^+$ lymphocytes and antibodies specific to VAA were remarkably increased in pVAA-IRES or pVAA-IRES-IL2 immunized fish. Moreover, the co-expression of IL2 enhanced the immune response in response to VAA DNA vaccination, as shown by the higher percentages of $sIgM^+$, $CD4\text{-}1^+$, $CD4\text{-}2^+$ lymphocytes and production of specific antibody. Importantly, the RPS in pVAA-IRES-IL2 and pVAA-IRES groups reached 64.1% and 51.3%, respectively, when compared with the 97.5% cumulative mortality in pIRES group. Furthermore, the number of *V. anguillarum* in liver, spleen and kidney of pVAA-IRES or pVAA-IRES-IL2 immunized flounder after *V. anguillarum* challenge was significantly reduced, as compared to that in pIRES group. These suggest that the bicistronic DNA vaccine can be an effective immunization strategy in inducing immune response against *V. anguillarum* infection and IL2 has the potential as the adjuvant for VAA DNA vaccine.

1. Introduction

Flounder (*Paralichthys olivaceus*) is an important economic marine species that widely raised in China. *Vibrio anguillarum*, a vital pathogenic bacterium, invades a wide range of fish species and causes huge loss in major marine fish worldwide [1,2]. The bacteria infected fish exhibited the classical symptoms of vibriosis, including lethargy, swollen in the abdomen and skin lesion and even ulceration [3]. Prevention of bacterial disease through the use of antibiotics raised the problems of multidrug resistance and concerns of environmental contamination, vaccination is becoming the most acceptable measure for controlling infectious diseases [4].

DNA vaccines offer several advantages over traditional vaccines modalities in practical and immunological points of view, which make it become the next generation vaccines [5]. Inoculation of the foreign antigen-encoding DNA plasmid induce both antigen-specific humoral and cellular immune responses to protect against viral and bacterial infections [6,7]. Moreover, DNA based immunization has the advantages of simple and identical production processes, and relatively easy and inexpensive to manufacture. Previously, a DNA vaccine containing the VAA gene of *V. anguillarum* was constructed and proved to increase the percentages of $CD4\text{-}1^+$, $CD4\text{-}2^+$ and $sIgM^+$ lymphocytes, induce the production of specific antibodies and confer moderated immune protection against *V. anguillarum* challenge [8]. Considering the relatively low efficacy of pVAA vaccine, there is an urgent need to further enhance the immune efficacy generated by VAA based DNA vaccine.

One feasible strategy to augment the immune effect of DNA vaccines is the co-administration of cytokines as eukaryotic plasmid. Cytokines, the low molecular weight glycoproteins and polypeptides that play a

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https://doi.org/10.1016/j.fsi.2019.08.052
Received 9 April 2019; Received in revised form 16 August 2019; Accepted 22 August 2019
Available online 22 August 2019

central role in modulating the fish immune response, have been widely proved as the ideal choice to enhance the immunogenicity of DNA vaccines [9]. Interleukin-2 (IL2), a key cytokine in stimulating T cell proliferation, mediating B cell proliferation and immunoglobulin synthesis [10], has been identified in salmonid [11], rainbow trout [12] and flounder [13]. The potential benefit of IL2 as molecular adjuvant has been investigated in chicken and human against bursal disease virus, hepatitis C virus and *Eimeria acervulina* [14–16]. However, there is no report related to IL2 as adjuvant of DNA vaccine in fish model.

Here, a bicistronic DNA vaccine encoding the antigenic gene (VAA gene of *V. anguillarum*) and adjuvant gene (IL2 gene of flounder) was constructed to further enhance the immune efficacy of VAA based DNA vaccine and demonstrated to express both VAA and IL2 proteins in transfected HINAE cell lines. Then, the constructed DNA plasmid was intramuscularly injected into flounder to evaluate the cellular and humoral immune response. Moreover, the bacterial burden in immune tissues and the protective effect were evaluated after *V. anguillarum* challenge.

2. Materials and methods

2.1. Ethics statement

All of the artificial experiments were conducted following the ethical standards and the guidelines of "Regulations for the Administration of Affairs Concerning Experimental Animals" documented by the State Science and Technology Commission of Shandong Province. The investigations were also verified by the Committee of the Ethics on Animal Care and Experiments at Ocean University of China.

2.2. Plasmid, bacteria, cells and fish

The pIRES bicistronic plasmid (Clontech, Mountain View, USA), contains two multiple cloning sites located on each side of the internal ribosome entry site (IRES), was used as the vector to construct the monocistronic and bicistronic DNA plasmids.

The pathogenic *V. anguillarum* was isolated from disease fish and stored at −80 °C in our laboratory [1]. The bacteria strain was cultured in 2216E broth medium at 28 °C for 18 h, harvested by centrifugation at 8000 × *g* for 20 min, and re-suspended with 0.01 M phosphate-buffered saline (PBS). The bacteria with the concentration of 1.0×10^7 CFU/mL was used for challenge assay.

The HINAE cell lines were kindly provided by Dr. Ikuo Hirono, professor of the Tokyo University of Marine Science and Technology. The cells were seeded in 6-well plates, cultured in Leibovitz's L-15 (L15) medium (Invitrogen, Carlsbad, CA, USA) containing 20% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and used for transfection assay.

Flounder, weighing 35 ± 5 g, were purchased from a marine fish farm without the history of *V. anguillarum* occurrence. The fish were cultured in tanks with aerated seawater at 21 ± 0.5 °C and fed with commercial dry food pellet twice a day.

2.3. Construction of the recombinant plasmid

The whole genome of *V. anguillarum* was extracted using TIANamp bacteria DNA Kit (Tiangen, Beijing, China) following the manufacturer's protocol and used as the template for VAA gene (GeneBank accession no. WP_013857004.1) amplification with VAA specific primers (Table 1). After purification with TIANgel Midi Purification Kit (Tiangen, China), the purified PCR product of VAA were digested with *Kpn* I and *Xho* I and then sub-cloned into the pIRES vector downstream of the CMV promoter to yield the monocistronic DNA plasmid pVAA-IRES. The sequence of the interest target gene was verified by specific PCR amplification and DNA sequencing to confirm the appropriate insertion of VAA gene into pIRES. The pVAA-IRES plasmid was digested with *Sal* I and *Bam*H I, to gain the liner plasmid in a 50 μL reaction. The reaction mixture contained 5 μL of pVAA-IRES (200 ng/μL), 1 μL each of *Sal* I and *Bam*H I, 5 μL of 10 × NEBuffer (NEB, Evry, France) and 38 μL H_2O. Digestion was performed for 2 h at 37 °C and confirmed by 1.0% agarose gel electrophoresis. The liner plasmid was extracted with Universal DNA Purification Kit (Tiangen, China).

Table 1
Primers used in this paper.

