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Immune effects of a bivalent expressed outer membrane protein to American eels (*Anguilla rostrota*)

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ABSTRACT

The specific and non-specific immune parameters and protection of American eels (*Anguilla rostrata*) were evaluated after immunized eels with a bivalent expressed out membrane protein (OMP) of porin II of *Aeromonas hydrophila* and ompS2 of *Edwardsiella tarda*. One hundred eighty eels were distributed into 3 equal groups and intraperitoneal (i.p) injection with phosphate-buffered saline (PBS group), formalin-killed-whole-cell (FKC) of *A. hydrophila* and *E. tarda* (FKC group) or the bivalent OMP (OMP group). The lymphocytes and red blood cells collected on 14, 21 and 42 days post-vaccination were used to evaluate the stimulation index (SI) and the sera collected on 14, 21, 28 and 42 days were used to assize the titers of specific antibody as well as lysozyme activity. Lysozyme activities in skin mucus, suspension of liver and kidney were also recorded on 14, 21 and 28 days. On 28 d post-vaccination, eels from all three groups were challenged by i.p injection of live *A. hydrophila* or *E. tarda*. The results show that, compared with the PBS group, proliferation of lymphocytes in OMP group was significantly ($P < 0.05$) enhanced on 21 days, and the serum titers of anti-*A. hydrophila* and anti- *E. tarda* antibody in eels of FKC and OMP group were significant increased ($P < 0.05$ or $P < 0.01$) on 14, 21 and 28 days. Activity of the lysozyme in serum, skin mucus, liver and kidney were significant changed ($P < 0.05$ or $P < 0.01$) between the three groups. Relative Percent Survival (RPS) after challenged with *A. hydrophila* on 28 days post immunization in two vaccinated groups vs. PBS group were 50%, and the RPS challenge *E. tarda* in FKC and OMP vs. PBS group were 50% and 37.5% respectively. These results suggest that American eels immunized with the bivalent OMP would positively affect specific as well as non-specific immune parameters and protect against infection by the two pathogens in freshwater farming.

1. Introduction

Aeromonas hydrophila and *Edwardsiella tarda* are two common pathogenic bacteria in freshwater farming of eel and other fishes. It has caused incalculable losses in the aquaculture industry [1–6]. In the spring and summer, it causes many highly infectious diseases, such as hemorrhagic septicemia, liver bleeding, gill-rot disease and dermorrhagia in European eel (*Anguilla anguilla*), Japanese eel (*Anguilla japonica*) and American eel (*Anguilla rostrata*) [5–7]. Due to long-term application of antibiotics in treating these diseases, drug resistance among pathogens has developed less and less available drugs [8,9]. Recently, many studies report the whole inactivated bacteria vaccine [10,11], live bacteria vaccine [12] and subunit vaccine [13–15] of *A. hydrophila* and *E. tarda*, but immunogenicity of bivalent expressed out membrane protein (OMP) from two pathogens has not been reported [16]. From 2002 to 2012, 87 strains of pathogens (11 species in 3 genera) were isolated from more than 70 batches of diseased American, European and Japanese eels showing various symptoms, and more than half of which (46 strains) were identified as *A. hydrophila* or *E. tarda* by biochemical and molecular methods [7,16,17]. Based on some studies [7,12,18], it was found that eels (50 g eel^{-1}) challenged (i.p) with 1.0×10^7 cfu of *A. hydrophila* or 1.0×10^6 cfu of *E. tarda* caused 100% death. As eels farming in China are easy subject to the two above mentioned pathogens in the summer, it is necessary to develop a bivalent vaccine with low toxicity and high efficiency. In this study, based on the connection of two DNA partial sequences of OMP porin II of *A. hydrophila* (see Attachment I, from 211 bp to 1032 bp) and ompS2 of *E. tarda* (see Attachment II, from 418 bp to 1188 bp), an expression vector (pGex-2T-OMP-S2-porin II) was constructed and a bivalent OMP was expressed through an *Escherichia coli* (DE3) BL21 and purified by affinity chromatography

