Fish and Shellfish Immunology 86 (2019) 618–626

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Intramuscular administration of a DNA vaccine encoding OmpK antigen induces humoral and cellular immune responses in flounder (*Paralichthys olivaceus*) and improves protection against *Vibrio anguillarum*

Hongsen Xu[a], Jing Xing[a,b,*], Xiaoqian Tang[a,b], Xiuzhen Sheng[a], Wenbin Zhan[a,b]

[a] Laboratory of Pathology and Immunology of Aquatic Animals, KLMME, Ocean University of China, 5 Yushan Road, Qingdao, 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, No. 1 Wenhai Road, Aoshanwei Town, Qingdao, 266071, China

ARTICLE INFO

Keywords:
OmpK DNA vaccine
Vibrio anguillarum
Paralichthys olivaceus
Humoral immune response
Cellular immune response
Immune protection

ABSTRACT

Outer membrane protein K (OmpK) is an immunogenic protein that could act as subunit vaccine candidate for *Vibrio anguillarum*. In this study, a DNA vaccine encoding the OmpK gene of *V. anguillarum* was constructed and confirmed to express OmpK *in vitro* and *in vivo*. To evaluate the potential of pcDNA3.1-OmpK (pOmpK) as vaccine candidate, the humoral and cellular immune responses, and protective effects were analyzed in flounder model. The results showed that the transcription and translation of OmpK gene occurred in both transfected hirame natural embryo (HINAE) cells and injected fish muscles, indicating the functionality of pOmpK to express OmpK. Fish immunized with pOmpK showed significant increase of surface IgM positive ($sIgM^+$), CD4-1^+, CD4-2^+ lymphocytes and production of specific anti-*V. anguillarum* or anti-rOmpK antibodies, which indicate the activation of humoral and cellular immune responses after vaccination. Moreover, a relative percent survival (RPS) rate of 50.00% against *V. anguillarum* infection was obtained for flounder immunized with pOmpK. In conclusion, this study indicates that pOmpK is able to induce humoral and cellular immune responses and can be used as a DNA vaccine candidate.

1. Introduction

Aquaculture is one of the fastest growing sector of agriculture that provide food to the expanding world population. One of the main hurdles to sustainable fish production in many regions is the management and control of infectious disease. *Vibrio anguillarum* is a Gram-negative bacterium that could widely infect many fish species [1,2]. Fish affected by this bacterial showed typical signs of vibriosis, including a generalized septicemia with hemorrhage on the base of fins, red spots on the ventral and dark skin lesions with ulceration and blood [3]. Occurrences of this vibriosis have a certain relationship with chemical stress, biological stress and physical stress, and mortality in farm stocks can be up to 100% [4]. Great economic losses have been reported because of *V. anguillarum* infection in aquaculture [5]. Due to the fact that the mortality of *V. anguillarum* infected fish is high and the therapy using antibiotic is a challenge from ecological and economic points of view, vaccination is becoming an effective measure in protecting fish from disease [6].

It's well known that outer membrane proteins, important factors for Gram-negative bacteria, play a vital role in bacteria colonization, bile resistance and infection [3,7,8]. Numerous studies have been reported that outer membrane proteins are generally very immunogenic due to their exposed epitopes on the cell surface [9] and have the potential of vaccine candidate for fish against *V. anguillarum* [10], *Vibrio parahaemolyticus* [11,12], *Vibrio harveyi* [13], *Edwardsiella tarda* [14,15] and *Aeromonas hydrophila* [16,17] infection. In our previous research, recombinant OmpK (rOmpK) was demonstrated to induce the production of specific antibodies, increase the percentage of $sIgM^+$ lymphocytes and confer a RPS of 62.16% for flounder [18].

DNA vaccines, compared to traditional antigen vaccines, offer several advantages in practical and immunological points of view, which attract the interest of researchers, manufacturers and investors and are considered as the next generation vaccine approach following the administration of subunit vaccines [19]. The DNA vaccines are easy to produce and relatively inexpensive because of the identical processes of production, and relatively stable for DNA structure; also, DNA vaccines induce strong and long-lasting humoral and cellular immune responses that are similar with live vaccines [20]. Considering the high immune

* Corresponding author. Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao, 266003, China.
E-mail address: xingjing@ouc.edu.cn (J. Xing).

https://doi.org/10.1016/j.fsi.2018.11.073
Received 31 July 2018; Received in revised form 20 November 2018; Accepted 30 November 2018
Available online 01 December 2018
1050-4648/

protection provided by rOmpK and advantage of DNA vaccine, we are interested in whether OmpK, in form of DNA, would induce immune protective effect for flounder (*Paralichthys olivaceus*).

Nowadays, many vaccines are still under development and some have been licensed for commercial production [20–22]. However, the development of fish vaccines is hampered by dearth of information about fish immune system, and the vaccine efficacy is evaluated mainly empirical. At present, since the lack of specific antibodies against T cell markers, researchers evaluated the cellular immune response in fish largely by analyzing the expression profile of immune related genes [23–25]. In our previous study, we produced specific polyclonal antibodies against CD4-1 or CD4-2 [26], which provided the tools for us to analyze the variation of CD4-1$^+$ or CD4-2$^+$ lymphocytes and then reveal the T cell immune response induced by DNA vaccine at cell level.

In the present study, a DNA vaccine encoding OmpK gene of *V. anguillarum* was constructed and successfully expressed *in vitro* and *in vivo*. The immune protective efficacy was tested after *V. anguillarum* challenge in flounder (*P. olivaceus*) model. Meanwhile, the humoral and cellular immune responses of flounder, including the production of specific serum antibodies and the percentage of surface IgM positive (sIgM$^+$), CD4-1$^+$, CD4-2$^+$ lymphocytes in peripheral blood (PBL), spleen and head kidney, were investigated after immunization.

Overall, this study not only reveals that pOmpK could be a DNA vaccine candidate, but also contributes to the understanding of humoral and cellular immune responses triggered by DNA vaccination in flounder model, and provides such benefit of rich in the evaluation platform of vaccine efficacy.

2. Materials and methods

2.1. Ethics statement

Investigations were conducted strictly with 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. These studies were also allowed by the Committee of the Ethics on Animal Care and Experiments at Ocean University of China.

