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Immune responses and immune-related gene expression profile in orange-spotted grouper after immunization with *Cryptocaryon irritans* vaccine

Xue-Ming Dan [a,1], Tuan-Wei Zhang [b,1], Yan-Wei Li [b], An-Xing Li [b,*]

[a] College of Animal Science, South China Agricultural University, Guangzhou, Guangdong Province 510642, PR China
[b] Key Laboratory for Aquatic Products Safety of Ministry of Education, State Key Laboratory of Biocontrol, The School of Life Sciences, Sun Yat-sen University, 135 Xingang West Street, Haizhu District, Guangzhou, Guangdong Province 510275, PR China

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ABSTRACT

In order to elucidate the immune-protective mechanisms of inactivated *Cryptocaryon irritans* vaccine, different doses of *C. irritans* theronts were used to immunize orange-spotted grouper (*Epinephelus coioides*). We measured serum immobilization titer, blood leukocyte respiratory burst activity, serum alternative complement activity, and serum lysozyme activity weekly. In addition, the expression levels of immune-related genes such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), major histocompatibility complexes I and II (MHC I and II), and transforming growth factor-β1 (TGF-β1) were determined in spleen and gills. The results showed that the immobilization titer, respiratory burst activity, and alternative complement activity of immunized fish were significantly increased, and the levels of the last two immune parameters in the high-dose vaccine group were significantly higher than in the low-dose vaccine group. Serum lysozyme activity in the high-dose vaccine group was significantly higher than in the PBS control group. Vaccination also regulated host immune-related gene expression. For example, at 2- and 3- weeks post immunization, IL-1β expression in the high-dose vaccine group spleen was significantly increased. At 4-weeks post immunization, the fish were challenged with a lethal dose of parasite, and the survival rates of high-dose vaccine group, low-dose vaccine group, PBS control group, and adjuvant control group were 80%, 40%, 0%, and 10% respectively. These results demonstrate that inactivated *C. irritans* vaccination improves specific and nonspecific immune responses in fish, enhancing their anti-parasite ability. These effects are vaccine antigen dose-dependent.

1. Introduction

Cryptocaryon irritans is an obligate parasite of tropical and subtropical marine fish that causes white spot disease [1]. It has a very wide host range and can infect almost all marine teleosts [2,3]. In recent years, the annual outbreak of white spot disease in marine fish culture zones of the southern and eastern Chinese coasts has posed very serious harm to the Chinese marine aquaculture industry [4]. Therefore, preventing *C. irritans* infection has become a core issue in this field. Chemical immersion is only effective during the free-living stage (tomonts and theronts), not in the parasitic stage (trophonts) [1,2,5–7], and growing concern about food and environmental safety has led to strict chemical limitation. Studies on oral drug resistance to *C. irritans* are limited. Medium-chain fatty acids (caprylic acid) are useful to a certain degree, but the effect is highly variable [8]. Other methods such as freshwater immersion, heat treatment, drying treatment, ozone treatment, ultraviolet radiation, and culture container rotation have proven to be effective [2,7,9,10], but these methods can only be carried out in a closed, re-circulating aquaculture system, they cannot be applied in an open environment (e.g., cages and ponds).

Since fish-acquired immune-protection against *C. irritans* was demonstrated [11,12], it has been recognized that vaccination can effectively prevent *C. irritans* parasitism [1]. In recent years, *C. irritans* immune prophylaxis has become a research hotspot, and there have been numerous basic research studies in this field [13–17]. An inactivated *C. irritans* vaccine has also been developed, which was shown to provide effective protection in fish [13,14,17].

To date, evaluating the effects of parasite vaccines has focused on specific immune responses, such as mucus or serum antibody

* Corresponding author. Tel./fax: +86 20 84115113.
E-mail address: lianxing@mail.sysu.edu.cn (A.-X. Li).
[1] Xue-Ming Dan and Tuan-Wei Zhang contributed equally to this paper.

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titers [13,14,17]; few investigations have assessed innate immunity [18]. However, the fish innate immune system plays an important role in defending against invading micro-organisms [19,20]. Several studies demonstrated that innate immune factors and immune-related genes participate in anti-parasitic immunity [19–22]. In the present study, we sought to elucidate the protective mechanisms of an inactivated *C. irritans* vaccine. Orange-spotted grouper (*Epinephelus coioides*) were immunized with vaccine containing 10,000 or 30,000 theronts per fish, and serum immobilization titers and non-specific immune factor activities (blood leukocytes respiratory burst activity, serum alternative complement activity, and serum lysozyme activity) were determined weekly. We also measured the expression levels of immune-related genes (IL-1β, TNF-α, MHC I, MHC II, and TGF-β1) in spleen and gill. Four-weeks post immunization, the fish were challenged with a lethal dose of theronts to assess the protective effects of different vaccine doses.

