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Dynamic distribution of formalin-inactivated *Edwardsiella tarda* in olive flounder (*Paralichthys olivaceus*) post intramuscular injection

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ABSTRACT

Intramuscular (i.m.) injection is one of the common delivery methods of vaccination in aquaculture, which could induce an ideal immune protection to fish. In the present study, the olive flounders (*Paralichthys olivaceus*) were injected intramuscularly with 200 μl of three concentrations of formalin-inactivated *Edwardsiella tarda* bacterin (10^7, 10^8, 10^9 CFU ml^{-1}) to investigate the transportation and dynamic distribution of antigen uptake in tissues by absolute real-time quantitative PCR (qPCR). The amount of uptaken antigen increased firstly, and then decreased. The peak occurred first in the blood at 6–9 h after i.m. injection, and in the spleen and head kidney at 9–15 h, then in the liver, gill and muscle at 15–24 h, finally in the skin and intestine at 36 h. The amount of uptaken antigen was highest in the head kidney, followed by in the spleen, blood, gill, and liver, and lowest in the muscle, skin and intestine. Among the three dose groups, the amount of uptaken antigen in all tested tissues became higher with the increasing dose of injected bacterin. Moreover, the tissue distribution of antigen uptake was investigated by indirect immunofluorescence assay (IIFA) at 15 h after i.m. injection with 200 μl of 10^8 CFU ml^{-1} *E. tarda* bacterin. The distribution of antigen was mainly observed in the head kidney, then in the spleen, blood, liver, gill and muscle, and least in the skin and intestine, which correlated with the results of absolute qPCR detection. Furthermore, the expression levels of MHC Iα, MHC IIα, CD4-1 and CD8α were detected by RT-qPCR. The expression of these four genes peaked highest in the head kidney, followed by in the spleen, liver, blood and gill, and lowest in the muscle, skin and intestine, and the levels increased in parallel with the increasing dose of injected vaccine. All these results provided an important insight into the dynamic transportation of antigen uptake, and also deepened the understanding of immune response to the i.m. injection.

1. Introduction

Edwardsiella tarda is a Gram-negative intracellular pathogen, causing a systemic disease called edwardsiellosis, which has resulted in enormous economic losses in cultured seawater and freshwater fish around the world (Xu and Zhang, 2014). Currently, vaccines are considered as one of the most economic, safety, environmentally friendly strategy to prevent this disease, and a number of highly successful vaccines have been developed, for example, live attenuated vaccine, natural avirulent strain vaccine, formalin-killed vaccine and ghost bacteria vaccine (Lan et al., 2007; Takano et al., 2010; Castro et al., 2008; Yamasaki et al., 2015; Kwon et al., 2007). Nowadays, immersion, oral and injection are the common used method to deliver these different type of vaccines in aquaculture. Immersion immunization is probably the simplest method of vaccination with minimum damage, but the immune responses are less robust. Oral immunization is stress-free and not labor intensive, but the vaccine is easily damaged. In contrast, intramuscular (i.m.) injection vaccination offers a regional delivery advantage in fish, which could evoke adaptive immune response and accomplish immunological memory (Plant and Lapatra, 2011). However, the knowledge about the dynamic change and the transportation of antigen in teleost fish post i.m. injection is insufficiently elucidated.

It has been reported that the amount of antigen uptake in tissues will affect the effectiveness of vaccination, and which could be an ideal indicator for evaluating the effect of vaccination method (Nakanishi et al., 2002). Therefore, the study of the transportation and dynamic change of antigen will contribute to the understanding of the antigen uptake mechanism and the optimization of vaccination strategy. In the recent year, antigen uptake has been investigated using different type of antigens including lipopolysaccaride (LPS) and protein of bacteria, inactivated or live bacteria, live attenuated bacteria and alum-precipitated bovine serum albumin (AP-BSA) (Stensvåg et al., 1999; Khimmakthong et al., 2013; Liu et al., 2014; Ototake and Nakanishi,

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1992). Several methods like absolute quantitative PCR (qPCR), plate culture, electron microscopy and immunohistochemistry have been employed to detected the antigen uptake (Gao et al., 2016; Zapata et al., 1987; Vervarckea et al., 2005; Khimmakthong et al., 2013). In mammals, when vaccine was intramuscularly administrated to organisms, the antigen was taken up by cells at the administration site firstly, and then be redistributed to cells and lymph with blood circulation (Dupuis et al., 2000; Winegar et al., 1996). A major part of the vaccine was cleared and degraded, and a very small amount of the administered vaccine was distributed in another tissues. In teleost fish, the research of the antigen uptake was mainly on the tissue distribution. However, little was known concerning the fate of the vaccine and the dynamic change of antigen. Therefore, it is of greatest importance to investigate the antigen transportation and dynamic distribution in the major immunological organs to have a better understanding of the antigen uptake mechanism.

In the present study, an absolute qPCR was developed to investigate the dynamic change of uptaken antigen in various tissues of olive flounder after i.m. injection with 200 μl of three concentrations (10^7, 10^8, 10^9 CFU ml^{-1}) of formalin-inactivated *E. tarda* vaccine. Moreover, indirect immunofluorescence assay (IIFA) was used to examine the distribution of antigen in tissues. Furthermore, a reverse transcription quantitative PCR (RT-qPCR) was developed to determine the expression levels of the four antigen presentation-related immune genes (MHCIα, MHCIIα, CD4-1 and CD8α).

