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A live attenuated *Vibrio anguillarum* vaccine induces efficient immunoprotection in Tiger puffer (*Takifugu rubripes*)

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ABSTRACT

Tiger puffer (*Takifugu rubripes*) is becoming an economic promising aquaculture species in China. However, the development of Tiger puffer breeding industry is restricted by some serious aquatic disease such as vibriosis. An effective live attenuated vaccine MVAV6203 was developed in our previous studies by curing the virulence plasmid pEIB1 and unmarked inframe-deletion of the *aroC* gene from the virulent *V. anguillarum*. Here, we evaluated whether this live vaccine was suitable for Tiger puffer against disease caused by *Vibrio* genus. The live vaccine show virulence attenuation in both juvenile and adult fish vaccinated with either a single or high dose (50-fold single dose). In addition, administration of the live vaccine shew limited growth in fish and did not affect fish body weight significantly, with no adverse impact on growth between vaccinated and saline control fish. Furthermore, increased expression of cytokines involved in pro-inflammatory (IL-1β, TNFα and IL-6), cell-mediated immunity inducing (IL-12p35, IL-12p40 and IL-18), antiviral/intracellular pathogen killing (I-IFN-1, IFN-γ and Mx), peripheral T cell expansion and survival controlling (IL-2, IL-7 and IL-15) and antigen processing maker and anti-inflammatory (MHC I and IL-10) were elicited significantly after the vaccination. These cellular responses correlate with protection against virulent strain challenge and high RPS of 90.67% and 80.31% in juvenile and adult fish were obtained, respectively. These data indicated for the first time that the live attenuated *V. anguillarum* vaccine is suitably applied for the development of an effective and safe vaccine for prevention of vibriosis in Tiger puffer aquaculture industry.

1. Introduction

Nowadays, vaccination becomes a common strategy to prevent disease [2]. So far, different types of vaccine have been designed for farmed fish such as inactivated vaccines, attenuated live vaccines, purified recombinant subunit vaccines and DNA vaccines. Live vaccines, with high immune efficiency and mimicking natural infection, become one of the disease-prevention strategies against fish pathogens. A live attenuated vaccine was developed in our laboratory previously by curing the virulence plasmid pEIB1 and unmarked inframe-deletion of the *aroC* gene from a virulent *Vibrio anguillarum* [1]. The vaccine protects *Scophthalmus maximus* [2], *Paralichthys olivaceus* [1] and *Danio rerio* [3] efficiently either by immersion or injection administration, and exhibits excellent cross immune protection against *Vibrio harveyi* in *Epinephelus coioides* [1] and *Vibrio alginolyticus* in *Pseudosciaena crocea* [4]. However, whether the live vaccine can be applied to more economic fish is unknown.

Generally, an effective vaccine is expected to be recognized by host immune system. After the recognization, some signalling pathways are activated and some inflammatory mediators are released [5]. Cytokines are kinds of mediators in the immune system by binding to specific receptors at cell membrane [6]. They drive inflammatory signals that regulate the capacity of resident and newly arrived phagocytes to destroy the invading pathogen [7]. A cytokine network in teleost has been described [7], providing convenience for understanding fish immunology and investigating immune responses induced by pathogens and vaccines.

Tiger puffer (*Takifugu rubripes*) becomes a raising economic farming species in China [8]. Since vibriosis is a common disease in course of Tiger puffer farming, we determined the safety and efficiency of a live attenuated *V. anguillarum* vaccine on Tiger puffer in this study. In addition, the impact on growth and immune responses induced by the vaccine were evaluated as well.

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2. Materials and methods

2.1. Fish maintenance

Healthy Tiger puffer at different ages (weighing 45.0 ± 5.0 g and 450.0 ± 50.0 g respectively) were obtained from a commercial farm (Tianzheng Corp., Dalian, China) and maintained in aerated tanks that were supplied with a continuous flow of sand-filtered seawater at 25 ± 1 °C. Fish were fed twice daily with commercial feed and acclimated at least one week. Before experiments, fish were randomly selected for health examination. Several indexes such as body colour, motility, feeding habit of the fish were considered. Then fish were taken for inspection and dissection. Besides, whether the fish were infected with *V. anguillarum* was analyzed by plating tissues including liver, kidney and spleen on thiosulfate citrate bile salts sucrose agar (TCBS, Difco, USA). Before vaccination, fish were anaesthetized with MS-222 (100 ng/ml) in seawater. All fish experiments were carried out according to the guidelines and approval of the Animal Research and Ethics Committees of East China University of Science and Technology.