Primer	Primer sequence (5′-3′)	Acc. no.
VAA-F	GGGGTACCGCCACCATGAACAGTACTTTTATCGTC (*Kpn* I)	NC_015633.1
VAA-R	CCGCTCGAGTTACACTTCTAATATCACGCG (*Xho* I)	
IL2-F	GCGTCGACGCCACCATGGAGCACTTTATTGGGATTC (*Sal* I)	KY307833.1
IL2-R	CGGGATCCTGGAGAAGCTTTGCATGGTT (*Bam*H I)	
rpoS-F	GAAGATGCCAAAGAAGGGTTT	VAA_RS12590
rpoS-R	GAGCATTTGCGTACTAGCTTT	

The underlined letters represent the restriction enzyme sites.

The total RNA was extracted from kidney of flounder using TRIZOL reagents (Invitrogen, USA) as per the manufactures' instructions and quantified by Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The genomic DNA contaminant was removed using RNase-free DNase I (Promega, Madison, WI, USA). The first-strand cDNA was synthesized using 1 μg of total RNA with the assistance of Reverse Transcriptase M-MLV kit (TaKaRa, Dalian, China) following the manufacture's protocol. This cDNA was further used as template for PCR amplification of IL2 with specific primers designed using primer 5.0 software from the available NCBI sequences (GeneBank accession no. KY307833.1) (Table 1) in a 25 μL reaction with Taq DNA polymerase (5 U) (Thermo Fisher Scientific, USA). The cyclic condition for amplification of IL2 was 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, with a further final extension at 72 °C, for 5 min. Amplified PCR product was run in 1.0% agarose gel and subsequently purified by gel extraction using TIANgel Midi Purification Kit (Tiangen, China). After that, the PCR products were digested with *Sal* I and *Bam*H I and then ligated into the liner pVAA-IRES plasmid, to construct the bicistronic DNA plasmid pVAA-IRES-IL2. The sequence of the bicistronic plasmid was verified by specific PCR amplification and DNA sequencing, and then digested with *Kpn* I/*Xho* I and *Sal* I/*Bam*H I to confirm the appropriate insertion of IL2 and VAA genes into pIRES, respectively. The Endotoxin-free recombinant monocistronic and bicistronic DNA plasmids were prepared using EndoFree plasmid kit II (Tiangen, China) and quantified the concentration with Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, USA). The plasmids with concentration of 500 ng/μL and 200 ng/μL were used for transfection and immunization, respectively.

2.4. Immunofluorescence staining of VAA and IL2 expressed in HINAE cells

The potential of recombinant plasmid to express exogenous gene was verified *in vitro* transfected HINAE cell lines by indirect immunofluorescence assay (IFA) using specific rabbit anti-rVAA polyclonal antibody and mouse anti-rIL2 polyclonal antibody prepared in our laboratory.

The HINAE cells were seeded on coverslips and cultured in L15 medium. When the confluence arrives 70–80%, the cells were transfected with 500 ng of pIRES, pVAA-IRES or pVAA-IRES-IL2 using Lipofectamine® 3000 (Thermo Fisher Scientific, USA) reagent following the manufacture's instruction. Forty-eight hours after transfection, the cells were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature, washed three times with PBS, and then permeabilized by incubation for 10 min with 0.1% (v/v) Triton X-100 at room temperature. After washing thrice with PBS, the cells were blocked with 3% BSA

at 37 °C for 1 h, and incubated with rabbit anti-rVAA polyclonal antibody (1:5000) and mouse anti-rIL2 polyclonal antibody (1:1000) at 37 °C for 1 h in a humid chamber. After washing thrice as before, Alex Fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, USA, diluted 1:1000) and Alex Fluor 647-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, USA, diluted 1:1000) were added for incubation of 1 h at 37 °C. The nucleus of cells was stained with 4′, 6-diamidino-2- phenylindole (DAPI) (Invitrogen, USA) in a humidified chamber for 10 min. After washing with PBS as before, the coverslips were mounted onto sliders and imaged under EVOS FL Auto 2 (Thermo Fisher Scientific, USA). Cells transfected with pIRES were used as a negative control.

2.5. Vaccination, challenge and sampling

Six hundred flounder were randomly assigned to three groups (200 fish/group), named as pIRES, pVAA-IRES and pVAA-IRES-IL2, respectively. After acclimation for 1 week, fish were administrated in the epaxial muscle with 100 μL of recombinant plasmids containing 20 μg of pIRES, pVAA-IRES and pVAA-IRES-IL2, respectively.

For assessment of vaccine efficacy, forty flounder were randomly chosen from each group at 7th week after immunization and used for challenge studies as previous report [17]. Briefly, each fish was intraperitoneally injected with 1.0×10^6 CFU live virulent *V. anguillarum* and observed daily following 15 days. The dead fish were removed as soon as they dead. The relative percent survival (RPS) was calculated following the equation: RPS = {1 - (% mortality in vaccinated fish/% mortality in control fish)} × 100% [18].

The lymphocytes were isolated from peripheral blood at 1st, 2nd, 3rd, 4th, 5th, 6th and 7th weeks post immunization as previous report [19,20]. Briefly, blood was sampled from the caudal vein, blended with RPMI-1640 containing 20 IU/mL of heparin immediately, and store at 4 °C for 1 h. The partial red blood cells were removed by centrifugation at 100 × *g* for 15 min. Then, the cell suspensions were layered over a discontinuous Percoll (Pharmacia, USA) gradient (1.020/1.070 g/cm^3) followed centrifugation at 840 × *g* for 30 min. The lymphocytes were isolated from the interface, washed with PBS by centrifugation at 680 × *g* for 5 min to remove the Percoll and then used for flow cytometry (FCM).

Blood was drawn from the caudal veins (N = 3) at weeks 1, 2, 3, 4, 5, 6 and 7 post immunization for serum isolation. After setting at room temperature for 1 h, the blood was stored at 4 °C overnight and centrifugation at 3000 × *g* for 10 min. Serum was separated into aliquot and stored at −80 °C for future use.

At 7th day after-challenge, liver, spleen and kidney of flounder (N = 5) were sampled for qPCR and preparation of cryosections. Simply, one part of tissues was kept in RNAlater® (Invitrogen, USA) and stored at −20 °C for qPCR; the other part of tissues was immersed in OCT compound (Miles, Elkhart, IN) and frozen in −80 °C. The 7 μm thick frozen sections were cut using a Leica CM 1900 microtome (Leica, Nussloch, Germany), fixed with precooled acetone for 10 min, air-dried for 15 min and then stored at −20 °C prior to IFA.