2.2. Construction of pcDNA3.1-OmpK

To construct pcDNA3.1-OmpK (pOmpK) plasmid (Fig. S1A), OmpK gene (GenBank: FJ705222.1) was amplified by PCR with specific primers (OmpK-F: 5′-GGGGTACCACCATGGCTGACTATTCAGATGGCG-3′, underlined sequence, *Kpn I* site, OmpK-R: 5′-CGGAATTCTTAGAACTTGTAAGTCACAGC-3′, underlined sequence, *EcoR I* site). The PCR products were digested with *Kpn I* and *Eco*R I restriction enzyme and ligated into pcDNA3.1 vector, resulting in pOmpK plasmid. The recombinant plasmid was verified by specific PCR amplification and DNA sequencing, and then digested with *Kpn I* and *EcoR I* to confirm the appropriate insertion of OmpK gene into pcDNA3.1 (Fig. S1B). Endotoxin-free pOmpK plasmid was extracted using EndoFree plasmid Kit (Tiangen, Beijing, China) following the manufacture's protocol. The concentration of these plasmid was measured by Nanodrop 8000 Spectrophotometer (Thermo-Fisher, MA, USA). The plasmid was resuspended in sterile PBS with a final concentration of 200 ng/μl and diluted in sterile water with a final concentration of 500 ng/μl for vaccination and transfection, respectively.

2.3. Antibodies, cells, bacteria and animals

The protein of recombinant OmpK (rOmpK) was expressed in and purified from *Escherichia coli* and used for production of mouse anti-rOmpK polyclonal antibody following previous reports [24,25]. Briefly, 200 μl of purified rOmpK (1 mg/ml) emulsified with Freund's complete adjuvant (1:1) was injected in BALB/C mice. The second week, mice was immunized with the same volume of rOmpK combined with Freund's incomplete adjuvant at equal volume. At third and fourth week, boost immunization was performed by injecting 200 μl rOmpK in the tail vein, respectively. The antiserum was collected from the immunized mice at one week after the last boost immunization. Western blot was used to analyze the immunogenicity and specificity of polyclonal antibodies and found that antiserum could specifically bind to purified rOmpK (Fig. S2). The polyclonal antibodies were diluted into 1:1000 with PBS and used in immunofluorescence assay (IFA) and FCM assay. Monoclonal antibodies against flounder IgM (FIgM-Mab) and rabbit anti-flounder CD4-1 or CD4-2 polyclonal antibodies (FCD4-1-Pab, FCD4-2-Pab) were previously produced in our laboratory [26,27]. The ascites fluids of FIgM-Mab were diluted into 1:1000 and used in FCM, Western blot and ELISA. FCD4-1-Pab and FCD4-2-Pab, diluted into 1:1000 and 1:1500, respectively, were used in FCM assay.

Hirame natural embryo (HINAE) cells were kindly provided by Dr. Ikuo Hirono, the professor of Tokoyo University of Marine Science and Technology [28]. The cells were plated in 6-well plates, cultured with Leibovitz's L-15 medium (Thermo-Fisher, MA, USA) containing 20% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin, and used for transfection to analyze the expression of OmpK proteins.

Vibrio anguillarum was isolated in our previous study and stored in our lab [2]. The bacteria were grown at 37 °C on Luria Bertani (LB) medium for 12 h and measured the concentration using an Accuri C6 cytometer. The bacteria with a concentration of 1.0×10^7 CFU/ml was used in challenge and ELISA.

Flounder (*P. olivaceus*, apparently healthy without prior history of any infection, average weight 35 ± 5 g), were purchased from a fish farm in Rizhao, Shandong province, China. The fish were randomly sampled for the examination of bacterial recovery from spleen and kidney, and no bacteria were discovery from both of the tested tissues. The fish were cultured in 336-L tanks containing 200 L re-circulating aerated seawater at 21 ± 0.5 °C, and fed commercial dry food (Shandong Sheng-suo Fish Feed Research Center, Shandong, PR China) twice a day.

2.4. Expression of pOmpK in vitro

HINAE cells, about 70–80% confluence, were transfected with 500 ng of pOmpK vector or pcDNA3.1 (negative control) using Lipofectamine® 3000 (Thermo-Fisher, MA, USA) following the manufacturer's instructions. After the cells were kept at 24 °C for 48 h, one part of cells was collected and evaluated for the transcription of OmpK gene by RT-PCR as previous described [28], the other part of cells was harvested by digestion with pancreatin and fixed with 4% (w/v) paraformaldehyde for 20 min. Cells were incubated with mouse anti-rOmpK antibody and then stained with goat-anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC, 1:256, Sigma, USA). The percentage of positive cells which could express OmpK was analyzed with Accuri C6 cytometer (BD Accuri™, Piscataway, NJ, USA). In parallel, cells were grown on coverslips and transfected with 500 ng of DNA plasmid as mentioned above to confirm the expression of OmpK gene by IFA, as described previously [29, 30]. Briefly, the mouse polyclonal antibody directed against rOmpK and a 488-conjugated goat anti-mouse IgG secondary antibody (1:1000, Molecular Probes, USA) were inoculated with transfected cells, successively. The cells were stained the nuclei with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Invitrogen, Carlsbad, USA) at room temperature for 10 min and the specific fluorescence was imaged through epifluorescence microscopy IX71 (Olympus, Japan). Cells transfected with pcDNA3.1 were served as negative control group.

2.5. Vaccination and sampling

Three hundred flounder were randomly divided into three groups. After acclimation for 1 week, fish were intramuscularly administrated

in the epaxial muscle below the dorsal fin with 100 μl (200 ng/μl) of pOmpK, pcDNA3.1 or PBS, respectively. Fish were anaesthetized or over-anaesthetized with MS-222 (Sigma, Beijing, China) prior to the experimental manipulations such as injection, blood and tissue collection.

To investigate the transcripts of OmpK gene, three fish from the different groups were killed at 3rd, 5th, 7th, 9th and 14th days post-vaccination and muscles surrounding the injection site were sampled and placed into RNA later (TaKaRa, Dalian, China). In parallel, the muscle tissues from fish at 7th days after vaccination were taken, embedded in tissue freezing medium (Leica, UK), immediately frozen in −80 °C and used to investigate OmpK expression by IFA.

For detection of CD4-1$^+$ and CD4-2$^+$ T lymphocytes, six fish were randomly sampled in each group at 3rd and 14th days post vaccination. The lymphocytes in PBL, spleen and head kidney were separated by Percoll gradient density, confirmed using Giemsa staining as previous reported [27,31], and then applied on FCM assay.

For detection of sIgM$^+$ B lymphocytes, three fish were randomly sampled at 4th and 5th weeks after immunization. The lymphocytes in PBL, spleen and head kidney were separated as mentioned above and used in FCM.