2.2. Bacterial strains

The live attenuated *V. anguillarum* strain MVAV6203 was constructed in our previous work [1]. Stock cultures were maintained at −80 °C in a suspension of LB20 (Luriae Bertani mediums supplemented with 2% NaCl) containing 20% (v/v) glycerol. MVAV6203 was cultured on TCBS at 28 °C for 36 h. Colonies from fresh TCBS were subcultured into LB20 and harvested by centrifugation at 5000 rpm for 10 min. Then, cells were washed twice in sterile physiological seawater (PSW, NaCl, 20g/l; KCl, 0.7g/l; $MgCl_2{\cdot}6H_2O$, 4.8g/l; $NaHCO_3$, 0.11g/l; $MgSO_4{\cdot}7H_2O$, 3.5g/l; and $CaCl_2{\cdot}2H_2O$, 1.6g/l) and diluted into specific optical density at 600 nm. Finally, bacterial cells were diluted with PSW and ultimate concentration was determined by spreading plate method. Wild-type (WT) strain *V. anguillarum* MVM425 (O1 serotype) used in this study was isolated from diseased fish suffering from vibriosis epizootic in a fishery in the Yellow Sea of China [9]. Culture conditions were similar to vaccine strain.

2.3. Vaccine safety evaluation

To evaluate safety of the vaccine, 150 juvenile fish weighing 45.0 ± 5.0 g were randomly divided into five groups (30 fish/group): V_S, fish vaccinated with 2×10^6 CFU of vaccine (single dose) and revaccinated with the same dose 2 weeks later; C_S, fish mock-vaccinated with sterile PBS and remock-vaccinated with PBS 2 weeks later as a control; V_H, fish vaccinated with 1×10^8 CFU of vaccine; C_H, fish mock-vaccinated with sterile PBS as a control; I, fish injected with 1×10^8 CFU of WT. All fish received an intramuscular injection (IM) with a volume of 0.1 ml. Cumulative survival rate was recorded after vaccination. Meanwhile, 150 adult fish weighing 450.0 ± 50.0 g were administrated with the same manipulation. The experiment was performed in triplicate.

2.4. The impact on pufferfish growth of the vaccine

To determine the impact on fish growth of the vaccine, 100 juvenile fish weighting 45.0 ± 5.0 g were randomly divided into two groups (50 fish/group): V, fish vaccinated with 2×10^6 CFU of vaccine; C, fish mock-vaccinated with sterile PBS. At 0, 1, 2, 3, 4, 5 and 6 w p.v., average weight of fish was calculated. The experiment was performed in triplicate.

2.5. Persistent carrier state of the live vaccine in tissues

To determine persistent carrier state of the vaccine in tissues, 100 juvenile fish weighing 45.0 ± 5.0 g were randomly divided into two groups (50 fish/group): V, fish vaccinated with 2×10^7 CFU of MVAV6203; C, fish injected with 2×10^7 CFU of WT. Visceral organs (liver, kidney and spleen) from three fish in each group were sampled, weighed, and homogenized in 1 ml PSW. Homogenates were serially diluted and plated on TCBS at 28 °C for 48 h. Colonies that featured with yellow centers were counted. Bacteria counts were calculated by dividing the weights of the tissues and from the mean of three samples in triplicate experiments.

2.6. Vaccine effectiveness evaluation

To evaluate the effectiveness of the vaccine, 100 juvenile fish weighing 45.0 ± 5.0 g were randomly divided into two groups (50 fish/group): V, fish vaccinated with 2×10^6 CFU of vaccine; C, fish mock-vaccinated with sterile PBS. All fish received an IM injection with a volume of 0.1 ml. At 4 w post vaccination (p.v.), 30 fish from each group were IM challenged with 1×10^7 CFU of WT. Cumulative survival rate was recorded for lasting 21 days and relative percent survival (RPS) was calculated according to the following formula devised by Amend (1981). $\mathrm{RPS} = \left(1 - \frac{\%\ \text{mortality of vaccinated fish}}{\%\ \text{mortality of control fish}}\right) \times 100\%$. At 1, 2, 3 and 4 w p.v., liver and spleen from three vaccinated fish and control fish were sampled for RT-qPCR. Meanwhile, adult fish were also used for vaccine effectiveness evaluation but not for RT-qPCR assay. Both vaccination and challenge were conducted in triplicate.

2.7. RNA extraction and real-time quantitative PCR (RT-qPCR)

Sample RNA was extracted and RT-qPCR was carried out as previously described [10]. Primers used for RT-qPCR were listed in Table 1. Most primer sequences were referred to the previous report [11]. At the end of each PCR, melting curve analysis was performed to confirm PCR product amplified. The relative expression of each immune-relative gene was determined by the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$ method) with β-actin as the reference gene.

2.8. Statistical analysis

Independent-sample t-tests were performed with SPSS software (Version 11.5, SPSS Inc.) to determine statistical significance. Significant differences were considered at $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results

3.1. Safety profiles of the live attenuated vaccine

Safety of the live attenuated vaccine to Tiger puffer was tested. The scheme was shown in Fig. 1. Briefly, fish were vaccinated with single dose repetitively or with high dose (50-fold single dose).

3.1.1. Safety evaluation of vaccine with single and repetitive dose

Both juvenile fish and adult fish were injected with 0.1 ml vaccine containing 2×10^6 CFU cells or PBS, and repeat vaccinations were performed 2 weeks later. As the results shown in Table 2, no death was observed after fish injected with the vaccine for the second time. Only 1.11% juvenile fish died in control group. It suggested that the live attenuated vaccine is safety with single and repetitive vaccination dose in Tiger puffer.