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# Protective efficacy of six immunogenic recombinant proteins of *Vibrio anguillarum* and evaluation them as vaccine candidate for flounder (*Paralichthys olivaceus*)

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ABSTRACT

*Vibrio anguillarum* is a severe bacterium that causes terminal haemorrhagic septicaemia in freshwater and marine fish. Virulence-associated proteins play an important role in bacterial pathogenicity and could be applied for immunoprophylaxis. In this study, six antigenic proteins from *V. anguillarum* were selected and the immune protective efficacy of their recombinant proteins was investigated. VirA, CheR, FlaC, OmpK, OmpR and Hsp33 were recombinantly produced and the reactions of recombinant proteins to flounder-*anti-V. anguillarum* antibodies (fV-ab) were detected, respectively. Then the recombinant proteins were injected to fish, after immunization, the percentages of surface membrane immunoglobulin-positive (sIg+) cell in lymphocytes, total antibodies, antibodies against *V. anguillarum*, antibodies against recombinant proteins and relative percent survival (RPS) were analyzed, respectively. The results showed that all the recombinant proteins could react to fV-ab, proliferate sIg + cells in lymphocytes and induce production of total antibodies, specific antibodies against *V. anguillarum* or the recombinant proteins; the RPS of rVirA, rCheR, rFlaC, rOmpK, rOmpR and rHsp33 against *V. anguillarum* was 70.27%, 27.03%, 16.22%, 62.16%, 45.95% and 81.08%, respectively. The results revealed that rHsp33, rVirA and rOmpK have good protections against *V. anguillarum* and could be vaccine candidates against *V. anguillarum*.



## 1. Introduction

*Vibrio anguillarum* is a halophilic bacterium that can infect many economic important fishes with a world-wide distribution [1–5]. Fish affected by this pathogen shows a symptom of a generalized septicaemia with haemorrhage on the base of fins, exophthalmia and corneal opacity [6]. Due to the lack of effective prevention and treatment measures, especially the limit of commercial vaccines, *V. anguillarum* has caused colossal economic losses in aquaculture industry.

Subunit vaccines are attractive for vaccination purposes due to their characteristics of safety, nonviability and avirulence. During recent years, studies have reported the use of subunit vaccines, in form of recombinant proteins, as vaccine candidates against *V. anguillarum* [7–9]. At present, a number of antigenic factors associated with bacterial pathogenicity, including motility [10], chemotaxis [11], adhesion [12] and virulence [13], have been reported in *V. anguillarum*. However, the immune protective potential of these proteins has not been investigated in flounder (*Paralichthys olivaceus*) model.

Based on the complete genome sequence of *V. anguillarum*, it is possible to obtain the gene sequences of immunogenic proteins, which have been reported as antigenic factors [14,15]. Using bioinformatics analysis, the subcellular localization and other bioinformations of these proteins, also their potential as possible protective antigens could be predicted. In order to elucidate new *V. anguillarum* immunogenic proteins with protective properties, in this paper, six *V. anguillarum* proteins as antigenic factors were

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investigated and the protective efficacy of recombinant proteins was evaluated in *P. olivaceus*.

## 2. Materials and methods

### 2.1. Vibrio anguillarum

The pathogenic *V. anguillarum* strain was stored in our lab [5]. The strain grew in 2216E marine medium and was incubated at 28 °C for 24 h, the bacterial suspension was obtained after the procedure of centrifugation, washes and re-suspension, and its' concentration was measured and adjusted to $2.5 \times 10^9$ CFU/ml for production of Formalin killed cells (FKC) or $1.0 \times 10^7$ CFU/ml for challenge experiment. FKC ($1.0 \times 10^8$ CFU/ml) were prepared as described previously [16] and used for immunization.

### 2.2. Fish

Flounder (*Paralichthys olivaceus*, 35 ± 5 g) were obtained from a fish farm (Rizhao, Shandong, China) and maintained in tanks with continuous aerated seawater to acclimate the lab environment at least for one week. The fish were confirmed free from *V. anguillarum* before experimental manipulation, fed twice per day with commercial dry pellets, and then used in fish vaccination and challenge experiments. Those experiments were conducted in accordance with the guidelines of "Regulations for the Administration of Affairs Concerning Experimental Animals" published by the State Science and Technology Commission of Shandong Province.

### 2.3. Antibodies

Monoclonal antibodies against flounder IgM (fIgM Mab) were produced previously in our laboratory [17], in this paper, the ascites diluted 1:2000 in PBS were employed in Western blotting (WB), Flow cytometry (FCM) and enzyme-linked immunosorbent assay (ELISA).

The flounder-*anti-V. anguillarum* antibodies (fV-ab) were produced by immunizing the flounder with FKC, the sera were diluted at 1:100 into PBS and used in WB.

