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# FlaC supplemented with VAA, OmpK or OmpR as bivalent subunit vaccine candidates induce immune responses against *Vibrio anguillarum* in flounder (*Paralichthys olivaceus*)

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ABSTRACT

In our previous study, ten candidate proteins have been identified with immunogenicity and protection against *Vibrio anguillarum* in flounder (*Paralichthys olivaceus*). Among them, FlaC is the important outer protein in the flagellum with immunogenicity; VAA, OmpK and OmpR are protection proteins against *V. anguillarum*. In this paper, FlaC supplemented with VAA, OmpK or OmpR as bivalent subunit vaccine candidates, and their immune response of flounder and protective effects were evaluated, respectively. Recombinant(r) proteins of FlaC were mixed with rVAA, rOmpK and rOmpR, respectively, rVAA + rFlaC (AF), rOmpK + rFlaC (KF) and rOmpR + rFlaC (RF); formalin-killed cells (FKC) or PBS were injected to flounder, respectively. After immunization, the percentages of $CD3^+$ T lymphocytes and surface membrane immunoglobulin-positive ($sIg^+$) B lymphocytes in peripheral blood lymphocytes (PBLs), total antibodies (TA), specific antibodies against *V. anguillarum* (VA), specific antibodies against bivalent recombinant proteins (PA), the expression of immune-related genes and relative percent survivals (RPS) were measured, respectively. The results showed that three bivalent vaccines candidates and FKC could induce the proliferation of $sIg^+$ B lymphocytes and $CD3^+$ T lymphocytes in PBLs. The TA, VA and PA induced in bivalent vaccines candidates and FKC groups were significantly higher than that of the control group. CD3, IgM, CD4-1, CD4-2, CD8α and CD8β genes were up-regulated. After challenge with *V. anguillarum*, RPS in AF, KF, RF and FKC groups exhibited 62.6 ± 2.33%, 78.95 ± 3.01%, 75.45 ± 0.97%, and 56.71 ± 2.15% respectively. The results revealed that three bivalent vaccines candidates and FKC could induce the immune response in flounder, and have good protection against *V. anguillarum*, and KF can be an efficient bivalent subunit vaccine candidate.



## 1. Introduction

*Vibrio anguillarum*, a rod shaped Gram-negative bacterium, is one of the important pathogenic vibrios. It widely exists in aquatic environments around the world [1,2], could cause bacterial disease in cultured fish, thereby, leads to enormous economic losses in the aquaculture industry [3].

Vaccination is optimal strategy for preventing fish disease [4]. Currently attenuated live vaccines, subunit vaccines, and DNA vaccines were reported in studies of fish diseases. Mutating virulence-related genes of *Edwardsiella tarda* as a live attenuated vaccine candidate against edwardsiellosis in zebrafish (*Danio rerio*) model were analyzed [5]. Recombinant outer membrane proteins (OMPs) or molecular chaperone protein GroEL as subunit vaccines candidates induced strong immune response of flounder [6,7]. A DNA vaccine of α-enolase against *Streptococcus iniae* was developed in Nile tilapia (*Oreochromis niloticus*) [8]. Moreover, combination of protein types and antigen sites as bivalent or multivalent vaccine could improve the immune protection [9]. A divalent DNA vaccine based on Sia10 and OmpU induced cross protection against *Streptococcus iniae* and *V. anguillarum* in flounder [10]. Bivalent DNA vaccine plasmids pDV (pDegQ + pVhp1) exhibited a higher survival rate (20%) than monovalent ones against *Vibrio harveyi* in flounder [11].

In *V. anguillarum*, a formalin-inactivated *V. anguillarum* had a protective effect against *V. anguillarum* in zebrafish [12], outer membrane protein (OMP) K as a subunit vaccine against *V. anguillarum* in Indian carp (*Labeo rohita*) [13], FlaA as a subunit vaccine

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against *V. anguillarum* in tilapia [14], and a DNA vaccine candidate of mutated zinc-metalloprotease protection against *V. anguillarum* in flounder [15]. In our previous studies, ten immunogenic proteins in *V. anguillarum* from flounder serum or GenBank have been identified, their recombinant proteins induced the immune response of flounder and showed significant relative percent survivals (RPS) against *V. anguillarum* challenge [16,17]. Among them, FlaC is important protein of outer protein in flagellum, which was reported as vaccine candidate protein [18,19], VAA, OmpK and OmpR are located at different positions of the bacteria cell, considering the various RPS, in this paper, recombinant (r) FlaC supplemented with rVAA, rOmpK or rOmpR as three bivalent vaccines candidates were produced, and the immune response of flounder and immune protection effect against a *V. anguillarum* challenge were evaluated, respectively.

## 2. Materials and methods

### 2.1. Fish

Flounder (*Paralichthys olivaceus*, 35 ± 5 g) were obtained from a fish farm (Rizhao, Shandong, China) and acclimated in the wet lab with continuous aerated seawater for two weeks. To ensure disease free, fish were randomly sampled for the 16s DNA detection and then used in the following experiments.

This study was carried out strictly in line with the procedures 81 in the Guide for the Use of Experimental Animals of the Ocean University of China in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU84 2010/63). All efforts were dedicated to minimize suffering.