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[16]. Eels were injected (i.p) with 0.2 ml the bivalent OMP (100 μg eel^{-1}), formalin-killed *A. hydrophila* and *E. tarda* (2×10^8 cfu eel^{-1}) or 10 mM phosphate-buffered saline (PBS, pH = 7.4) respectively to evaluate the immunogenicity and protection of the bivalent OMP. The effects of such OMP immunization on the immune functions and the Relative Percent Survival (RPS) rate of American eels (*Anguilla rostrata*) were studied [19]. The preliminary results are quite positive which calls for further studies in this new expressed bivalent OMP as a vaccine in fish farming.

2. Materials and methods

2.1. Fish and bacteria

One hundred eighty American eels (*Anguilla rostrota*, mean body weight 50 g) were brought to the laboratory from an eel farm located at Xiamen, China. After quarantined for seven days, the eels were randomly divided into three groups of 60 each in aquarium tanks of 1000 L capacity and acclimated to the laboratory conditions for one week. Fish were fed with commercial pellet feed (Fuqing Tianma Feed Co., Ltd in Fujian province in China) twice a day. The fish feces and salvage were siphoned out 3 h after feeding. A strain of *A. hydrophila*, isolated from the liver of diseased eels with ulcers on the peduncle, and another strain of *E. tarda*, isolated from the kidney of diseased eels with hepatomegaly and bleeding, was used in this study [12,20]. Two strains were tested for 96 characteristics by the Biolog GeneIII system and got 94.4% and 96.6% probability as *A. hydrophila* and *E. tarda* respectively. The strain of *A. hydrophila* showed 99% similarity to the 16S rRNA sequence of *A. hydrophila* (JQ034593, JQ034594) and *E. tarda* showed 100% similarity to the 16S rRNA sequence of *E. tarda* (FJ405292, EU259317, EU231639). The bacteria were stored at −80 °C in saline with 20% glycerol added. Strains were pathogenic as determined by infections experiments and the lethal median dose (LD_{50}) by i.p injection were 5.0×10^4 and 2.6×10^3 colony forming units (cfu) g^{-1} body weight for *A. hydrophila* and *E. tarda* respectively [12,20].

2.2. Reagent

① RPMI 1640 medium (pH = 7.0): Roswell Park Memorial Institute 1640 (RPMI 1640, HyClone) solution with 0.5% $NaHCO_3$, 0.05 mM mercaptoethanol and 10 mM hepes (HyClone) ② ConA: dissolving 2.0 mg Concanavalin A (ConA, Sigma) in 20 ml RPMI 1640 medium, then storing at −20 °C. ③ MTT: dissolving 1.0 g 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Biomal) in 200 ml 10 mM PBS (pH = 7.4), then storing at 4 °C in the dark. ④ Heparin sodium: diluting 0.1 g of heparin sodium (The Second Reagent Factory of Shanghai) in 10 ml physical saline, then storing at −20 °C. Four reagents prepared above were filtrated by 0.22 μm filter membrane before stored. ⑤ Phosphate Buffered Saline (PBS): the desired concentration and pH of the solution was prepared by the various proportion mixture of solution A (contains 0.2 M Na_2HPO_4) and B (contains 0.2 M NaH_2PO_4). ⑥ Coating solution: 0.15 g Na_2CO_3 and 0.29 g $NaHCO_3$ were dissolved in 100 ml double distilled water. ⑦ Washing solution (PBST): 10 mM PBS (pH 7.4) with 0.05% (v/v) tween-20. ⑧ Blocking solution: 1% BSA (The Second Reagent Factory of Shanghai) in PBST.

2.3. Preparation of formalin killed cells (FKC) of A. hydrophila and E. tarda

Bacteria *A. hydrophila* and *E. tarda* were cultured in Tryptone soya broth (TSB) at 28°Cfor 24 h, and the cells were harvested by centrifuging at 5000× *g* for 10 min. The pellets were washed three times with 10 mM PBS (pH = 7.4). Then formaldehyde was added to the suspension to a final concentration of 0.4% (v/v). The suspension was incubated at 28 °C for 24 h and was tested by adding dilution on Tryptone soya agar (TSA) plates for completely inactivation. The cells were adjusted to a concentration of 1.0×10^9 cfu ml^{-1}. That's the formalin-killed-whole-cell (FKC) prepared for immunize the fish as a positive control.