On days 28 and 35 post-immunization, five fish from each group were killed and blood were drawn from the caudal vein. After clot at 4 °C overnight, serum from immunized and non-immunized fish was obtained by centrifugation at 3000 × *g* for 10 min, stored at −20 °C, and used in ELISA and Western blot.

2.6. Expression of pOmpK in vivo

To verified the transcript of OmpK gene, total RNA was extracted from tissues using TRIZOL reagent (Baosheng, Dalian, China) and measured by Nanodrop 8000 Spectrophotometer. Single-strand cDNA was synthesized with Reverse Transcriptase M-MLV kit (TaKaRa, Japan) according to manufacturer's instructions and used as the template in PCR amplification. 18S rRNA gene was used as an internal control. The specific primers used for RT-PCR of OmpK were mentioned above.

Cryosections (7 μm in thickness) of muscle tissues were prepared on a Leica CM 1900 microtome (Leica, Germany) and used to examine whether OmpK was expressed in vaccinated fish by IFA. Briefly, sections were transferred to microscope slides treated with poly-L-lysine. Microscope slides were treated with precooled acetone for 10 min, air-dried for 15 min and blocked with 3% BSA overnight at 4 °C. Then the slides were subsequently incubated with mouse anti-rOmpK polyclonal antibody (1:1000) and goat-anti-mouse IgG-488 antibodies (1:3000) at 37 °C for 1 h each and stained with DAPI in a moisture chamber for 10 min. Finally, the slides were washed 3 times and mounted in buffered glycerin for observation with fluorescence microscopy IX71 (Olympus, Japan). Muscles from pcDNA3.1 injected fish were used as negative control group.

2.7. FCM assays

We next investigated the percentage of sIgM$^+$, CD4-1$^+$ and CD4-2$^+$ lymphocytes in PBL, spleen or head kidney after immunization. Briefly, for detection of CD4-1$^+$ or CD4-2$^+$ T lymphocytes, lymphocytes were incubated with FCD4-1-Pab (1:1000) or FCD4-2-Pab (1:1500), respectively, and then incubated with 647-conjugated goat anti-rabbit IgG (1:1000, Thermo-Fisher Scientific, USA); for detection of sIgM$^+$ B lymphocytes, lymphocytes were incubated with FIgM-Mab (1:1000), and then incubated with FITC-conjugated goat-anti-mouse Ig (1:256, Sigma, USA). After that, the cell suspensions were analyzed with Accuri C6 cytometer as previous reported [27,31]. Briefly, lymphocytes with the similar cell granularity and cell size, which were displayed by side- and forward-scatter (SSC and FSC) parameters, respectively, were gated in R1 scope of the dot plots. Then, the percentage of cells distributed among R1 was analyzed in fluorescence histograms in which FITC-labeled cells and Alexa Fluor® 647 labeled cells were reflected by fluorescent light (FL)-1 and FL-4, respectively. Myeloma culture supernatant or rabbit negative serum instead of the aforementioned primary antibodies was used as negative control, respectively.

2.8. ELISA

The titers of specific anti-rOmpK or anti-*V. anguillarun* antibody were assessed by ELISA as described previously [30]. Briefly, microplates (96-wells, Costar, NY, USA) were coated with 100 μl V. *anguillarum* (1.0×10^7 CFU/ml) or purified rOmpK (100 μg/ml) at 4 °C overnight and then blocked with 3% BSA, after washing with PBST (phosphate buffered saline containing 0.05% Tween 20) for three times, 100 μl of fish anti-serum, which was diluted into 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048 with PBS, respectively, was added in triplicate and incubated at 37 °C for 1 h. After washing with PBST as before, the plates were then incubated with 100 μl of fIgM Mab and goat-anti-mouse IgG conjugated with alkaline phosphatase (AP) (1:5000, Merck Millipore, Darmstadt, Germany) as secondary and third antibodies at 37 °C for 1 h, respectively. The binding antibodies were visualized by adding p-nitrophenyl phosphate (pNPP, Sigma, MO, USA) dissolve in 50 mM carbonate-bicarbonate buffer (pH 9.8) containing 0.5 mM $MgCl_2$. The absorbance was read at 405 nm with an automatic ELISA reader. Endpoint titers were the corresponding ultimate dilutions at which the P/N value still higher than 2.1. The serum from pcDNA3.1 or PBS immunized group instead of that from pOmpK vaccinated fish was used as negative control.

2.9. Western blot

The immune-specificity of sera from vaccinated fish was verified by Western blot. Briefly, the purified rOmpK was electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darm-stadt, Germany). After blocking with 3% BSA overnight at 4 °C, the membrane were successively incubated with fish anti-serum (1:100, collected from pOmpK immunized fish at 35th days post vaccination), fIgM Mab and AP conjugate goat-anti-mouse IgG (1:5000) at 37 °C for 1 h. The color reaction was developed by incubating membranes with prepared substrate solution (100 mM NaCl, 100 mM Tris and 5 mM $MgCl_2$, pH 9.5) containing 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma) and nitroblue tetrazolium (NBT, Sigma) for 5 min, and stopped by washing with distilled water. Serum from PBS or pcDNA 3.1 immunized fish instead of pOmpK vaccinated fish was performed as negative control.

2.10. Challenge

To determine the protection efficacy afforded by pOmpK plasmid, flounder (N = 30) immunized with PBS, pcDNA3.1 or pOmpK were challenged intraperitoneally with a lethal dose of 1.0×10^6 CFU (10 × LD50) live virulent *V. anguillarum* at 6th week post immunization. The dead fish were removed timely and the cause of death was analyzed as previously reported [2]. Survival of each group was monitored daily up to 15 days after the challenge, and relative percent survival (RPS) was calculated according to the following method [32]:

RPS = {1 - (% mortality in vaccinated fish/% mortality in PBS injected fish)} × 100%.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad software, Inc. San Diego, CA, USA). All data were expressed as mean ± standard deviation (SD) and analyzed with one-way analysis of variance (ANOVA). Statistical differences were analyzed by