### 2.2. Vibrio anguillarum

The pathogenic strain of *V. anguillarum* SJ060621 used in this study was stored in our laboratory [3]. It was cultured in 2216E marine medium at 28 °C for 24 h, then bacteria cells were collected and washed with 0.01 M phosphate buffered saline (PBS, pH 7.2) by centrifuging at 8000 ×*g* for 10 min at 4 °C. *V. anguillarum* suspension at concentration of $1.0 \times 10^7$ CFU $ml^{-1}$ was used for challenge experiment and Enzyme-linked immunosorbent assay (ELISA). And formalin-killed cells (FKC) of *V. anguillarum* were prepared as described previously [20], $1.0 \times 10^8$ CFU $ml^{-1}$ FKC was used in immunization experiment.

### 2.3. Antibodies

Mouse anti-flounder IgM monoclonal antibodies (FIgM-Mab) and the rabbit anti-flounder CD3 polyclonal antibodies (FCD3-Pab) were produced previously in our laboratory [21,22], and in this paper, the ascites fluids of FIgM-Mab diluted into 1:1000 with PBS were used in Flow cytometry (FCM) and ELISA, and FCD3-Pab (dilution of 1:500 in PBS) was used in FCM.

### 2.4. Recombinant proteins production and bivalent vaccine candidates preparation

Recombinant proteins (r) VAA, FlaC, OmpK and OmpR from *V. anguillarum* have been constructed in our previous study [16,17]. The processes of production recombinant proteins were the same as our previous study [23]. Briefly, *E. coli* BL 21 containing recombinant plasmid was induced by Isopropyl β-D-1-thiogalactopyrano side. His-tagged recombinant proteins were purified using His Trap™ HP Ni-Agarose (GE healthcare, China) followed by the manufacturer's instruction. The purified protein was dialyzed for 24 h against PBS and treated with Triton X-114 to remove endotoxin [24]. The protein was analyzed by SDS-PAGE and visualized after staining with Coomassie brilliant blue R-250. The concentrations of recombinant proteins were determined using the Bradford method, and they were adjusted to 2 mg $ml^{-1}$ with PBS. RFlaC was mixed 1:1 (v/v) with rVAA, rOmpK and rOmpR, respectively, then the bivalent vaccines candidates, rVAA + rFlaC (AF), rOmpK + rFlaC (KF) and rOmpR + rFlaC (RF) were used in immunization experiment. For ELISA, 50 μg $ml^{-1}$ of bivalent vaccines candidates were used as coating antigens.

### 2.5. Immunization and sampling

Flounders were randomly divided into five groups, 280 fish per group. AF, KF, RF, FKC and PBS were emulsified with Freund's complete adjuvant (cat No. F5881, Sigma, USA) at equal volume, respectively. And then 100 μl was immunized intraperitoneally to each fish.

For $CD3^+$ T lymphocytes detection, three fish were randomly sampled in each group at $1^{st}$, $3^{rd}$, $5^{th}$, $7^{th}$, $14^{th}$, $21^{st}$ post immunization day, respectively. The peripheral blood was collected, and the peripheral blood lymphocytes (PBLs) were separated according to our previous study [21], and then they were adjusted into $1 \times 10^6$ cells $mL^{-1}$ with PBS, and used in FCM.

For $sIg^+$ B lymphocytes and antibody detections, three individuals were randomly sampled in each group at $1^{st}$, $2^{nd}$, $3^{rd}$, $4^{th}$, $5^{th}$, $6^{th}$ and $7^{th}$ post immunization week, respectively. The PBLs were collected same as mentioned above and used in FCM. The immunized serum (IS) was isolated as described previously [25], then used in ELISA for the total antibodies (TA), specific antibodies against *V. anguillarum* (VA) and specific antibodies against bivalent recombinant proteins (PA) determination.

Pre-experiments were performed for the dilutions of IS used in specific antibodies against bivalent recombinant proteins (PA) determination, the IS from control group and immunized groups at $4^{th}$, $5^{th}$ and $6^{th}$ week (around the peak time) were collected, their titers were determined, and then serial dilutions of IS at 10-, 20-, 40-, 80- and 160-fold were used to test the specific antibody level using ELISA, respectively. Finally, dilution at 1:80 of IS was used in specific antibody detection.

For immune related genes analysis, three individuals were sampled in each group at $12^{th}$, $24^{th}$, $48^{th}$ and $96^{th}$ hour post immunization. The spleen was collected, the total RNA was extracted following Trizol method, and the RNA integrity was detected using 1% agarose gel electrophoresis. RNA concentration and purity were measured with NanoDrop 8000 (Thermo Fisher Scientific, USA). Then, cDNA was synthesized using the PrimeScriptTM reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions; cDNA concentrations were measured with NanoDrop 8000, adjusted into 100 ng $ml^{-1}$, and then used for Quantitative PCR (Q-PCR).

### 2.6. Flow cytometry

For $CD3^+$ T lymphocytes detection, the PBLs were incubated with FCD3-Pab for 1 h at 37 °C. Subsequently they were washed three times with PBS containing 5% (v/v) Newborn Calf Serum, incubated with goat-anti-rabbit Ig-Alexa Fluor® 647 (1:1000, Thermo Fisher Scientific) for 1 h in the dark at 37 °C, and then washed again. The cell suspensions were analyzed with a cytometer (BD Accuri, USA). For $sIg^+$ B lymphocytes detection, PBLs were incubated with FIgM-Mab for 1 h at 37 °C, and then incubated goat-anti-mouse Ig-FITC (1:256, Sigma) for 1 h in the dark at 37 °C. And then the cell suspensions were analyzed with cytometer. In each successive step, the samples were washed as described above. Fluorescent light (FL)-1 and FL-4 were used to determine FITC-labeled cells and Alexa Fluor® 647 labeled cells, respectively.