2.6. FCM

FCM was used to evaluate the variation of CD4-1$^+$, CD4-2$^+$ and sIgM$^+$ lymphocytes in the peripheral blood from immunized flounder as described previously [17]. For detection the percentage of CD4-1$^+$ and CD4-2$^+$ lymphocytes, cells (1.0×10^6 cells/mL), sampled from peripheral blood, were incubated with 500 μL of rabbit anti-flounder CD4-1 polyclonal antibodies (FCD4-1-Pab, 1:1000) or rabbit anti-flounder CD4-2 polyclonal antibodies (FCD4-2-Pab, 1:1500) at 37 °C for 1 h. After washing thrice with PBS by centrifugation at 680 × *g* for 5 min, 500 μL of Alex Fluor 647 conjugated goat anti-rabbit IgG (1:1000, Thermo Fisher Scientific, USA) was added at 37 °C for 1 h. To measure the proportion of sIgM$^+$ lymphocytes, the sampled lymphocytes were incubated with monoclonal antibodies against flounder IgM (fIgM-Mab, 1:1000) at 37 °C for 1 h. Then the cells were washed three times with PBS by centrifugation at 680 × *g* for 5 min, and incubated with 500 μL of Alex Fluor 488 conjugated goat anti-mouse IgG (1:1000, Thermo Fisher Scientific, USA) for 1 h at 37 °C. After washing as above, the cell suspensions were analyzed with the Accuri C6 cytometer (BD Biosciences, Piscataway, NJ, USA). In short, the identified population of lymphocytes exhibited in side- and forward-scatter (SSC and FSC) represent cell granularity and cell size characters, respectively. The lymphocytes with similar characters were gated in the R1 scope of the dot plots and used for analyzing the fluorescent histograms in which Alexa Fluor 488 labeled cells and Alexa Fluor 647 labeled cells were displayed by fluorescent light (FL)-1 and FL-4, respectively. Myeloma culture supernatant or rabbit negative serum were used as negative controls, instead of the aforementioned primary antibodies.

2.7. ELISA

ELISA was performed to analyze the production of specific antibody as described previously [17]. In brief, the 96-well plates (Costar, Cambridge, MA, USA) were coated with 100 μL purified rVAA (100 μg/mL) and incubated at 4 °C overnight. The wells were washed three times with PBST and blocked with 5% BSA in PBS for 1 h at 37 °C. After washing three times as before, 100 μL of flounder sera (1:100) from immunized fish at weeks 1, 2, 3, 4, 5, 6 and 7 post immunization was added into each well and incubated at 37 °C for 1 h. The plates were washed three times with PBST and incubated with 100 μL per well of fIgM-Mab (1:1000). Then the plates were washed as above and incubate with 100 μL of AP conjugated goat-anti-mouse IgG (1:5000). Following washed thrice with PBST, the color reaction was developed by adding p-nitrophenyl phosphate (pNPP, Sigma, MO, USA) and the absorbance was determined at 405 nm using an automatic ELISA reader (TECAN, Männedorf, Switzerland). The serum from pIRES immunized fish instead of primary antibody was used as the negative control.

2.8. IFA

IFA was performed to detect the presence of *V. anguillarum* in liver, spleen and kidney from flounder at 7th day after challenge. Briefly, the cryosections were hydrated with PBST for 5 min and blocked with 5% BSA at 37 °C for 1 h. The sections were labeled with anti-serum (1:50) from pVAA-IRES-IL2 administrated fish at 5th week post immunization for 1 h at 37 °C. After washing with PBST for thrice, the sections were incubated with fIgM-Mab (1:1000) at 37 °C for 1 h. After three washes with PBST, the sections were incubated with Alex Fluor 488 conjugated goat anti-mouse IgG (1:1000) at 37 °C for 1 h. Evans blue dye (EBD) (Fluka, Lyon, France) (1 μg/mL) was added as the counterstain for 10 min at room temperature. Three washes as before, images were acquired with EVOS FL Auto 2 microscope.

2.9. qPCR

In order to further investigate the number of *V. anguillarum* in liver, spleen and kidney, tissues were removed from flounder at 7th day after challenge, and rpoS (GeneBank accession no. AY695433) was chosen as the target gene to quantify the *V. anguillarum* burden by qPCR as previously described [8]. Briefly, the whole genome was extracted from tissues of flounder (N = 5) using TIANamp DNA Kit (Tiangen, China) according to the manufacturer's instruction and used as the template for qPCR amplification. The qPCR was run using a LightCycler® 480 real-time PCR system (Roche Life Science, USA). The reaction mixtures include 10 μL SYBR® Select Master Mix, 2 μL of extracted DNA samples, 0.4 μL each of forward and reverse primers and 7.2 μL RNase-free water. The condition includes an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of 15 s at 95 °C, and annealing/elongation at