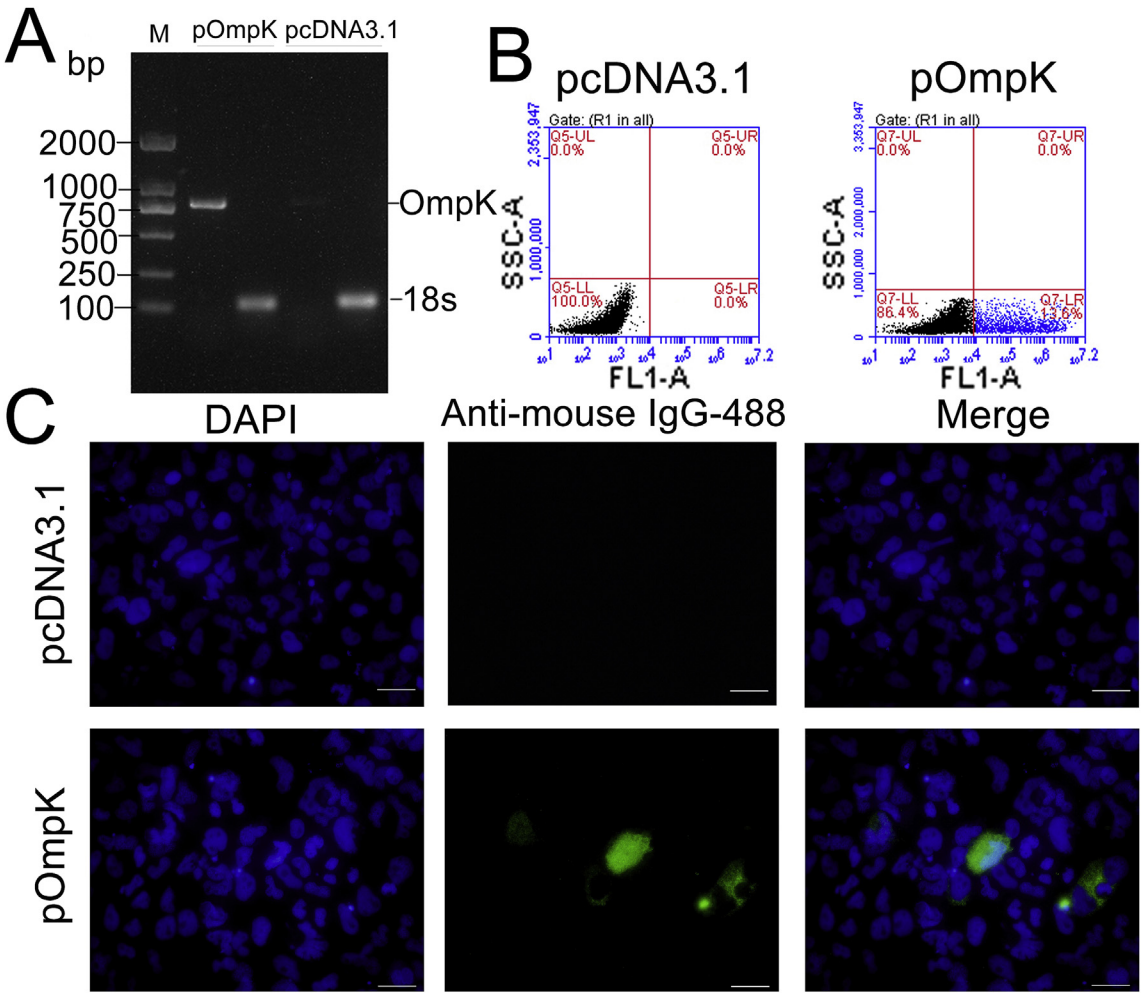

Fig. 1. Detection of OmpK expression in transfected HINAE cells. (A) RT-PCR analysis of OmpK gene transcripts in pcDNA3.1 or pOmpK transfected cells. 18S rRNA gene was used as internal control. lane M, DNA Marker. (B) Flow cytometric analysis of the percentage of cells expressing OmpK after transfection. The cells were stained with mouse anti-rOmpK polyclonal antibodies and then incubated with FITC labeled goat anti-mouse IgG. (C) Immunofluorescence microscopy assay of OmpK. The transfected cells were incubated with primary (mouse anti-rOmpK polyclonal antibodies) antibodies and secondary (goat anti-mouse IgG-488) antibodies to detect the expression of OmpK. 4′,6-diamidino-2-phenylindole (DAPI) staining shows cell nuclei, (Magnification at × 40, scale bar: 20 μm). pcDNA3.1 transfected cells were used as negative control.

Tukey's multiple pair wise comparison. Significant differences among fish immunized with pOmpK, pcDNA3.1 and PBS are performed with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), respectively.

3. Results

3.1. OmpK was successfully expressed in pOmpK transfected HINAE cells

RT-PCR was applied to examine the OmpK gene transcription in transfected cells. The results showed that a specific band was detected in pOmpK transfected cells. While no OmpK band was visible in pcDNA3.1 transfected cells. 18S rRNA transcript was observed in both pOmpK and pcDNA3.1 transfected cells (Fig. 1A). The flow cytometry (FCM) result revealed that percentage of cells that could express OmpK after transient transfection with pOmpK plasmid was about 13.6%, and no cell that express OmpK was observed in pcDNA3.1 transfected cells (Fig. 1B). IFA showed that HINAE cells transfected with the pOmpK plasmid showed a specific green fluorescence, and no fluorescence was observed in the cells that were transfected with the same amount of pcDNA3.1 plasmid (Fig. 1C).

All these results indicated that OmpK gene was successfully transcribed and translated in pOmpK transfected HINAE cells, confirming the functionality of pOmpK plasmid.

3.2. OmpK was expressed in flounder muscles after intramuscular injection of pOmpK

RT-PCR was applied to examine the OmpK gene transcription in immunized fish and revealed that OmpK gene transcripts were detected in muscle of pOmpK vaccinated fish at 3rd, 5th, 7th, 9th and 14th days post immunization, while no specific band was observed in pcDNA3.1 immunized fish (Fig. 2A). To examine whether OmpK was successfully expressed in vaccinated fish, we performed the IFA at 7th days post immunization and found that a specific green fluorescence was observed in muscle sections from pOmpK administrated fish. In contrast, no specific fluorescence was detected in the sections from pcDNA3.1 injected fish (Fig. 2B).

3.3. T cell immune response was induced by pOmpK vaccine

The potential of the pOmpK vaccine to induce T cell immune response was evaluated in terms of the percentages of CD4-1$^+$ and CD4-2$^+$ T lymphocytes in PBL, spleen and head kidney.

