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Different concentrations of *Edwardsiella tarda* ghost vaccine induces immune responses *in vivo* and protects *Sparus macrocephalus* against a homologous challenge

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ABSTRACT

Bacterial ghosts (BGs) can be generated by the controlled expression of the *PhiX174* lysis gene E in gram-negative bacteria. They are intriguing vaccine candidates since ghosts retain functional antigenic cellular determinants often lost during traditional inactivation procedures. Here we prepared *Edwardsiella tarda* ghost (ETG) and tested different concentrations in vaccination trials. The results showed that serum IgM antibody titers were significantly higher in three different concentration immunization groups than control group ($P < 0.05$), However, there was no significant ($P > 0.05$) difference between the immunized groups. The phagocytic percentage (PP) was significantly higher ($P < 0.05$) in ETG immunized groups than in the control group from 3 days post-treatment. The PP continued to rise with time until day 21, when the values of three ETG immunized groups were 45.7%,51.2% and 50.7%, respectively. In addition, phagocytic index (PI) was significantly higher ($P < 0.05$) in ETG immunized groups than in the control group after 7 days post-treatment. However, there was no significant ($P > 0.05$) difference of PP or PI between immunized groups. In addition, non-specific immune immunity, such as acid phosphatase, alkaline phosphatase, superoxide dismutase and lysozyme activities displayed a similar pattern in all immunized groups, all immunized fish showed significantly higher activities than control group fish ($P < 0.05$). Most importantly three ETG immunized groups were all significantly more protected against the *E. tarda* challenge (19/25, 76% survival), (21/25, 84% survival) and (20/25, 80% survival) respectively, compared to (9/25, 36% survival) survival in the control group, but there was no significant ($P > 0.05$) difference of survival rate (SR) or relative percent survival (RPS) between immunized groups. All these results suggest that an ETG could stimulate cellular and humoral immunity, and could be used as a vaccine candidate in *S.m*. In summary, ETG can protect fish from Edwardsiellosis, and there is no significant difference in SR and RPS when three different concentrations of ETG are used, so it can easily be developed as a vaccine for mechanical and artificial operations.

1. Introduction

Sparus macrocephalus (*S.m*) belongs to Sparus, and is mainly distributed in the western Pacific, extending from Hokkaido, Japan in the north to Taiwan and Hainan Island in the south of China. It is an economically important fish cultured in parts of Asia including Japan, Korea, China, and some other countries of Southeast Asia [1]. With the scale development of the aquaculture, the frequent occurrence of diseases has become one of the key points which restricts the development of aquaculture industry. It has been confirmed that *Edwardsiella tarda* (*E. tarda*), is the main pathogen of *S.m* [2]. As we know, *E. tarda* is the intracellular, rod-shaped Gram-negative, non-capsulated, motile, facultative anaerobic bacteria, and is widely distributed in aquatic environments and is infectious to a variety of animals including humans, fish, amphibians, reptiles, and birds [3].

In recent years, chemotherapy has been used effectively in

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controlling fish infections [4], however, there is significant concern regarding food safety following chemotherapeutic interventions in addition to the danger of selecting for antibiotic-resistant *E. tarda* isolates which have been reported worldwide [5]. These concerns have prompted the development of novel vaccination strategies for the control of *E. tarda* infections. In order to better prevent and control the infection of *E. tarda*, a biological method, such as vaccines, must be developed to control it.

Over the past ten years, vaccines have become one of the most important methods to prevent the infection of fish and other animals [3]. As it is reported, vaccines for prevention of *E. tarda* are various and show different protective efficiency. These vaccines, in common use, are formalin treated or heat-inactivated, effecting the physical and chemical structure of bacterial surface antigen, changing the original anti immunogenicity and affecting the immune effects of vaccine [6,7]. In recent years, the bacterial ghost vaccine, a new type of vaccine, has attracted the considerable attention of research workers. Bacterial ghosts may be generated by the controlled expression of the *PhiX174* lysis gene E in Gram-negative bacteria to form the free cytoplasm and reproduction of bacterial shell. Thus it preserves the original bacterial cell morphology, bacterial surface antigen, and adhesion properties. At the same time, bacterial ghosts contain LPS, adipose, peptidoglycans, and other natural immunostimulating complexes. It can induce stronger humoral immunity, cellular immunity, and mucosal immune response without the use of the adjuvant [3].