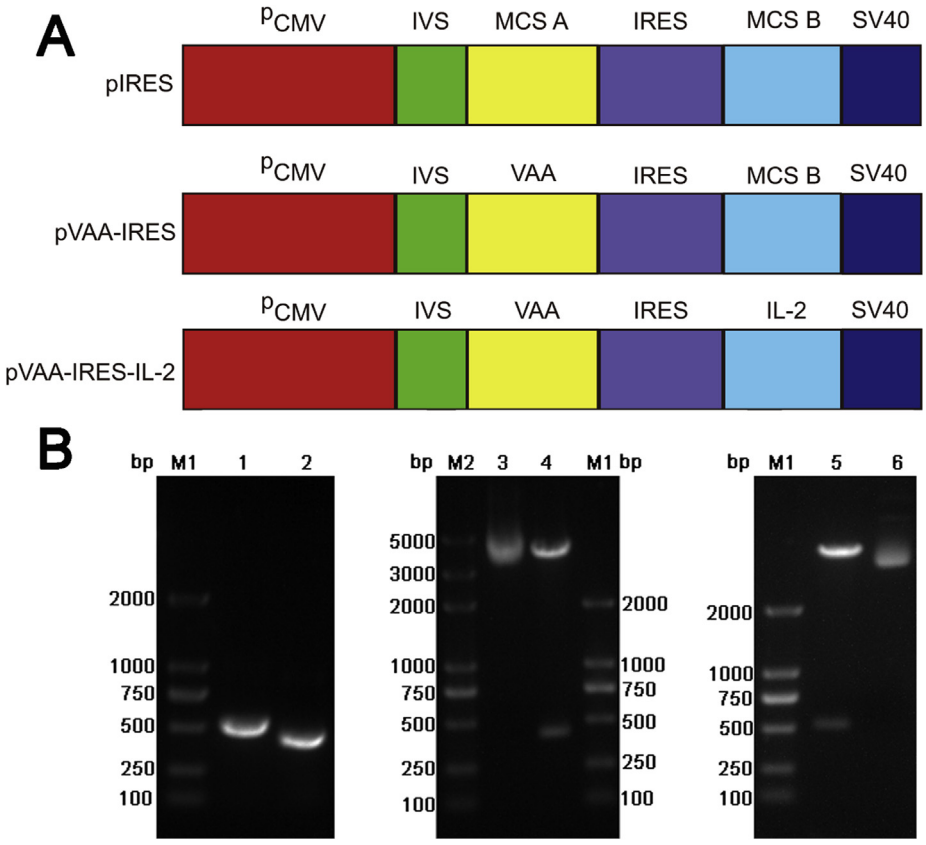

Fig. 1. Construction of pVAA-IRES and pVAA-IRES-IL2 plasmids. (A) Schematic structure of pIRES, pVAA-IRES and pVAA-IRES-IL2 plasmids. VAA gene was cloned into IRES containing vector alone or together with flounder IL2. pCMV, cytomegalovirus immediate early promoter; IVS, intervening sequence; MCS A, multiple cloning sites A; IRES, encephalomyocarditis virus (ECMV) internal ribosome entry site; MCS B, multiple cloning sites B; SV40, polyadenylation signals. (B) Amplification and digestion of recombinant plasmid. Lane1, VAA PCR product amplified by pVAA-IRES-IL2 plasmid; Lane 2, IL2 PCR product amplified by pVAA-IRES-IL2 plasmid; Lane 3 and 5, pVAA-IRES-IL2 plasmid; Lane 4, pVAA-IRES-IL2 plasmid digested by *Kpn* I and *Xho* I; Lane 6, pVAA-IRES-IL2 plasmid digested by *Sal* I and *Bam*H I.

60 °C for 60 s. The Ct values were read and converted into *V. anguillarum* counts following the previous established equation: $Y = -3.1043X + 32.15$. All experiments were performed in triplicate.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad software, Inc. San Diego, CA, USA). The results were shown as mean ± standard deviation (SD) and the differences among pIRES, pVAA-IRES and pVAA-IRES-IL2 groups in FCM, ELISA and qPCR were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple pair wise comparison. The significance level was defined as $P < 0.05$.

3. Results

3.1. Generation of pVAA-IRES and pVAA-IRES-IL2

As shown in Fig. 1A, in pIRES plasmid, two different multiple cloning sites (MCS A and MCS B) are located either upstream or downstream of the internal ribosome entry site (IRES). In pVAA-IRES plasmid, the VAA gene was inserted into the MCS A, downstream of the immediate early promoter of cytomegalovirus (CMV). In pVAA-IRES-IL2 plasmid, the VAA and IL2 gene were inserted into MCS A and MCS B, respectively.

PCR amplification and double digestion with restriction enzyme were used to verify the construction of pVAA-IRES-IL2 plasmid. In PCR assay, two specific bands, corresponding to target VAA (513 bp) and IL2 (423 bp) amplicon, respectively, were observed in the agarose gel following gel electrophoresis, confirming the pVAA-IRES-IL2 containing VAA and IL2 gene. Moreover, we verified the fidelity of the cloning by double digestion using *Kpn* I and *Xho* I; as well as by *Sal* I and *Bam*H I. The expected digestion products of each reaction were observed in agarose gel, indicating the VAA and IL2 are correctly ligated into the corresponding restriction enzyme site which resulted in the generation of pVAA-IRES-IL2. (Fig. 1B).

3.2. Expression of VAA and IL2 gene in transfected HINAE cell

The potential of recombinant plasmid to express VAA or IL2 protein was investigated in transient transfected HINAE cell lines by IFA. The results showed that a specific red fluorescence was detected in pVAA-IRES transfected cells, suggesting the expression of VAA protein. In pVAA-IRES-IL2 transfected cell lines, both green and red specific fluorescence were observed on the same cell, which reveal that the cells have the ability of expressing VAA and IL2 proteins after transfection for 48 h, simultaneously. In contrast, neither green nor red specific fluorescence was detected in the control group. All these results confirmed the potential of recombinant plasmid to express certain proteins *in vitro* transfected HINAE cell lines. (Fig. 2).

3.3. Cellular immune response was induced by recombinant plasmid

The potential of recombinant plasmid to increase the percentages of CD4-1$^+$ and CD4-2$^+$ lymphocytes in peripheral blood of immunized fish was analyzed by FCM, at weeks 1, 2, 3, 4, 5, and 6 post immunization. The fluorescence scatter plot showed the proportion of CD4-1$^+$ or CD4-2$^+$ T lymphocytes detected in one exemplary sample of three experimental fish at week 2 after immunization (Fig. 3A).

In pVAA-IRES and pVAA-IRES-IL2 immunized fish, the variation of CD4-1$^+$ lymphocytes showed a tendency of increasing first, reached the highest level, and then declined to the normal level. Specifically, the percentage of CD4-1$^+$ lymphocytes in pVAA-IRES immunized fish increased gradually at 1st week post immunization, reached the maximum level (6.7 ± 0.6%) at 2nd after immunization ($P < 0.05$), and retained to the normal level following 3rd after immunization. In pVAA-IRES-IL2 vaccinated flounder, the percentage of CD4-1$^+$ lymphocytes reached the peak level (10.5 ± 0.8%) at week 2 post immunization

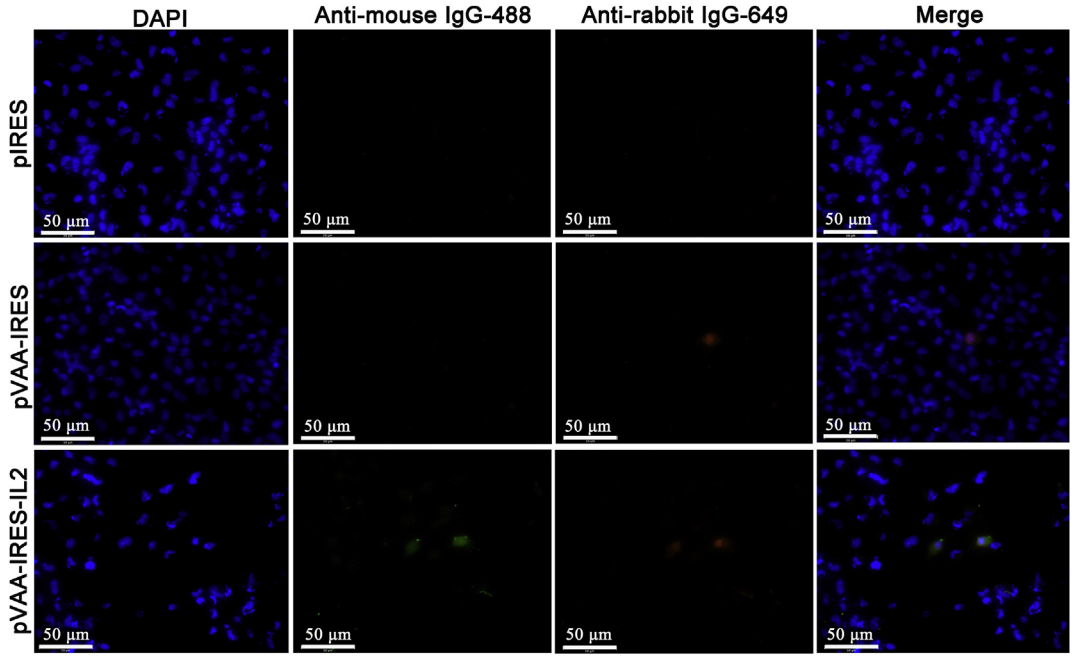

Fig. 2. The expression of recombinant plasmid in transfected HINAE cell lines. After transfection with recombinant plasmids for 48 h, HINAE cell lines were incubated with rabbit anti-rVAA antibody/mouse anti-IL2 antibody, and then incubated with goat anti-rabbit IgG-647 antibody/goat anti-mouse IgG-488 antibody to visualize the expression of VAA and IL2. pIRES was used as the control. DAPI panel show the control staining of cell nuclei. Scale bar: 50 μm.