The percentages of CD4-1$^+$ or CD4-2$^+$ T lymphocytes in PBL, spleen and head kidney were detected by FCM and showed in Fig. 3. In pOmpK vaccinated group, an extremely significant ($p < 0.001$) induction of CD4-1$^+$ T lymphocytes was observed in PBL (Fig. 3A, 8.2 ± 0.8%) and

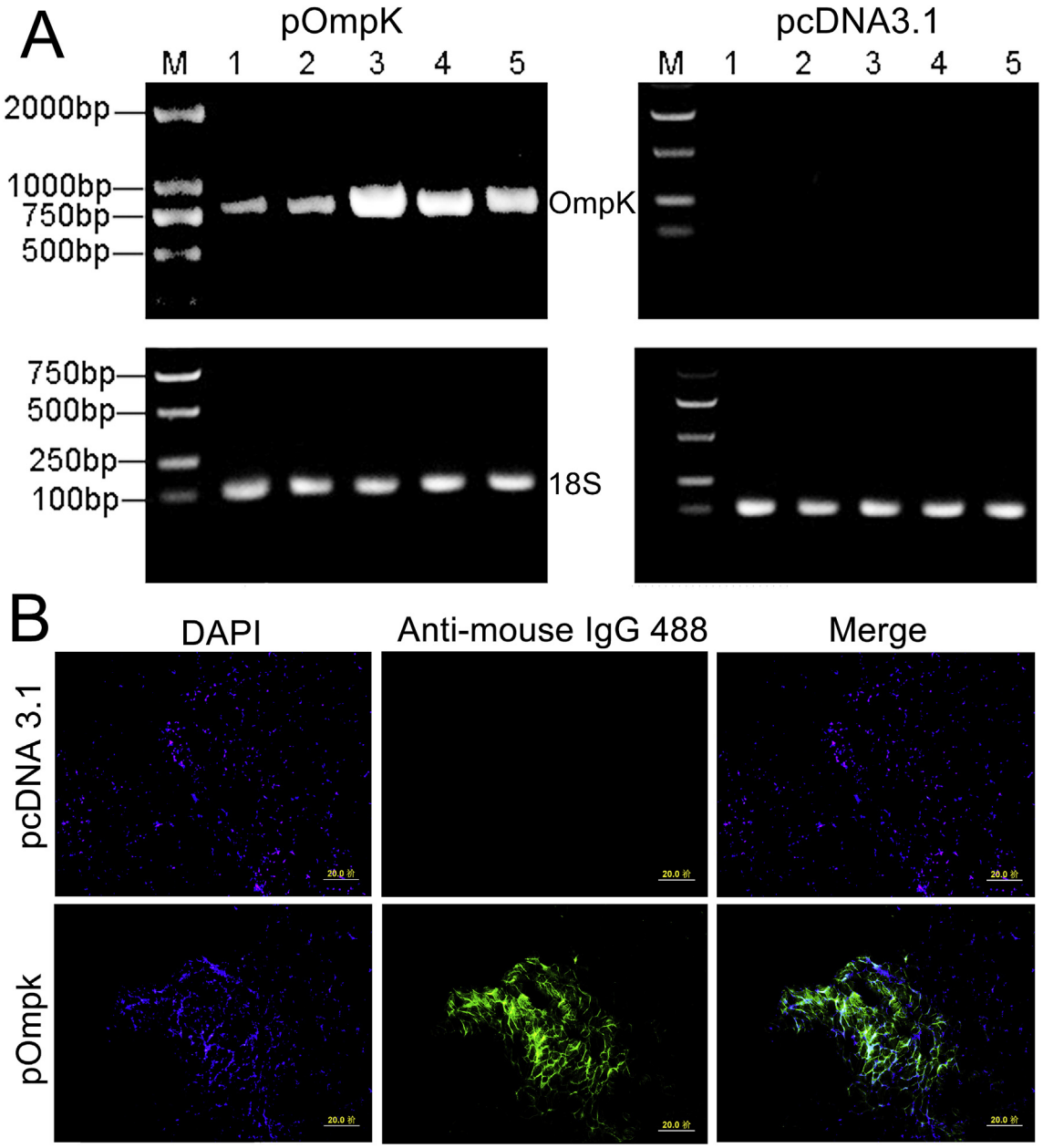

Fig. 2. Detection of OmpK expression in vaccinated fish. (A) RT-PCR detection of OmpK gene transcripts in muscle of pOmpK or pcDNA3.1 immunized fish, 18S rRNA gene was used as internal control. Muscle was taken from vaccinated flounder at 3rd, 5th, 7th, 9th and 14th days (lanes 1, 2, 3, 4 and 5) post vaccination, lane M, DNA marker. (B) Expression of pOmpK were detected by IFA. Flounder were intramuscularly injected with the plasmid. Seven days after immunization, sections of the muscles were incubated with mouse polyclonal antibody against rOmpK and then incubated with goat anti-mouse antibody coupled to Alexa 488. Immunofluorescence was analyzed by epifluorescence microscopy at a magnification of 20 × . Scale bar: 200 μm. Section from pcDNA3.1 injected fish was used as negative control.

spleen (Fig. 3B, 17.2 ± 1.2%) at 14th day post vaccination and a very significant ($p < 0.01$) percentage of CD4-1$^+$ T lymphocytes was observed in head kidney (Fig. 3C, 19.7 ± 1.0%) at 14th day post vaccination. While there was no significant induction of CD4-1$^+$ T lymphocytes in PBS and pcDNA3.1 injected fish at 3rd and 14th days post injection. As for CD4-2$^+$ T lymphocytes, a very significant induction ($p < 0.01$) was observed in PBL (Fig. 3D, 6.9 ± 0.8%) at day 14 post vaccination, and a significant induction ($p < 0.05$) was shown in head kidney (Fig. 3F, 17.5 ± 0.9%) at the meantime. Interestingly, there was a very significant induction ($p < 0.01$) and extremely significant ($p < 0.001$) in spleen at 3rd (6.0 ± 0.7%) and 14th (13.3 ± 1.2%) days post inoculation, respectively (Fig. 3E). However, no significant increase of CD4-2$^+$ T lymphocytes was observed in PBS and pcDNA3.1 injected group.

These results revealed that, compared with PBS and pcDNA3.1, pOmpK specifically induce the percentages of CD4-1$^+$ and CD4-2$^+$ T lymphocytes in PBL, spleen and head kidney, which indicated the elicitation of T cell immune response.

3.4. Humoral immune response was elicited by pOmpK vaccine

The ability of pOmpK plasmid vaccine to elicit humoral immune responses were investigated by means of sIgM$^+$ B lymphocytes percentage and specific antibody production.