### 2.4. Bioinformatics analysis of V. anguillarum antigenic proteins

Six previously reported proteins which were vital for *V. anguillarum* survival and virulence, including virulence protein A (VirA), outer membrane protein K (OmpK), outer membrane protein R (OmpR), heat shock protein 33 (Hsp33), chemotaxis methyl ransferase (CheR) and flagellin C (flaC), were selected. Their gene sequences were obtained from *V. anguillarum* 775 (Genbank No. NC_015633.1) and *V. anguillarum* M3 (Genbank No. CP006699.1), and bioinformations were analyzed by the bioinformatic softwares. The softwares of HMMTOP and TMHMM 2.0 were used to search for transmembrane helix, programs of PSORTb 3.0.2 and SOSUI-GramN were available to predict protein subcellular location, ExPASy Compute pI/Mw for prediction of pI and Mw.

### 2.5. Production of recombinant proteins

The genes of selected proteins were cloned by specific primers listed in Table 1 and the recombinant proteins, rVirA, rCheR, rFlaC, rOmpK, rOmpR and rHsp33, were produced as describe early [18]. Briefly, the PCR amplified products were purified using PCR purification kit (Taraka, China), and ligated into PEASY-E1 vector with pEASY-E1 Expression Kit (Taraka) followed by the manufacturer's instruction. The ligation product was transformed into *E. coli* BL21 (DE3) by heat shock at 42 °C and positive clones were selected on Luria-Bertani (LB) agar plates with 50 μg/ml ampicillin and confirmed by sequencing. The *E. coli* BL21 (DE3) carrying the recombinant plasmid was cultured in LB broth medium to a mid-logarithmic phase ($OD_{600} = 0.6$) and induced with 100 mM isopropyl-β-D-thiogalactoside (IPTG) for 8 h. Bacterial cells were harvested by centrifugation and His-tagged recombinant proteins were purified using His Trap™ HP Ni-Agarose (GE healthcare China, Beijing, China) as recommended by manufacturer's instruction. The concentration of purified proteins was quantified by Bradford method and adjusted to 50 μg/ml for ELISA, 1 mg/ml for WB, and 2 mg/ml for vaccination.

### 2.6. Western blotting

The purified recombinant proteins were separated by SDS-PAGE and transferred onto Poly vinylidene fluoride (PVDF) membranes (Millipore, USA). Then fV-ab, fIgM Mab and goat-*anti*-mouse IgG-alkaline phosphatase (AP) antibodies were used as primary, secondary and third antibodies, respectively. The antibody-bound proteins were visualized by incubating PVDF membrane with substrate solution (100 mM NaCl, 100 mM Tris and 5 mM MgCl2, pH 9.5) containing 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma, USA) and nitroblue tetrazolium (NBT, Sigma) for 5 min, and stopping by washing with distilled water. PBS instead of fV-ab as primary antibodies was performed as negative control.

### 2.7. Fish vaccination and sampling

Flounder were randomly allocated to eight groups (150 fish/group). On the day of immunization, purified recombinant proteins, FKC and PBS were emulsified with Freund's complete adjuvant (Sigma) at an equal volume, and then intraperitoneally injected to fish (100 μl per individual), respectively. At 1st, 2nd, 3rd, 4th, 5th, 6th and 7th weeks after immunization, six fish from each group were sampled for preparation of sera and lymphocytes in peripheral blood leukocytes (PBL).

Lymphocytes were prepared according to previously described procedure [20], and applied in FCM to analyze the percentage of surface membrane immunoglobulin-positive (sIg+) cells. Sera, used in ELISA for determination the titers of total antibodies, antibodies against *V. anguillarum* and antibodies against recombinant proteins, were produced as described previously [19].

### 2.8. Flow cytometry

Lymphocytes ($1 \times 10^7$ cells/ml) were incubated with fIgM Mab at 37 °C for 1 h. After washed three times with PBS containing 5% (v/v) Newborn Calf Serum by centrifugation at 640 × *g* for 5 min, goat-*anti*-mouse IgG-FITC antibodies (1:256, Sigma) were added into each well and incubated for 1 h at 37 °C. After washing three times as above, the percentages of sIg + cells in lymphocytes were analyzed with Accuri C6 cytometer (BD Accuri™, Piscataway, NJ, USA). In control group, myeloma culture supernatant was substituted for incubating with lymphocytes.

### 2.9. ELISA

Antibody titers of immunized fish were measured by ELISA as described previously with little modification [19] at week 1–7 after injection. Briefly, for the determination of total antibodies, 100 μl fV-ab (dilutions of 1:200) were coated, and incubated with fIgM Mab as primary antibodies and goat-*anti*-mouse IgG-AP as secondary antibodies for 1 h at 37 °C, successively. For determinations of antibodies against *V. anguillarum* or antibodies against