($P < 0.05$), and maintained the significant difference till 3rd after immunization, then declined to the untreated level following 4th week post immunization. In the control group, the proportion of CD4-1$^+$ lymphocytes maintained relative invariant during the experimental period. (Fig. 3B).

The percentage of CD4-2$^+$ lymphocytes exhibited the similar tendency as the variation of CD4-1$^+$ lymphocytes proportion in peripheral blood. In pIRES injected fish, the percentage of CD4-2$^+$ lymphocytes maintained the normal range over the entire experimental period. In pVAA-IRES immunized fish, the percentage of CD4-2$^+$ lymphocytes reached the peak level of 7.1 ± 0.4% at 2nd week post immunization, and finally recovered to the normal level at 4th week. In pVAA-IRES-IL2 group, the percentage significantly increased at 1st week ($P < 0.05$), reached the summits at 2nd week, which was 11.5 ± 0.7%, maintained the significance till 4th week, and followed a gradually descent at week 5 post immunization. Moreover, the percentage of CD4-2$^+$ lymphocytes in pVAA-IRES-IL2 immunized fish exhibited the significant increase at weeks 2, 3 and 4, compared to that in pVAA-IRES group. (Fig. 3C).

3.4. Humoral immune response was elicited by recombinant plasmid

The ability of recombinant plasmid to induce humoral immune response was analyzed by the increase of sIgM$^+$ lymphocyte percentage and production of specific antibodies. Lymphocytes were removed from the peripheral blood of immunized fish at week 1–7 after immunization, and the proportion of sIgM$^+$ lymphocyte was analyzed by FCM.

The fluorescence scatter plot represented the percentage of sIgM$^+$ lymphocyte in one of three experimental fish at week 6 post immunization (Fig. 4A).

In pVAA-IRES group, the percentage of sIgM$^+$ lymphocyte exhibited a significant increase at 2nd week ($P < 0.05$), reached the maximum lever of 24.1 ± 1.8% at 6th week, then declined stably. In pVAA-IRES-IL2 group, the percentage augmented significantly at week 2 after immunization ($P < 0.05$), get the summit level of 31.7 ± 3.7% at 6th week, and maintained the significant at 7th week ($P < 0.05$). Compared with the percentage of pVAA-IRES fish, the percentage in pVAA-IRES-IL2 group exhibited a significant increase at 4th, 5th, 6th and 7th weeks post immunization ($P < 0.05$). Moreover, in pIRES group, the proportion showed relative lower invariant during the experimental period. (Fig. 4B).

The production of specific anti-rVAA antibody elicited by recombinant plasmid was evaluated by ELISA with rVAA as the capture antigen. The results showed that the specific antibody has a tendency to enhance fist, reach the maximum, then decrease gradually in recombinant plasmid groups. However, in pIRES group, the antibody level kept at the relatively stable level throughout the experimental period. The antibodies against rVAA were significant higher than the control group at 3rd week, reached the highest level at 6th week, and then decreased steadily, in pVAA-IRES and pVAA-IRES-IL2 groups. Compared with the pVAA-IRES group, the antibody level exhibited significant increase at 4th, 5th, 6th and 7th week post immunization in pVAA-IRES-IL2 group. (Fig. 4C).

3.5. The recombinant plasmid inhibits the colonization of V. anguillarum

The *V. anguillarum* burden in liver, spleen and kidney at 7th day after challenge was analyzed by IFA and qPCR.

As presented in Fig. 5A, an increase of specific green fluorescence signal indicating the exist of *V. anguillarum* was observed in the tissue sample from pIRES group compared to pVAA-IRES or pVAA-IRES-IL2 vaccinated fish.

The qPCR results revealed that the bacterial load was significantly decreased in the liver, spleen and kidney of pVAA-IRES or pVAA-IRES-IL2 immunized fish compared with the pIRES injected group, at 7th day after *V. anguillarum* challenge. Moreover, the bacterial burden in pVAA-IRES and pVAA-IRES-IL2 group exhibited no significant difference. (Fig. 5B).

All these results indicating that both pVAA-IRES and pVAA-IRES-IL2 inhibit the colonization of *V. anguillarum* in the liver, spleen and kidney.

3.6. The recombinant plasmid confers immune protective efficacy for flounder

The ability of pVAA-IRES and pVAA-IRES-IL2 to protect flounder against *V. anguillarum* challenge was evaluate by means of RPS. Two days' after challenge, the dead fish initially appeared in pIRES group