Although studies have reported the superiority of *E. tarda* bacterial ghost vaccine at home and abroad, their non-specific immune parameters have not been reported [3,8]. In this study, we analyze changes in specific and non-specific immune parameters in fish vaccinated with different concentrations of bacterial ghost vaccine, using *S.m*. These immunological techniques and concentration selection would be useful in the development of *E. tarda* ghost vaccine. The protective ability of *E. tarda* ghost vaccine was investigated on *S.m* to provide the theoretical basis for the application of *E. tarda* ghost vaccine and to provide new ideas for the immunization of marine fish bacterial diseases.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. tarda strain SM (GenBank accession number MH390702) was isolated from *S.m* which were cultured in the net cage in the Yueqing Gulf [2]. *E. tarda* was grown in tryptic soy broth (TSB) (Oxoid Ltd., Basingstoke, Hampshire, England) at 28 °C. Transformed *E. tarda* cells were grown in TSB containing 100 μg/ml ampicillin (Sigma, MO, USA) at 28 °C. Incubation temperatures for repression and expression of the lysis gene during transformation were 28 °C and 42 °C, respectively.

2.2. Production of bacterial ghost vaccine

Bacterial ghost vaccines were generated following our earlier research paper using the same method [3]. Morphological features of *E. tarda* and ETG were examined by scanning electron microscopy (Hitachi S-2400) and transmission electron microscopy (7650; Hitachi) as previously described [3]. There were 1×10^{10} cells per milliliter of ETG vaccine were prepared.

2.3. Immunization protocol and challenge infection

S.m were fed in Zhejiang Marine Fisheries Research Institute. They were the same size and had no diseases, their weight was 150 g ± 10 g. These fish were fed daily using the compound feed bought from Fuzhou Haima Feed Co. Ltd. The water was changed every morning and the water was kept running for 1 h. The temperature of the water was at about 24 °C. The final experiment was carried out after two-weeks of feeding observation.

Fish were divided into four groups; three immunization groups (A to C groups were respectively immunized with ETG at 1×10^5 CFU/fish, 1×10^6 CFU/fish, 1×10^7 CFU/fish, n = 60 for each group), and control fish (group D), treated with phosphate-buffered saline (PBS), respectively. Before the experiment, fish were not fed for 24 h. Fish in immunization groups were intraperitoneally injected immunization with 0.2 ml bacterial ghost vaccine while fish in the control group were injected with 0.2 ml saline. Immune responses were measured after 0, 3, 7, 14, 21, 28 days of treating. From each group, six fish were taken to draw blood from the caudal vein (on day 0, two fish were taken randomly from each group before treatment). The blood of each fish was divided into two parts. One part of the blood was allowed to stand at room temperature for 2 h and was centrifuged at 4000 r/min for 10 min at 4 °C. After that, the upper serum was collected for the determination of serum antibody level. The other part of blood was made to the anticoagulant with heparin for the determination of phagocytic activity of blood leucocytes.

During the experiment, the water was controlled at about 24 °C, salinity at 25–26, pH 8.2, and the air was continuously aerated.

2.4. Phagocytic activity

Staphylococcus aureus was inoculated into common broth agar slants for 24 h, inactivated by 0.5% formaldehyde for 24 h, washed with sterile saline 3 times, adjusted to 1.0×10^8 CFU/fish and stored in 4 °C [10]. The phagocytosis was used as the phagocytic activity of leukocytes, which was determined using the same method following our earlier research [9]. Then 100 μL of anticoagulant was added to 100 μL of *S. aureus*. Then shacked it and put it into the water at 25 °C for 60min with shacking once every 10 min. After this, the mixture was drawn with a pipette on the slides, dried and fixed with methanol for 10 min, and then Giemsa stained for 1 h. Finally, slides were washed and dried to observe with an oil microscope.

The phagocytic percentage (PP) and phagocytic index (PI) were calculated according to the following equations.

$$\mathrm{PP} = \frac{\text{The number of cells involved in phagocytosis in one hundred phagocytes}}{100} \times 100\%$$

$$\mathrm{PI} = \frac{\text{The total number of bacteria in phagocytes}}{\text{The number of cells that had phagocyted bacteria}} \times 100\%$$

2.5. Antibody response assessment

The antibody level was determined by an enzyme-linked immunosorbent assay (ELISA) following our earlier research [3]. Briefly, 100 μL carbonate-bicarbonate buffer (pH 9.6) containing 1×10^5 formalin-killed *E. tarda* were added to respective microtiter plate wells and incubated for 20 h at 37 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 24 h at 4 °C with blocking buffer (0.5% BSA in PBS-T). After the plate was washed three times, sera from immunized and control fish was added to the plates, then incubated at 37 °C for 1 h and washed 3 times with PBS-T, then probed with 100 μL self-made rabbit anti-sparus-IgM antibody incubated at 37 °C for 1 h, and washed 3 times with PBS-T, added the horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at 37 °C. Plates were washed four times with PBS-T and binding visualized by adding TMB (Tiangen, Beijing, China) according to the manufacturer's instructions (100 μL/well). The plates were incubated at room temperature for 20 min and the reaction stopped with 100 μL of 2 M H_2SO_4 and the absorbance read at 450 nm.

2.6. Non-specific immune parameters assay

Acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD) and lysozyme (LZM) activity were determined at 0, 3,