Lymphocytes in PBL, spleen and head kidney were isolated from the immunized fish at 4th and 5th weeks post vaccination, and the

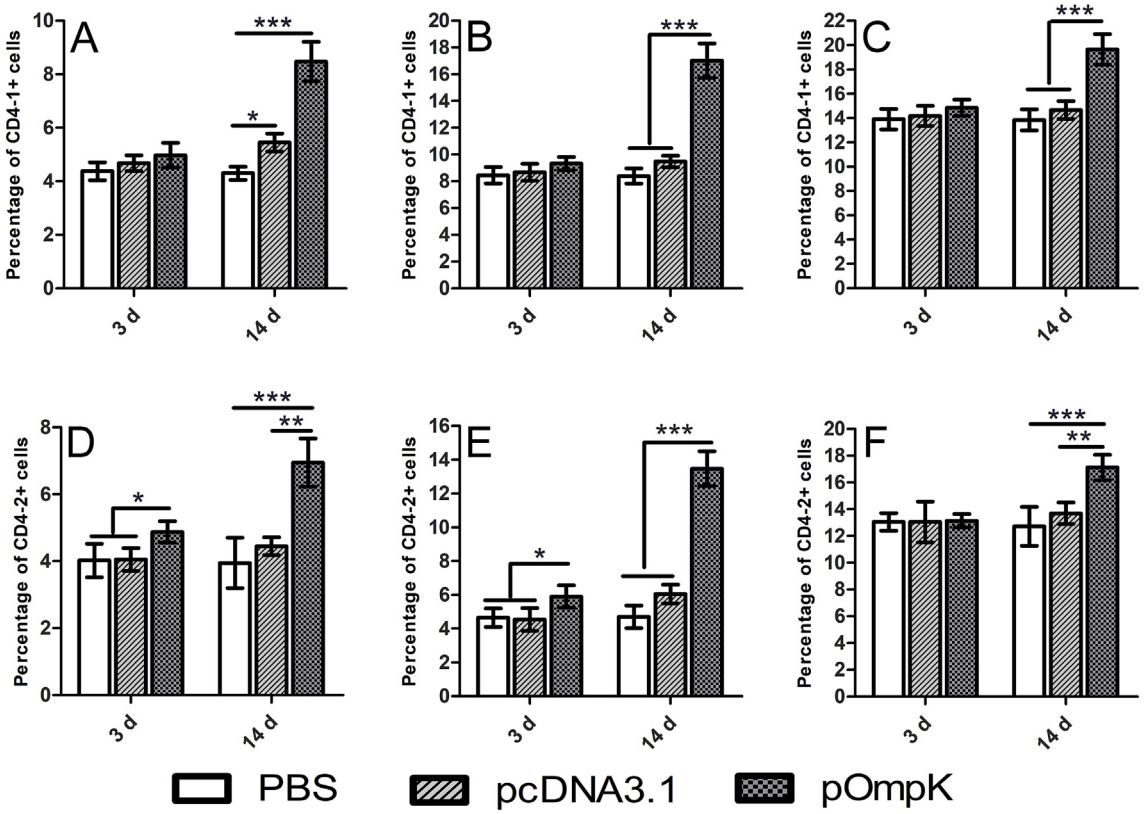

Fig. 3. Percentage of CD4 T lymphocytes at 3rd and 14th days post vaccination. (A) Variation of CD4-1$^+$ T lymphocytes in peripheral blood. (B) Variation of CD4-1$^+$ T lymphocytes in spleen. (C) Variation of CD4-1$^+$ T lymphocytes in head kidney. (D) Variation of CD4-2$^+$ T lymphocytes in peripheral blood. (E) Variation of CD4-2$^+$ T lymphocytes in spleen. (F) Variation of CD4-2$^+$ T lymphocytes in head kidney. Values are shown as means ± SD of three fish. Asterisks (*) on the bar represent the statistical significant difference, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

percentage of sIgM$^+$ lymphocytes was evaluated by FCM assay in Fig. 4A. Compared with the PBS and pcDNA3.1 injected fish, the percentage of sIgM$^+$ showed an extremely significant increase ($p < 0.001$) at 4th and 5th weeks post vaccination in PBL (Fig. 4A1), spleen (Fig. 4A2) and head kidney (Fig. 4A3).

Sera from immunized fish (N = 5) were sampled at 4th and 5th weeks after immunization and ELISA were performed for detection of specific anti-*V. anguillarum* or anti-rOmpK antibody titers. A significant induction in anti-*V. anguillarum* or rOmpK antibody was detected on 28th day, with a further slight increase noted on 35th day in pOmpK vaccinated fish. While the absorbance maintains the normal level in sera from PBS and pcDNA3.1 immunized fish at any dilutions (Fig. 4B1 and 4B2). Most importantly, sera from vaccinated flounder contained high levels of anti-*V. anguillarum* or rOmpK-specific antibodies as indicated by the endpoint titers (Fig. 4B1' and 4B2'). Furthermore, to verify the immune specificity of sera from vaccinated fish, we performed a Western blot analysis and found that sera from pOmpK immunized flounder could specifically bind to rOmpK protein. As expected, sera from pcDNA3.1 immunized fish failed to bind to the rOmpK protein (Fig. 4C).

These analyses revealed that, compared with pcDNA3.1 and PBS, pOmpK could increase the percentage of sIgM$^+$ B lymphocytes and production of specific antibody, which indicated the promotion of humoral response.

3.5. Immune protective effect of pOmpK vaccine

After challenge with *V. anguillarum*, the fish in PBS and pcDNA3.1 immunized groups began to die on day 2, and die quickly on 4th-7th days. However, a marked low accumulative mortality (46.67%) was observed in pOmpK vaccinated group compared to the PBS (93.33%) and pcDNA3.1 (86.67%) groups. Therefore, the protective efficacy of pOmpK, in terms of RPS, was 50.00% with PBS as the negative control (Fig. 5). This result revealed that pOmpK vaccine conferred the moderate efficacy to protect fish from *V. anguillarum* challenge.

4. Discussion

Outer membrane proteins are supposed to be important stimulators of host immune response since they are easily identified as heterologous antigen by the immune system of fish [11]. Hence, they have become attractive molecules for development of candidate vaccines. Many outer membrane proteins, in form of DNA vaccines, have been demonstrated to induce the production of specific antibodies and confer immune protection against *Vibrio. anguillarum, Aeromonas veronii, Paralabrax maculatofasciatus* and *Streptococcus iniae* [33–35]. In this study, we have constructed a DNA vaccine, pOmpK, encoding the OmpK gene of *V. anguillarum*. When the DNA vaccine (20 μg/fish) was intramuscularly injected into flounder, production of specific antibodies and percentage of CD4-1$^+$, CD4-2$^+$ and sIgM$^+$ lymphocytes were significantly induced. Moreover, pOmpK could protect flounder from *V. anguillarum* infection and provide a RPS of 50.00%. Compare with the RPS of 62.16% provided by rOmpK emulsified with Freund's complete adjuvant, the immune protection conferred by pOmpK seems less attractive. The antigen (dose, formulation, adjuvant and prime-boost regime), the route of administration and temperature of water have a certain relationship with vaccine efficacy [26,36–39]. It has been shown in fish that a high vaccine dose induces increased protection and lower temperature leads to a low antibody response against antigens [26,39]. Moreover, Freund's incomplete adjuvant could trigger early innate defense mechanisms and then assist in the generation of robust and long-lasting immune response to promote protective immunity against