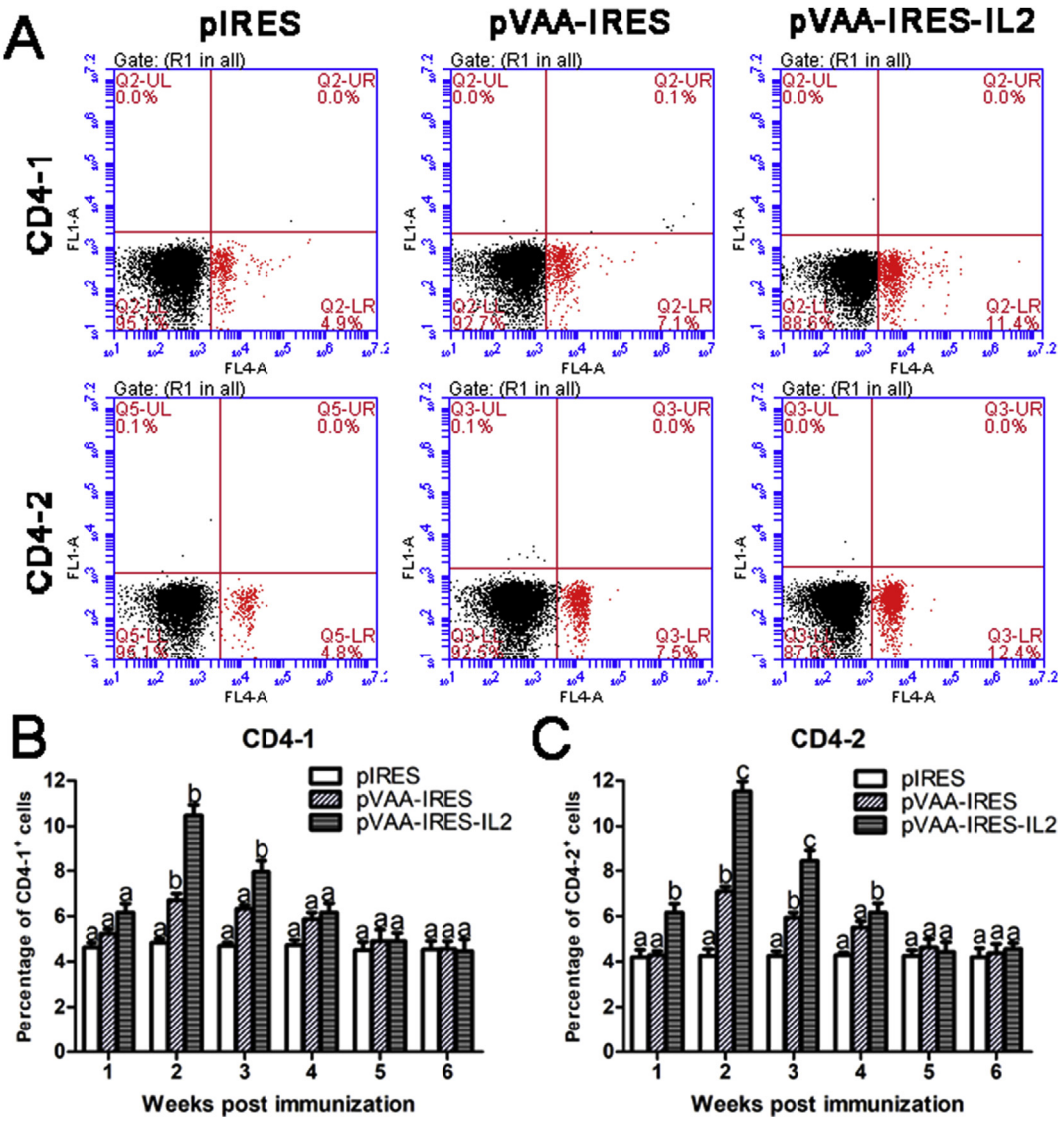

Fig. 3. Flow cytometric analysis of CD4-1$^+$ and CD4-2$^+$ T lymphocytes after immunization. (A) Plots show the percentages of CD4-1$^+$ and CD4-2$^+$ T lymphocytes in pIRES, pVAA-IRES and pVAA-IRES-IL2 immunized fish at week 2 post-immunization. FL4-A, fluorescence combined with Alex Fluor 647; FL1-A, fluorescence combined with Alex Fluor 488. (B) Changes of the percentage of CD4-1$^+$ T lymphocytes at 1st, 2nd, 3rd, 4th, 5th, and 6th weeks after immunization. (C) Variations of the percentage of CD4-2$^+$ T lymphocytes at 1st, 2nd, 3rd, 4th, 5th, and 6th weeks post immunization. All values are shown as means ± SD. Different letters (a, b and c) on the bar represent the statistical significance ($P < 0.05$) compared to each other at the same time point.

and died fast in the following 4th-7th days. Finally, only one fish was survived at the end of the experiment. The cumulative mortality in pIRES group was 97.5%. Comparatively, in pVAA-IRES and pVAA-IRES-IL2 vaccinated group, 21 and 26 fish were survived until the end of the experimental period. The cumulative mortality rate in pVAA-IRES and pVAA-IRES-IL2 groups were 47.5% and 35.0%, which corresponding to the RPS of 51.3% and 64.1%, respectively. (Fig. 6).

4. Discussion

The pIRES plasmid is a bicistronic eukaryotic vector that allows the simultaneous expression of two exogenous genes by cloning them into MCSA and MCSB of one plasmid. The IRES, derived from the encephalomyocarditis virus (EMCV), is an important component that permits the translation of two different open reading frames from the same mRNA. Both MCS's and the IRES are downstream of the immediate early promoter of CMV which could activate the expression two genes of interest at high level. Downstream of the MCSB, SV40 polyadenylation signals direct correct processing of the 3′ end of mRNA. The pIRES plasmid often been used to express two antigen genes or antigen gene in conjunction with cytokine, so as to construct bivalent vaccines or improve the effect of vaccines [21–23]. In recent years, researchers have used this plasmid to construct the bicistronic plasmid that simultaneously express antigen genes and cytokines in fish model, and investigate the potential of IL1β and IFNγ as vaccine adjuvants [24–26]. Moreover, the pIRES has been used as the based vector to co-express hepatitis B surface antigen and IL2 of mice [27]. Here, two recombinant plasmids, pVAA-IRES and pVAA-IRES-IL2 encoding VAA gene of *V. anguillarum* alone or plus with IL2 of flounder, were constructed based on the pIRES vector.

There are two *Bam*H I sites in the pIRES vector, which make the *Bam*H I is not suitable for use as the restriction enzyme in theory. However, in this research we used the *Bam*H I as the restriction enzyme in insertion of IL2 gene into pVAA-IRES plasmid, and the gel electrophoresis results revealed that the digestion occurred at the *Sal* I and *Bam*H I sites of the MCS. The possible reason was because that the relative distance between *Sal* I and *Bam*H I (in MCS) sites was near than that between *Sal* I and *Bam*H I (not in MCS) sites, so the digestion was

