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Immune responses of zebrafish (*Danio rerio*) induced by bath-vaccination with a live attenuated *Vibrio anguillarum* vaccine candidate

Zhihui Zhang, Haizhen Wu*, Jingfan Xiao, Qiyao Wang, Qin Liu, Yuanxing Zhang

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, PR China

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ABSTRACT

A fish vaccine candidate, live attenuated *Vibrio anguillarum*, which can protect fish from vibriosis, was established in our laboratory. In this study, the protective immunological mechanism of live attenuated *V. anguillarum* was investigated in zebrafish as a model animal. After bath-vaccinated with the live attenuated strain, zebrafish were challenged with wild pathogenic strain to test the immunoprotection of the live attenuated strain. As the results, specific antibody response of fish against *V. anguillarum* was found to gradually increase during 28 days post-vaccination, and remarkable protection was showed with a high relative protection survival (RPS) of about 90%. Moreover, the vaccination changed the expressions of several immune-related genes in the spleens and livers of zebrafish. Among them, the expressions of pro-inflammatory factors such as IL-1 and IL-8 were tenderly up-regulated with about 3–4 fold in 1–7 days post-vaccination, while MHC II rose to a peak level of 4-fold in 7th day post-vaccination. These results gave some important messages about the mechanism of specific protection induced by live attenuated *V. anguillarum* and showed the availability of zebrafish model in the evaluation of the vaccine candidate.

1. Introduction

In recent years, increasing infectious diseases of farmed fishes have brought huge loss to aquaculture. Within a variety of fish diseases, vibriosis is a widespread lethal hemorrhagic septicemia caused by *Vibrio anguillarum* [1], especially in China. A live attenuated *V. anguillarum*, a vaccine candidate against vibriosis, has been established in our laboratory by curing the virulence plasmid pEIB1 [2] and deletion of the *aroC* gene from the wild-type *V. anguillarum*. The live attenuated strain can protect *Paralichthys olivaceus* and *Epinephelus coioides* against *V. anguillarum* infection (US patent, No: 20080274136, 2010) though the mechanism of its protection is still unknown.

Due to its small size, rapid growing and easy manipulation [3], zebrafish has become a useful model animal as a link between cellular assay with mammals test [4]. The well-developed immune system and its available genome information [5] make zebrafish a good model for immunity research [6]. It was reported the pathogenesis of *Edwardsiella tarda* [7] and *Francisella* [8] were characterized in zebrafish model. Furthermore, with zebrafish, infection processes of some important bacterial pathogens have also been studied, such as *Aeromonas hydrophila* [9], *Salmonella typhimurium* [10], *V. anguillarum* [11], *Streptococcus iniae* [12] and *Mycobacterium marinum* [13]. It is also a powerful model in studies of viral infection caused by viral haemorrhagic septicemia virus (VHSV) [14] and nodavirus [15]. In addition to the usefulness of zebrafish as the infection model, an efficient antiviral immune response was demonstrated to arise in zebrafish vaccinated with the live attenuated VHSV [16]. Recently, it was reported that zebrafish was used as an animal model in screening of live attenuated vaccine candidates against mycobacteriosis [17] and edwardsiellasis [18]. Besides, several immune cytokines including interleukin-1β [19], interleukin-8 [20], lysozyme [21], interleukin-10 [22], interleukin-15 [23] and major histocompatibility complex (MHC) [24] have also been identified in zebrafish.

Ideally, live attenuated vaccine can be delivered to fish by bath for large scale intensive aquaculture with convenient administration and effective immunoprotection [25]. In the present work, zebrafish was bath-vaccinated with live attenuated *V. anguillarum*. A significant protection of vaccinated zebrafish was obtained comparable with that on *P. olivaceus*. The specific antibody response in zebrafish showed that the effective protection was involved in the humoral immune response. Moreover, the expression profiles of immune-related genes indicated the innate and adaptive immune responses provoked by the vaccine candidate.

* Corresponding author. Tel.: +86 21 64253065; fax: +86 21 64253025.
E-mail address: wuhzh@ecust.edu.cn (H. Wu).

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2. Materials and methods

2.1. Fish rearing

After purchased from a commercial fish market (Shanghai, China), 630 zebrafish were held in 10 l tanks for one week. The fish were reared on a 12-h light/12-h dark cycle and fed with commercial feed for aquatic animal twice per day. Water temperature was maintained at 24 °C during the cultivation. The zebrafish were anaesthetized by immersion in 100 ng ml^{-1} tricaine methanesulphonate (MS-222, Sigma, USA) before manipulations. For euthanasia, they were immersed in 300 ng ml^{-1} MS-222 for at least 10 min.

2.2. Vaccination, sampling and challenge

The wild-type *V. anguillarum* MVM425 preserved in our laboratory was isolated from turbot [26]. The attenuated *V. anguillarum* strain MVAV6203 was constructed in our previous work.

V. anguillarum MVAV6203 was grown in high-salt Luria-Bertani (LB) medium at 30 °C for 16 h and counted as colony forming units (CFU) by spreading plate method. The cells were harvested by centrifugation and rinsed twice in 3% saline. Finally, cells were resuspended and diluted to 1×10^8 CFU ml^{-1} with 3% saline. The 315 zebrafish of vaccinated group (V group) were immersed in the aerated cell-resuspended saline for 8 min. As control group (C group), the rest fish were immersed in 3% saline for 8 min. Water temperature in all experiments was maintained at 24 °C.