2. Materials and methods

2.1. Fish and parasites

Orange-spotted grouper (*E. coioids*) (36.4 ± 8.5 g, 15 ± 0.97 cm) were purchased from Daya Bay Aquaculture Center, Guangdong, China. All experiments were carried out in accordance with European Union Directive 2010/63/EU. To confirm whether the experimental fish had been infected with *C. irritans*, 10 fish were randomly selected, and serum immobilization titer was detected according to the method described in 2.4. The immobilization titer of all 10 fish was 0, confirming that they had not been previously infected by *C. irritans*. During the 2 weeks prior to experiment, the fish were habituated to the experimental conditions until eating and other activities were normal. Sand-filtered seawater was used as the culture water, with 29–32% salinity, ≥5.0 mg/l dissolved oxygen, pH of 7.9–8.3, <0.1 mg N/L nitrite content, and water temperature maintained at 27 ± 0.5 °C.

C. irritans used in the experiment was GD1 isolate stored in our laboratory, which was originally isolated from gills of five infected pompanos during an outbreak of white-spot disease in June, 2004 [23]. Parasite propagation was carried out according to a previously described method [23].

2.2. Vaccine preparation and immunization

Theronts counting and collection were carried out according to the method described by Dan et al. [23] with some modifications. Briefly, post counting, formalin was added to a concentration of 100 μl l^{-1} to inactivate the theronts. The solution was centrifuged at 2057 × g for 10 min to collect precipitate, and 0.1 M phosphate-buffered saline (PBS) was added to re-suspend the precipitate. The suspension was mixed with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) and thoroughly vortexed until a milky liquid that did not disperse in water was obtained.

Experimental fish were divided into four groups of 40 fish each. Vaccine I: each fish received an intraperitoneal (i.p.) injection with 0.1 ml vaccine containing 10,000 theronts; Vaccine II: each fish was injected i.p. with 0.1 ml vaccine containing 30,000 theronts; PBS control: each fish was injected i.p. with 0.1 ml PBS; Adjuvant control: each fish was injected i.p. with mixture of 0.05 ml PBS and 0.05 ml Freund's complete adjuvant.

2.3. Sampling

Sampling was carried out at weeks 0, 1, 2, 3, and 4 post immunization, with five fish per group each time. Prior to sampling, fish were anesthetized with 100 μl l^{-1} eugenol. We collected 0.1 ml of anticoagulated blood and 0.5 ml of non-anticoagulated blood from the tail vein of each fish. The former was used to measure blood leukocyte respiratory burst activity, and the latter was stored at 4 °C overnight, then centrifuged at 12,857 × g for 10 min to collect serum for determination of alternative complement activity, lysozyme activity, and immobilization titer. Meanwhile, the gills and spleen of each fish were collected and frozen in liquid nitrogen for RNA extraction and immune-related gene expression assessment.

2.4. Immobilization titer

Immobilization assays were carried out in 96-well tissue culture plates according to the method described by Luo et al. [14]. Briefly, 100 μl sterilized seawater was added to each well, followed by 100 μl of serum sample to the first well. The resulting solution was mixed, and 100 μl mixture was added to the second well, etc., to form serially diluted solutions. Finally, 100 μl theronts solution (containing 200 active theronts) was added to each sample-containing well. After incubation at room temperature for 1 h, the plates were observed under a microscope. The maximum dilution factor of the solutions that immobilized all theronts was determined to be the immobilization titer.

2.5. Non-specific immune factor activities

2.5.1. Respiratory burst activity

Blood leukocyte respiratory burst activity was detected according to the method described by Anderson et al. [24]. Briefly, an equal volume of 0.2% Nitro blue tetrazolium (NBT) buffer was added to 0.1 ml anticoagulated sample, and the mixture was incubated at room temperature (28 °C) for 30 min. Next, 0.05 ml of the mixture was added to a glass tube containing 1 ml dimethylformamide (DMF), then centrifuged at 8228 × g for 5 min. The supernatant was collected, and the absorbance value was detected at 540 nm.

2.5.2. Alternative complement activity

A fish ACH_{50} enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) was used for detection. In brief, 10 μl serum was added to the microtiter plates, diluted five times and incubated at 37 °C for 30 min. The plates were washed, and 50 μl HRP-conjugated anti-complement antibody was added and incubated at 37 °C for 30 min. Then the plates were washed again before chromogen solutions A (50 μl) and B (50 μl) were added, the plates were developed at 37 °C for 15 min, and the reaction was quenched with stop solution. Absorbance was measured at 450 nm, and sample complement activities were determined by comparing absorbance with a standard curve.

2.5.3. Lysozyme activity

Lysozyme activity was detected by with an LZM test kit (Nanjing Jiancheng Bioengineering Institute, China). In brief, 100 μl distilled water, standard liquid, and serum samples were added to 1 ml bacteria solution, mixed, incubated in a 37 °C water bath for 15 min, then immediately transferred to an ice bath for 3 min. Then, the solution was transferred into a 1-cm optically clear colorimetric dish for the transmittance (T_{15}) determination at 530 nm. The transmittance of the distilled water at 530 nm was adjusted to 100% before the detection. Lysozyme content of the samples was calculated by using the following formula:

Lysozyme content (μg/ml) = $(UT_{15} - OT_{15})/(ST_{15} - OT_{15})$ × standard concentration (100 μg/ml is namely 2000 U/ml) × sample dilution factor, where UT_{15} is test tube transmittance, OT_{15} is blank tube transmittance, and ST_{15} is standard tube transmittance.