2. Materials and methods

2.1. The experimental fish

Healthy olive flounders (*Paralichthys olivaceus*) with an average body length of 15–17 cm were obtained from a fish farm in Rizhao, Shandong province, China. The fish were reared in the laboratory with running dechlorinated and aerated water (temperature 20 ± 1 °C, dissolved oxygen concentration 6.5 ± 0.5 mg L^{-1}, water salinity 28 ± 1%) and fed daily with standard commercial pellets. After two weeks acclimation, the olive flounders were randomly sampled for microscopic and bacteriological examination to guarantee that the all the experimental fish were not infected by *E. tarda*.

2.2. Preparation of inactivated E. tarda bacterin and rabbit anti-E. tarda polyclonal antisera

The *E. tarda* strain HC01090721 was originally isolated from the ascites of olive flounder (Tang et al., 2010). *E. tarda* was inoculated onto brain heart infusion (BHI) agar plate at 37 °C for 48 h, and then single clones were selected and transferred into BHI-broth for shaking incubation for 24 h at 37 °C. After reaching the stationary phase (OD_{600} = 1.0), the bacteria were harvested and washed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and inactivated with 0.5% formalin (V/V) for 96 h at 4 °C. The safety of the inactivated *E. tarda* was checked by culturing the cells on BHI agar for 3 days at 37 °C. The inactivated *E. tarda* cells were washed three times with PBS and then pelleted by centrifuging at 8000 × g for 10 min. After the last wash, the concentration of bacteria was adjusted to 1.0×10^{10} CFU ml^{-1} for later use.

The formalin-inactivated *E. tarda* was used as immunogen to prepare the rabbit anti-*E. tarda* polyclonal antisera as described previously (Hong et al., 2009). The rabbit was immunized four times, and when the antibody titer reached to 1:20,000-50,000, the polyclonal antiserum was taken by drawing blood from heart under anesthesia. Meanwhile, the healthy rabbit serum was also obtained as control. Rabbit IgG was purified using protein G-agarose fast flow column (Sigma). Protein concentration and purity of the IgG fraction were estimated using BCA protein assay kit (Novagen) and SDS-PAGE, respectively.

2.3. Vaccination and sampling

The olive flounders were randomly divided into four groups, and each group contains 60 fish. In the three vaccination groups, the fish were injected with 200 μl of three concentrations (10^7, 10^8, 10^9 CFU ml^{-1}) of formalin-killed *E. tarda* bacterin by the intramuscular route in the dorsal muscle block, lateral to the dorsal fin. In the control group, the fish were intramuscularly injected with equal volume of PBS. Prior to sampling, the olive flounders were anesthetized with 300 ng ml^{-1} MS-222 (Sigma, USA) for 15 min. At 15 h post i.m. injection, 3 fish were randomly sampled from 10^8 CFU ml^{-1} group and PBS group. The intestine, skin, spleen, head kidney, liver, muscle and gill were collected and embedded in frozen section compound (Surgipath FSC22, Leica) and stored at −80 °C for IIFA. At 0, 3, 6, 9, 15, 24, 36, 48, 72 and 96 h post i.m. administration, 6 fish were randomly sampled from each group. After collecting blood from vena caudalis with heparinized syringes, the intestine, skin, muscle, gill, spleen, head kidney and liver were sampled and washed three times with sterilized PBS and placed into tubes containing RNAlater® (Ambion, Austin, Texas). The muscle was sampled at the dorsal muscle, 2 cm away from the injection site. All samples were stored at −80 °C until RNA and DNA extraction. The fish used in this study was carried out strictly in line with procedures in the Guide for the Use of Experimental Animals of Ocean University of China. In this study, the methods used in the animal experiments were approved by the Instructional Animal Care and Use Committee of the Ocean University of China (permit number: 20150101). All possible effort was dedicated to minimizing suffering.

2.4. DNA and cDNA preparation

Total DNA was extracted from each tissue using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions, and adjusted to 100 ng μl^{-1} using a NanoDrop ND-8000 (Thermo Scientific, USA).

Total RNA was extracted from each tissues by Trizol (Sigma, USA) according to the manufacturer's instructions. The concentration of RNA was detected by NanoDrop ND-8000 spectrophotometer, and the integrity of RNA were evaluated by 1.5% agarosegel. To get rid of gDNA (genomic DNA), 1 μg total RNA was incubated with 1 unit DNase I for 15 min at 37 °C. The cDNA single strand was synthesized by using Reverse Transcriptase M-MLV kit (TaKaRa, China) and stored at −20 °C for RT-qPCR.

2.5. Detection of uptaken E. tarda in different tissues

The absolute qPCR standard curve was established for quantifying the uptake of *E. tarda* in sampled tissues of olive flounder post i.m. injection described by Gao et al. (2016). 2 μg DNA was extracted from 3.3×10^8 *E. tarda* cells, and diluted in 10-fold serial dilution series as templates to create the standard curve for quantification. The absolute qPCR assay was performed in triplicate for each dilution using Roche480 real-time PCR system (LightCycler480, USA). Reaction mixtures (20 μl) containing 10 μl of SYBR Green (Roche, Sweden), 0.4 μl of forward and reverse primers (Table 1), 7.2 μl of RNase-free water and 2 μl of *E. tarda* DNA. The Ct values of each tissue were measured and converted to the initial counts of inactivated *E. tarda* using the following equation: $y = -3.515\ x + 43.78$ ($R^2 = 0.994$), Which was obtained based on the linear equation $y = a*x + b$ where y is Ct value and x is the logarithm of initial template quantity. The final values were converted into the amount of *E. tarda* cells in 1 μg total DNA extracted from each tissue.

2.6. Tissue cryosections and IIFA

Continuous 6 μm thick frozen sections of tissues selected from 10^8 CFU ml^{-1} group and PBS group were cut using a Leica CM1900 cryostat