To eliminate individual differences, three pools (10 fish in each pool) of the spleen and liver tissues in V group and C group were isolated in 0, 1, 3, 5, 7 and 14 days post-vaccination (dpv), respectively [27]. In 0, 3, 7, 14 and 28 dpv, blood of twenty-five fish of each group was collected from the caudal vein by placing a capillary tube after the caudal fin cut, with 3 μl/fish. The blood clotted at room temperature for 1–2 h and then at 4 °C overnight. Serum was collected after centrifugation at 750 *g* for 10 min, divided into several aliquots and stored at −20 °C until use. All fish for sampling were euthanized in 300 ng ml^{-1} MS-222 for at least 10 min.

The bath challenge was conducted in triplicate, and 20 zebrafish were used in each group in 28 dpv. All the zebrafish of V group and C group were immersed in 2 l water with 1×10^6 CFU ml^{-1} *V. anguillarum* MVM425, was grown in high-salt Luria-Bertani (LB) medium at 30 °C for 12 h. Mortality was checked several times daily for 28 days in each challenge experiment and dead fish was removed.

The cumulative percent mortality (CPM) was determined for lasting 28 days. Relative percent survival (RPS) was calculated according to the following formula:

$$\mathrm{RPS} = (1 - \%\ \text{mortality of immunized fish} / \%\ \text{mortality of control fish}) \times 100\%$$

2.3. Specific antibody detection

Antibody levels in zebrafish serum against *V. anguillarum* were determined from 3 dpv to 28 dpv by a modified ELISA method. For coating, microplate was coated overnight at 4 °C with 1×10^7 CFU ml^{-1} wild-type *V. anguillarum* MVM425 in 100 μl coating buffer (50 mM carbonate buffer, pH 9.6). Unbound antigen was removed by washing the plate three times with 300 μl washing buffer (0.05% Tween-20 in phosphate buffered saline (PBS, pH 7.2)). Blocking was performed by 2 h incubation with 100 μl blocking buffer (2% bovine serum albumin (BSA) in washing buffer). After blocking, the zebrafish sera were diluted with a 1:90 dilution in PBS with 0.3% BSA and added into the wells (100 μl $well^{-1}$) duplicatively. After incubated in room temperature for 3 h, the microplate was washed three times by 300 μl washing buffer and 100 μl mouse-anti-zebrafish IgM (Aquatic Diagnostics, UK, 1:30 dilution in washing buffer) was added into each well. After incubated for 1 h in room temperature, the microplate was washed three times by 300 μl washing buffer, followed by incubation in room temperature for 1 h with 100 μl $well^{-1}$ goat-anti-mouse IgG conjugated with HRP (Abgent, USA, 1:200 dilution in PBS with 1% BSA). Finally, the microplate was washed three times by 300 μl washing buffer and 100 μl $well^{-1}$ TMB was added. After incubated for 10 min in room temperature, 50 μl H_2SO_4 (2 M) as stop solution was added into each well, and then the intensity of ELISA reading was determined at OD of 450 nm with a microplate reader.

2.4. Quantitative real-time PCR

Total RNA was isolated from liver and spleen samples after grinded by Trizol (Invitrogen, USA), following the manufacturer's instructions. The RNA was dissolved in DEPC-treated water. The RNA was digested by RNase-free DNase I (Promega, USA) to eliminate genomic DNA contaminant. First strand cDNA was synthesized using 0.5 μg of total RNA by ReverTraAce reverse transcriptase (Toyobo, Japan) with oligo d(T) primer, and was stored at −80 °C. RT-qPCR was performed following the manufacture's instruction of SYBR green real-time PCR mix (Toyobo) using ABI 7500 Real-time Detection system (Applied Biosystems, USA). Primers used in PCR were designed with Primer express software (Applied Biosystems) (Table 1). The relative expression of each immune-relative gene was determined by comparative threshold cycle method ($2^{-\Delta\Delta Ct}$ method) with β-actin as reference gene.

2.5. Statistical analysis

Independent-sample *t*-test in the SPSS software (Version 11.5, SPSS Inc.) was used to determine statistical significance. Significant differences were considered at $P < 0.05$.

3. Results

3.1. Protection of immunized zebrafish from V. anguillarum challenge

Zebrafish were challenged with wild-type *V. anguillarum* MVM425 by immersion. The dose of challenge was determined based on LD_{50} with infection of untreated zebrafish with

Table 1
Primers used in this study.

Primer	GenBank accession No.	Sequence (5′–3′)	Efficiency (%)
β-actin	NM_131031.1	F: ATGGATGAGGAAATCGCTGCC R: CTCCCTGATGTCTGGGTCGTC	116
lyz	NM_139180.1	F: GATTTGAGGGATTCTCCATTGG R: CCGTAGTCCTTCCCCGTATCA	124
IL-1β	AY340959	F: TGGACTTCGCAGCACAAAATG R: GTTCACTTCACGCTCTTGGATG	96
MHC I	AF137534.1	F: GGAGTTCACCTTGCTTATGC R: CCCTCTGACCCATTCTTGT	120
MHC II	BQ261921.1	F: TGACTCAACTGTCCGTGATA R: CCATTAGCCATCTCCATAGTG	110
IL-8	XM_001342570.2	F: GTCGCTGCATTGAAACAGAA R: CTTAACCCATGGAGCAGAGG	120
IL-10	NM_001020785.2	F: TCACGTCATGAACGAGATCC R: CCTCTTGCATTTCACCATATCC	121
IL-15	NM_001039565.1	F: ACAGAGGAAGAAGCCTACAG R: GCGATGAAGACGAGAAAGAG	147