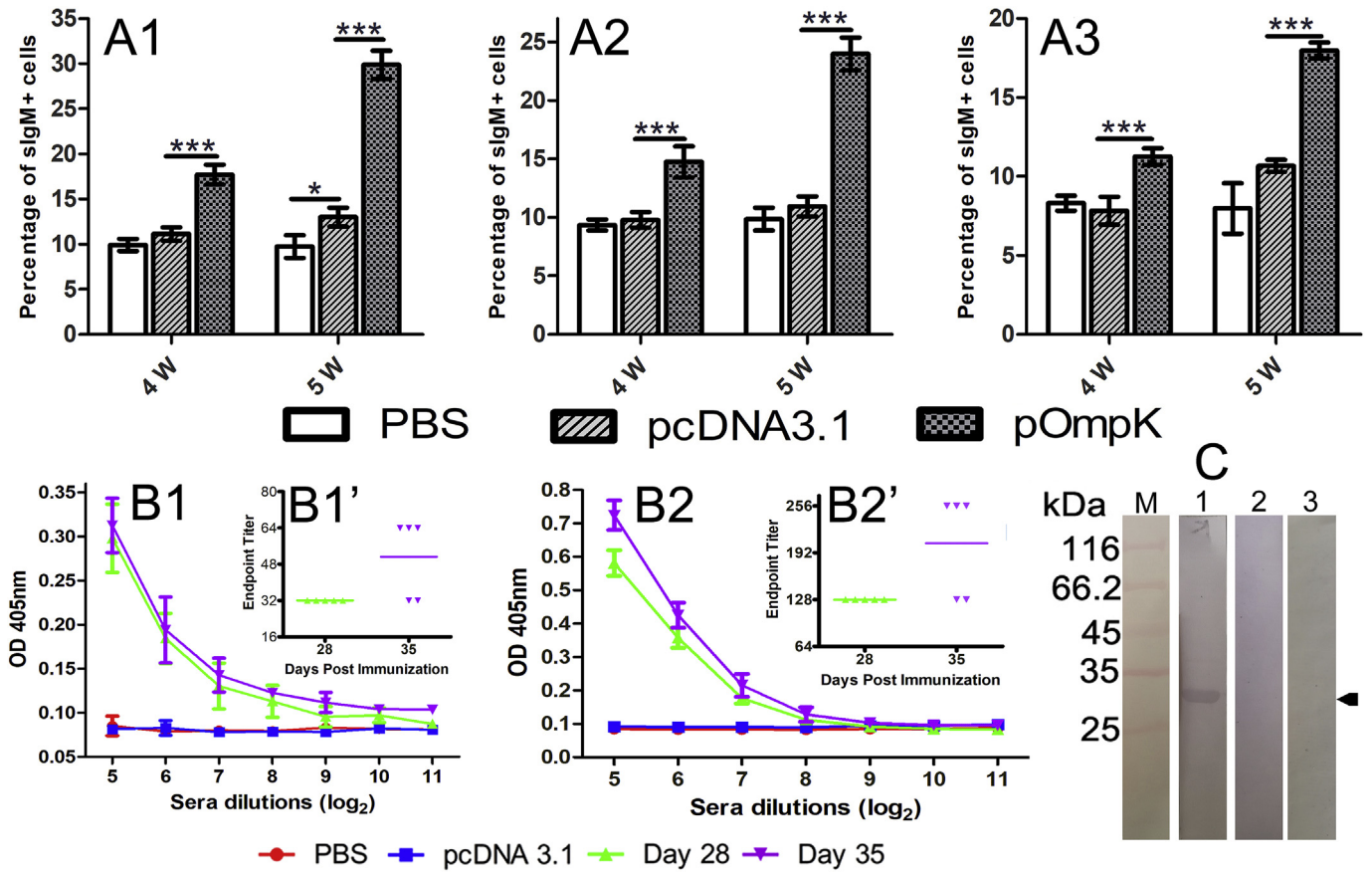

Fig. 4. Humoral immune response elicited by pOmpK vaccine. (A) Flow cytometric analysis of $sIgM^+$ cells in peripheral blood (A1), spleen (A2) and head kidney (A3) of immunized flounder at 4th and 5th weeks after immunization. (B) ELISA analysis measuring specific antibody production (measured by OD405 values) in immunized fish. Binding to *V. anguillarum* or rOmpK was analyzed with sera from animals at different time points (days 28 and 35 post immunization) at various dilutions. The data shown are representative of at least five separate experiments (N = 5). (C) Western blot analysis of rOmpK specific antibodies induced by pOmpK immunization. The rOmpK protein was electrophoresed on a 12% SDS polyacrylamide gel and analyzed by Western blot. Binding to rOmpK is indicated by the arrowhead. M: molecular mass marker; Lane 1: Western blot analysis using serum from pOmpK immunized fish (day 35 after immunization); Lane 2: negative control using the serum from pcDNA3.1 injected fish; Lane 3: negative control using the serum from PBS injected fish. Values are shown as means ± SD. Asterisks (*) on the bar represent the statistical significant difference, $*p < 0.05$, $***p < 0.001$.

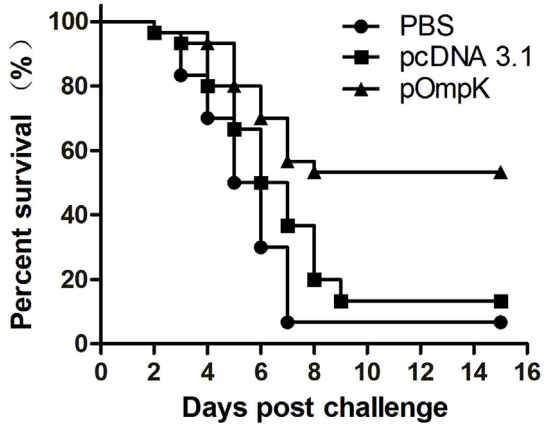

Fig. 5. Survival percentages of immunized fish after being challenged with *V. anguillarum*. Fish were intramuscularly injected with PBS, pcDNA3.1 or pOmpK. At 6th week post immunization, fish were challenged by intraperitoneally injecting 1.0×10^6 CFU live *V. anguillarum* and survival percentages were calculated at the end of the monitored period.

pathogens [40]. The ideal dose of DNA plasmids, efficient adjuvant and route of immunization need to be investigated to optimize the immune protection in future.