2.4. Preparation of the bivalent OMP

The lyophilized powder of a bivalent OMP expressed partial porin II of *A. hydrophila* and partial OmpS2 of *E. tarda* was provided by Wang Yu [16]. Preparation of the bivalent expressed OMP was summarized as following: after gene cloning by PCR, one PCR product of the DNA fragment of porin II of *A. hydrophila* (see Attachment I, from 238 bp to 1080 bp) which added a DNA adapter (5′tcgggcggtggcggctcgggtggcggatca3′) at the forward primer of the fragment was connected with another DNA fragment of OmpS2 of *E. tarda* (see Attachment II, from 460 bp to 1209 bp) which added a DNA adapter (3′gcatatccgagcccgccaccaccgagccca5′) at the reverse primer of the fragment by fusion PCR as following.

Reverse primer of the DNA fragment of OmpS2 + Adaptor 3′ctactcttactgaagtgggcacga + gcatatccgagcccgccaccaccgagccca5' 5′ tcgggcggtggcggctcgggtggcggatca + tccggtatcgccaagactgaatg 3′ Adaptor + Forward primer of DNA fragment of Omp porin II.

After the ligated sequence (1650 bp) of two above-mentioned fragments and a plasmid vector pGex-2T-His (adding a His tag to pGex-4T-1 at C end) [14] were digested by restrictive endonuclease enzymes BamHI and EcoRI simultaneously, the segments of the ligated sequence and pGex-2T-His were recycled by gel extract kit, and ligated using T4 DNA ligase at a molar ratio of 4:1 at 4 °C overnight. The resulting recombinant plasmids (pGex-2T-OMP-S2-porin II) were transformed to *E. coli* BL21 (DE3, Novagen) for ultimate expression. And the constructs pGex-2T-OMP-S2-porin II were confirmed by DNA sequencing (Invitrogen).

One-litre cultures of *E. coli* BL21 (DE3) transformed with pGex-2T-OMP-S2-porin II were grown in Luria broth (LB) at 37 °C from a 1/100 dilution of starter inoculum. Protein expression was induced with 0.25 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) at cell density (A600) of 0.7—0.9. The cell cultures continued to grow at 16 °C for 12 h. Due to C end of recombinant fusion antigen with six histidines, the recombinant fusion OMP was purified by using Ni^{2+}-NTA agarose resin. Briefly, 500 ml of bacteria cultivated suspension was prepared, centrifugated, resuspended with the buffer liquid (50 mM phosphate, 300 mM NaCl, pH 7.4), and sonicated with the energy of 700 W for 10 min, and ultracentrifugated for 5 min at 10 000 rpm at 4 °C.

The sonicated recombinant fusion protein was purified by Ni^{2+}-NTA agarose resin with abluent (20 mM phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.44) and lavation (20 mM phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.44) respectively, and then the purified OMP was renaturated and lyophilized. The purity of lyophilized protein was determined by gel electrophoresis and the molecular mass of the recombinant fusion protein revealed by SDS-PAGE (Bandscan software) was 87.1 kD. Before test, the lyophilized OMP was dissolved in 10 mM PBS (pH = 7.4) at a concentration of 1 mg/mL and mixed with equivalent Freund's incomplete adjuvant to a final concentration of 0.5 mg/mL.

2.5. Fish vaccination and sampling

In three groups of eels, bivalent OMP group were immunized (i.p) with 0.2 ml (100 μg) of the purified fusion protein (0.5 mg ml^{-1}); positive Control group and negative Control group was injected with 0.2 ml FKC (FKC group) and 0.2 ml sterile 10 mM PBS (pH 7.4, PBS group), respectively. On 14, 21, 28 and 42 days