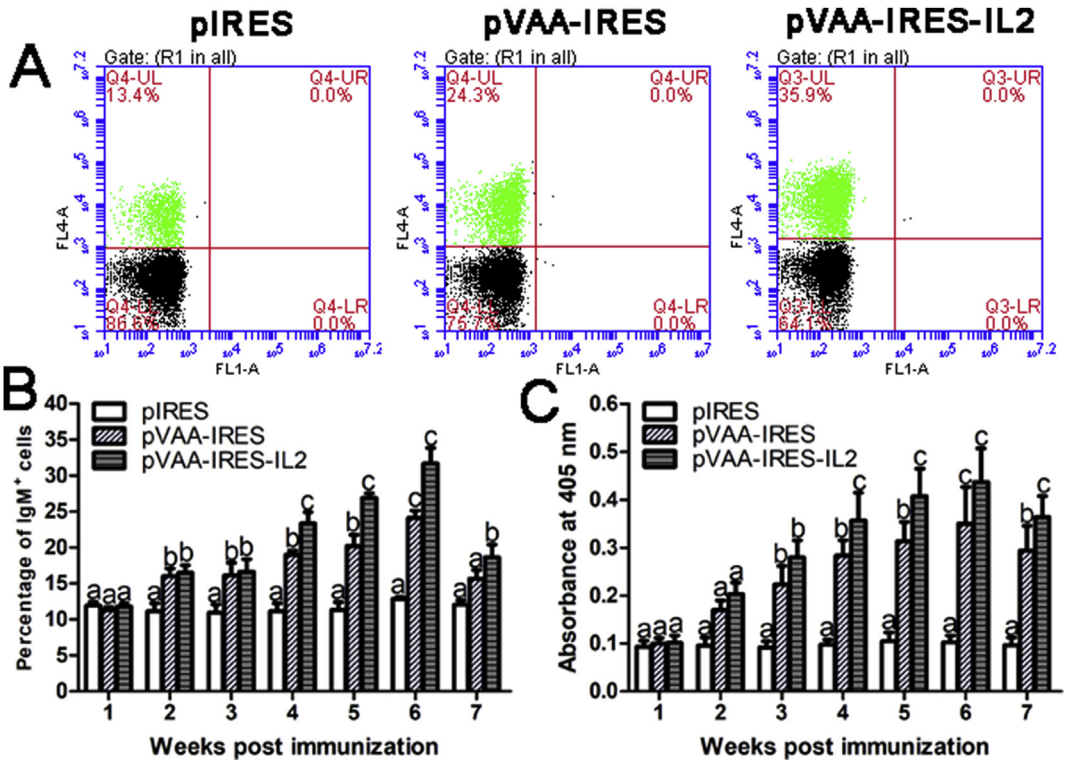

Fig. 4. The humoral immune response induced by bicistronic DNA vaccine. (A) FCM assay investigating the percentage of $sIgM^+$ B lymphocytes in peripheral blood of flounder. Plots show the percentage of $sIgM^+$ lymphocytes in pIRES, pVAA-IRES and pVAA-IRES-IL2 immunized fish at week 6 post-immunization. (B) Changes of the percentage of $sIgM^+$ lymphocytes at 1st, 2nd, 3rd, 4th, 5th, 6th and 7th weeks after immunization. (C) ELISA measuring the specific anti-rVAA antibody production in immunized fish at 1st, 2nd, 3rd, 4th, 5th, 6th and 7th weeks post immunization. All values are shown as means ± SD. Different letters (a, b and c) on the bar represent the statistical significance ($P < 0.05$) compared to each other at the same time point.

prefer to occurred at *Sal* I and *Bam*H I (in MCS) sites. Nevertheless, the *Bam*H I is not recommended to use as the restriction enzyme. The *Xba* I is better than *Bam*H I if the target gene does not contain the *Xba* I restriction site.

Vaccination is considered as a cost-effective measure to prevent the spread of infectious diseases in the global aquaculture industry. The introduction of vaccine will contribute to the reduction of antibiotics usage and disease outbreaks in fish farming. Multiple factors, such as antigen character and dose, primarily determined the efficacy of vaccines. Moreover, the introduction of adjuvants or immune stimulants is the commonly method to increase the vaccine efficiency [28]. Traditional adjuvants in fish farming, such as mineral oil and aluminum salts can sometimes offer desirable outcomes regarding the vaccine efficacy, but often they are discounted by the negative side effects that are associated [29,30]. Therefore, there is an ever-growing demand to develop effective adjuvants with minimal side effect.

Cytokines play a key role in the immune response of vertebrates and have been exploited as potential vaccine adjuvant that can stimulate stronger host adaptive immunities [31,32]. IL2, a cytokine with functions of stimulating T cell proliferation and mediating B cell proliferation, is the first cytokine used as an adjuvant to enhance the immune response in various animal models [33,34]. However, the use of recombinant IL2 as a vaccine adjuvant is quite restricted by its character of systemic toxicities and short *in vivo* half-life [35]. Therefore, the IL2, in form of DNA plasmid, is the most valuable way to augment the efficacy of DNA vaccine. At present, the plasmid IL2 has been used as the vaccine adjuvant to boost the humoral and cellular immune responses after inoculation of DNA vaccines in variety of small animal models [36–39]. Here, a bicistronic plasmid encoding IL2 as adjuvant was constructed to enhance the immune response of VAA DNA vaccine in flounder model.

The $CD4^+$ T cells, also known as T-helper (Th) cells, play a crucial role in orchestrating adaptive immune response against the invasion of pathogens and vaccines [40]. They will undergo numerous proliferation and differentiation into distinct Th cell subsets and then induce the immune response following the recognition of foreign antigen-derived peptide presented in the context of major histocompatibility complex (MHC) class II on APCs [41]. Therefore, the kinetics of CD4 gene expression, has a certain correction with protection conferred by vaccine and could be used to partially reflect the activation of cellular immune response in gene level following vaccination. In fish model, the change of $CD4^+$ T cells after vaccination was mainly detected by investigating the expression of CD4-1 and CD4-2 genes [42–45]. Recently, development of specific antibodies in flounder against the $CD4^+$ T cell markers (CD4-1 and CD4-2) [46] has allowed for a better characterization of cellular responses to hirame novirhabdovirus infection and DNA vaccination by the increased percentages of $CD4\text{-}1^+$ and $CD4\text{-}2^+$ lymphocytes [8,17,47]. Here, remarkable increase of $CD4\text{-}1^+$ and $CD4\text{-}2^+$ lymphocytes in peripheral blood was observed in pVAA-IRES and pVAA-IRES-IL2 group. Moreover, the percentage of $CD4^+$ lymphocytes in pVAA-IRES-IL2 group is higher than that of pVAA-IRES immunized fish, which indicate that IL2 has the potential to increase cellular immune response. The result is in line with the previous researches which indicating IL2 activates the proliferation of $CD4^+$ lymphocytes [48,49].

Antigen specific antibodies play a vital role in inhibition and elimination of invading pathogens and then protecting fish against pathogenic infections [50]. Production of antigen specific antibodies in response to immunization is the basic mechanism of immunoprotection. Previous researches have revealed that antibody response was activated by intramuscularly administrate DNA vaccination in studies of various fish models and suggested that antibody titer has a correction with immunoprotection conferred by vaccines [51–54]. In this study, remarkable production of specific antibodies against VAA was induced by either pVAA-IRES or pVAA-IRES-IL2 immunization. Importantly, co-