Compared with our previous study on subunit vaccine, the DNA vaccine were different from form and vaccine administration. Highly purified recombinant proteins mainly induce humoral response and DNA vaccines induce both cellular and humoral immune responses in mammals [20]. In our previous research, humoral immune response was observed after intraperitoneal administration of rOmpK. Specifically, the titer of antibody and percentage of $sIgM^+$ lymphocytes peaked at 5th week post-vaccination [18]. Interestingly, specific antibody titers and $sIgM^+$ lymphocytes reached the higher level in pOmpK immunized fish at 5-week post-vaccination, which shed light for us that rOmpk and pOmpK may induce similar patterns of humoral immune response. Similar result has also been observed in other research that compare the immune effect of rEta2 and pCEta2 from *E. tarda* [41]. In the percent study, cellular immune response in terms of the percentage of $CD4\text{-}1^+$ or $CD4\text{-}2^+$ lymphocytes was induced by pOmpK vaccine. However, the cellular immune response induced by rOmpK was not included due to the lack of specific anti-CD4-1 or CD4-2 antibodies at that time. The cellular immune response induced by rOmpK merits further investigation in the future to analyze weather subunit and DNA vaccine both elicit cellular immune response in fish model.

In the present study, pOmpK DNA vaccine based on pcDNA3.1, the common plasmid vector used for the construction of DNA vaccine, was constructed. The transcription and translation of OmpK in HINAE cell line at 48 h post transfection and in fish muscle at 7th day after vaccination were observed. Previous studies also demonstrated the positive results that other DNA vaccines could transcribe and translate in

transfected cell line and injected muscle tissues, and even then redistributed from the administration site to various tissues [42–46]. Compared with the high signal of OmpK in fish muscle, the transfection efficacy in HINAE cell line seems relatively low. Perhaps, the dose of DNA plasmid used for transfection and injection, and the time at which detected for protein expression contribute to the phenomenon.

It was well known that after intramuscular administration of DNA vaccine, the expressed antigen was presented to T cells via MHC I and MHC II molecules and subsequently activate the cellular immune response, which plays an important role in immunity to the control of pathogen infection [47]. In the present study, a significant increase of the percentage of CD4-1$^+$ or CD4-2$^+$ lymphocytes in PBL, spleen and head kidney was induced by pOmpK. Similarly, DNA vaccines encoding GRA17 and GRA23 gene from toxoplasmosis could increase the percentage of CD3$^+$CD4$^+$CD8$^-$ and CD3$^+$ CD8$^+$ CD4$^-$ in spleen of mice [48]. Numerous studies have demonstrated that the cellular immune response was induced by DNA vaccine in fish model at gene level [49–51]. The proliferation of antigen-specific T cells could specifically indicate the cellular immune response for the antigen and partial represent the potential of this antigen used for vaccine candidate. It was reported that the virus specific T cell immune response was induced by DNA vaccine from spring viremia of carp virus through the proliferation of Zap70$^+$ T cells [52]. This shed light for our future study about the evaluation of antigen specific T cell response.

Specific antibody production is an important parameter of humoral immune response after vaccination and mostly widely used correlates of protection for mammalian vaccines [53,54]. Previous studies have reported the ability of DNA vaccine in inducing specific antibodies. Specific antibodies against bacteria or recombinant proteins were measured as early as 14th days and maintained until 70th days after vaccination, and the highest level of antiserum appeared between 5th and 7th weeks post vaccination [34,43,55]. In this study, the specific antibodies against *V. anguillarum* or rOmpK were detected and significantly increased at 28th and 35th days after immunization, and the antibodies specifically bind to rOmpK were also demonstrated by Western blot.

In this work, we further investigate the percentage of sIgM$^+$ lymphocytes in vaccinated flounder to confirm the induction of humoral immune response at cell level and preliminarily reveal the functions of sIgM$^+$ lymphocytes after vaccinated with a DNA vaccine. We observed a remarkable induction of sIgM$^+$ B lymphocytes in peripheral blood, spleen and head kidney of pOmpK immunized fish at 4th and 5th weeks post vaccination, which confirm the activation of B cell immune response at the cell level and explore the functions of sIgM$^+$ B lymphocytes after DNA vaccination. This results were well supported by the previous study, which showed that the sIgM$^+$ B lymphocytes in PBL, spleen and pronephros was induced at 1st, 2nd, 3rd, 4th, and 5th weeks after immunization with DNA plasmid (pOmpC or pGroEL) of *E. tarda* [28,50].

In acquired immunity, T lymphocytes regulate the immune responses of B lymphocytes, including the production of antibody and proliferation of sIgM$^+$ B lymphocytes [31]. In our previous researches, the CD3$^+$ and sIgM$^+$ lymphocytes reached the maximum levels at 7th day and 4th week after immunization with subunit vaccine, respectively [56,57]. Therefore, the times that possibly corresponding to the significant increase of CD4-1$^+$, CD4-2$^+$ and sIgM$^+$ lymphocytes were selected to reveal the induction of cellular and humoral immune response.

It was well known that the route of challenge has an influence on immune response of fish and could lead to the possible difference in infection process [58]. Even though other challenge models, such as bath challenge and cohabitation, could better represent the natural route of infection, they required a large number of pathogens and more difficult to standardize the dose of infection pathogens. At present, intraperitoneal injections are often used as effective artificial challenge because of their benefits in guaranteeing the identical uptake of antigen and ease of standardization [59]. Thus, intraperitoneal injection of *V. anguillarum* was selected to evaluate the vaccine efficacy in our study. Moreover, the high dose (10 × LD50) of *V. anguillarum*, which could approximately cause the 100% death of flounder in 14 days, was used in challenge assay following previous research [60,61]. Further studies should focus on the protective efficacy of vaccines through a more scientific challenge model.

Together, our studies indicated that pOmpk could be a promising DNA vaccine candidate that induce the B cell mediated humoral and T cell mediated cellular immune response and evoke moderate protection against *V. anguillarum* infection.

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

Research reported in this publication was supported by the Fundamental Research Funds for the Central Universities (201822015), the National Natural Science Foundation of China (31730101; 31672684 and 31672685), NBRPC (2012CB114256), Key Research and Development Program of Shandong Province (2016GNC115001), the OUC-AU joint projects, the Open Foundation of Functional Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology (2016LMFS-A01), and Taishan Scholar Program of Shandong Province.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.073.

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