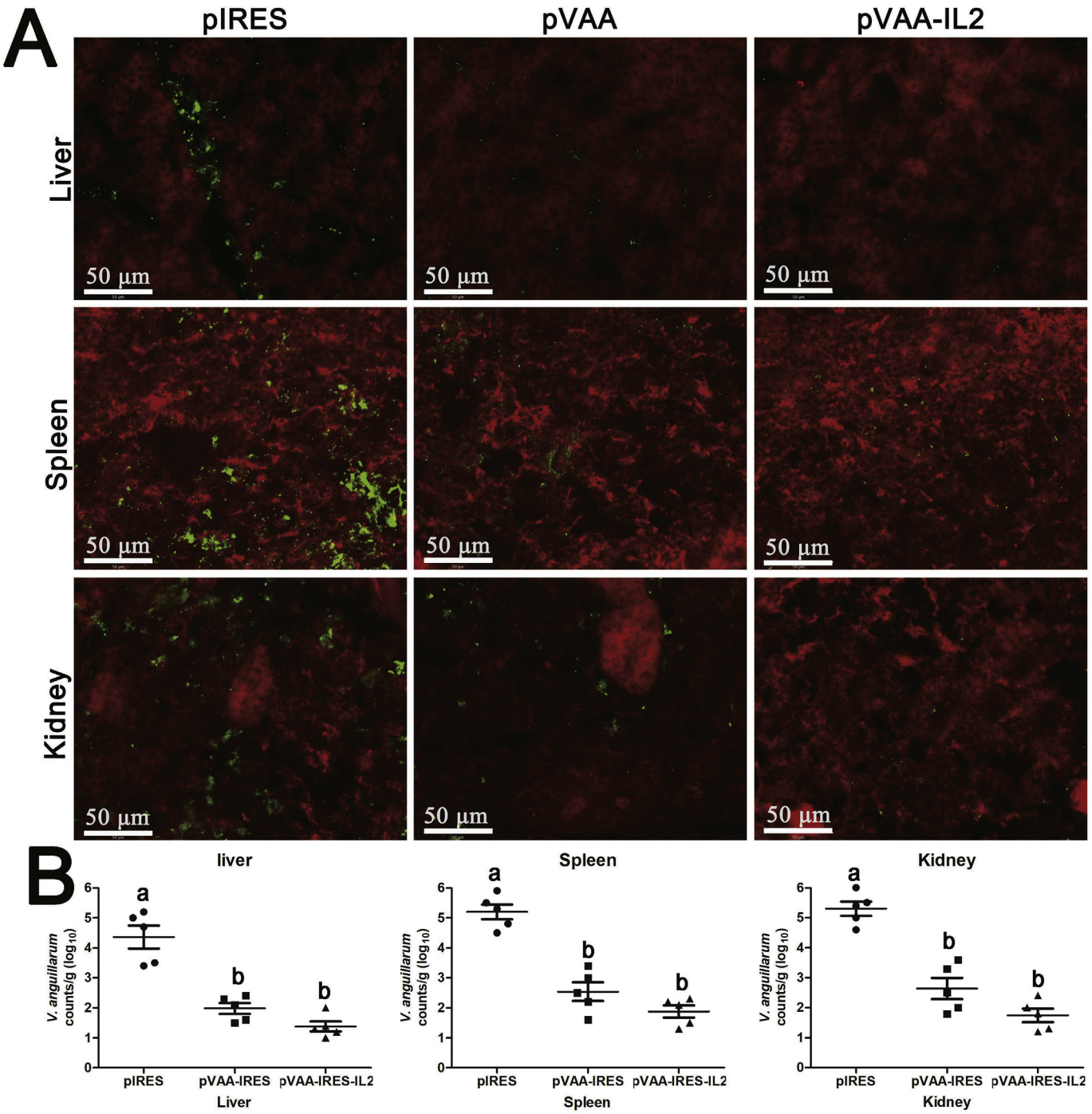

Fig. 5. Detection of bacterial burden after *V. anguillarum* challenge. (A) Tissue distribution of *V. anguillarum* in liver, spleen and kidney of flounder at day 7 post-challenge detected by IFA. *V. anguillarum* was stained with specific green fluorescence and tissues were stained in red by EBD. Scale bar: 50 μm. (B) The *V. anguillarum* burden in liver, spleen and head kidney of flounder at 7th day post challenge was detected by qPCR. All values are shown as means ± SD. Different letters (a, b and c) on the bar represent the statistical significance ($P < 0.05$) compared to each group at the same tissue. (N = 5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

delivery of a plasmid expressing IL2 along with VAA DNA vaccine significantly increased the level of antibodies than administration of the VAA DNA vaccine alone in flounder. The percentage of sIgM$^+$ B lymphocytes partially represent the activation of humoral immune response at cell level. Here, a significant increase of sIgM$^+$ B lymphocytes in peripheral blood was observed in pVAA-IRES or pVAA-IRES-IL2 group. Moreover, administration of the plasmid expressing IL2 with VAA DNA vaccine increase the percentage of sIgM$^+$ B lymphocytes than that induced by VAA DNA vaccine alone. All these results suggest that IL2 has a potential in enhancing humoral immune response of flounder fish.

The bacterial burden in tissues is an intuitive parameter representing the amount of pathogens in vaccinated fish after challenge, and can be used to evaluate the inhibition of bacteria in flounder protected by vaccination [55]. The qPCR, used rpoS as the target gene, is an accurate and species-specific method to detect the *V. anguillarum* burden in tissues of infected flounder [56]. In this study, the amount of *V. anguillarum* in liver, spleen and kidney, was analyzed by qPCR

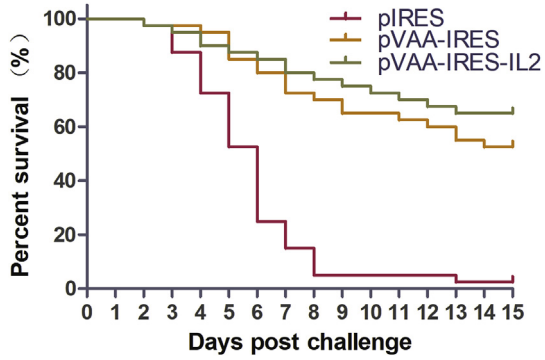

Fig. 6. Survival percentage of the immunized fish after challenge with *V. anguillarum*.

amplification of rpoS gene after *V. anguillarum* challenge. The results revealed that the number of *V. anguillarum* was significant decreased in pVAA-IRES or pVAA-IRES-IL2 group than that of the pIRES injected control group after expose to *V. anguillarum* infection. Moreover, the bacteria burden in liver, spleen and kidney showed a slight decrease in pVAA-IRES-IL2 group, compared to that in pVAA-IRES group. The IFA indicate the similar trend as qPCR. In addition, the RPS in pVAA-IRES-IL2 group is higher than those conferred by pVAA-IRES immunized group and pIRES control group. The decrease of bacteria burden and increase of RPS demonstrate that IL2 could be used as the adjuvant for VAA DNA vaccine to inhibit the bacteria burden and increase the RPS.

Together, our study showed that the constructed bicistronic DNA vaccine encoding VAA as antigenic gene and IL2 as immune adjuvant is a potential application for protection against *V. anguillarum* in flounder. Moreover, the co-expression of IL2 gene increased the humoral and cellular immune response induced by DNA vaccine, and enhanced the protective effect after challenge.

Conflicts of interest

All the authors read and approved this version of the final manuscript, confirmed the integrity of this work and declare no conflict of interest.

Acknowledgements

This work was funded by the National Key Research and Development Program of China (2018YFD0900503), the National Natural Science Foundation of China (31730101; 31672684 and 31672685), the Fundmental Research Funds for the Central Universities (201822015), the Director Foundation of Functional Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology (2018MFSD-01), NBRPC (2012CB114406), Key Research and Development Program of Shandong Province (2016GNC115001), and Taishan Scholar Program of Shandong Province.

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