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Comparative analysis of adaptive immune response after vaccine trials using live attenuated and formalin-killed cells of *Edwardsiella tarda* in ginbuna crucian carp (*Carassius auratus langsdorfii*)

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ABSTRACT

Edwardsiella tarda is an intracellular pathogen that causes edwardsiellosis in fish. Although vaccine trials with formalin-killed cells (FKC) have been reported, the vaccinations failed in protect against *E. tarda* infection. On the other hand, a live attenuated vaccine strategy is effective against edwardsiellosis; however, the mechanism underlying its effectiveness in fish is unclear. In the present study, we compared the adaptive immune responses in fish vaccinated with FKCs and live attenuated vaccines to elucidate the induction of adaptive immune responses following vaccination. After challenge with *E. tarda*, live cell (LC)-vaccinated fish showed high survival rates, high IFN-g and T-bet gene expression levels, and increased cytotoxic T lymphocytes (CTLs). In contrast, all FKC-vaccinated fish died following *E. tarda* infection. In addition, FKC vaccination induced high IL-4/13A and IL-10 expression levels and increased antibody titers, whereas Th1-like responses were suppressed. These results indicate that LC vaccination contributes to protection against *E. tarda* infection by inducing cell-mediated immunity (CMI). Thus our study findings could contribute to the development a vaccine that induces CMI against edwardsiellosis.

1. Introduction

Vaccination is one of the most effective methods for preventing diseases in fish. Currently, a number of vaccines are commercially available for use in the aquaculture industry. Although vaccines are expected to induce long-lived, lymphocyte-mediated immunoprotection in fish, little is known about the basic protective mechanisms elicited by fish immunization. The efficiency of a vaccine should not only be evaluated in terms of protection, but also in terms of its ability to elicit humoral and cell-mediated immune responses to target antigens [1]. Thus far, vaccine efficacy in fish has been evaluated on the basis of the relationship between antibody titer and survival rate during a vaccination challenge. However, accurate evaluation of vaccines is difficult in diseases in which cell-mediated immunity plays an important role.

Edwardsiellosis, caused by the bacterium *Edwardsiella tarda*, is a serious problem in cultured fish. Since the control of *E. tarda* using antimicrobial drugs is difficult, development of a vaccine that prevents edwardsiellosis is an important issue in aquaculture. Although vaccine trials with formalin-killed cells (FKCs) have been reported, the vaccinations were ineffective in protecting against *E. tarda* infection [2,3]. *E. tarda* is an intracellular parasitic bacterium, not only infects epithelial cells [4] but also resists the phagocytic and bactericidal activities of the phagosome [5–9]. Since antigen-specific antibodies are only effective in an extracellular environment, they are incapable of eliminating intracellular pathogens. On the other hand, cell-mediated immune mechanisms can eliminate pathogen-infected cells by inducing cell death, thereby removing the niche for intracellular pathogen replication [10]. Moreover, cell-mediated immune mechanisms also induce the bactericidal activity of macrophages. In mammals, CMI plays a

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major role in protection against intracellular bacterial infections [11–13]. Furthermore, our recent study indicated that CMI plays a crucial role in protection against intracellular bacterial infections in fish [14].

In fish, a live attenuated vaccine strategy is effective against intracellular bacterial infections such as those caused by *Edwardsiella ictaluri*, *Aeromonas salmonicida*, and *Aeromonas hydrophila* [15–17]. In addition, the survival rate of live attenuated *E. tarda* (LC)-vaccinated fish was higher than that of FKC-vaccinated fish after *E. tarda* infection [3]. However, the induction of cell-mediated immunity against *E. tarda* infection is unclear in LC- as well as FKC-vaccinated fish.

The triploid ginbuna crucian carp (*Carassius auratus langsdorfii*) is a naturally occurring gynogenetic fish that could be a potential model for investigating CMI in teleost fish [18]. The CMI- and humoral immunity-related genes such as T-bet, GATA-3 and IFNγ have been characterized in ginbuna crucian carp [19–21]. In addition, monoclonal antibodies (mAbs) against cell surface markers of CTLs and helper T cells (CD8α and CD4, respectively) have also recently been developed [22,23].

In the present study, we compared the adaptive immune responses between fish vaccinated with FKCs and live attenuated vaccines as a challenge in order to elucidate the induction of immune response following vaccination. We believed that this study would facilitate vaccine development for protecting fish against intracellular pathogens, such as *E. tarda*, *Mycobacterium* sp., and *Nocardia seriolae*.

2. Materials and methods

2.1. Fish

Clonal triploid ginbuna crucian carp (*C. auratus langsdorfii*), from the island of Okushiri, Japan (OB1 clone), were obtained from the National Research Institute of Aquaculture, Japan. The fish (weighing 5–30 g) were maintained in running water at a temperature of 25 °C ± 1 °C, and were fed commercial pellets on a daily basis.

2.2. Bacteria

The *E. tarda* FPC498 strain was isolated from the ascites of a naturally infected Japanese flounder (*Paralichthys olivaceus*). The bacteria were incubated for 48 h at 25 °C in tryptic soy agar (TSA; Nissui, Tokyo, Japan) before experimental use.

The low virulent *E. tarda*, SPM31 strain was made from a wild type virulent *E. tarda* FPC498 strain by using a pCHR81, a suicide delivery plasmid for derivation of the Tn5 transposon [3]. The bacteria were cultured for 48 h at 25 °C in TSA containing 50 μg/ml kanamycin before using it for vaccination.

For FKC preparation, *E. tarda* FPC498 strains were incubated with 0.5% formalin in phosphate buffered saline (PBS, pH7.2) for 24 h at 4 °C. FKCs were washed thrice with PBS and adjusted to a final volume of 1×10^9 CFU/ml in PBS. The suspensions were streaked on TSA for sterility check and stored at 4 °C until use.

2.3. LD_{50} determination

Fish were infected with an intraperitoneal (i.p.) injection of *E. tarda* FPC498 (10^5–10^8 CFU/100 g body weight [BW]) or SPM31 (10^5–10^8 CFU/100 g BW) in PBS. The survival rate was recorded daily for 4 weeks post-injection. The median lethal dose (LD_{50}) was calculated using the method described by Reed and Müench [24].

2.4. Vaccination and challenge

The experimental groups of 6 fish each were intraperitoneally vaccinated with 1×10^8 CFU/100 g BW of FKC or 1×10^7 CFU/100 g BW (0.2 LD_{50}) of the SPM31 strain (live cell; LC). A control group of 6 fish was injected with 1 ml/100 g BW of PBS.

The challenge was performed 30 days post vaccination. All experimental 6 fish were challenged with 1×10^7 CFU/100 g BW (20 LD_{50}) of *E. tarda* FPC498. Dead fish were observed for clinical signs and bacteria were isolated from their kidney, spleen, and hepatopancreas.

2.5. Low-dose Edwardsiella tarda infection and preparation of serum and kidney leukocytes

Low-dose *E. tarda* infection was induced 30 days post-vaccination. FKC- or LC-vaccinated and PBS-injected fish were injected with 1×10^5 CFU/100 g BW (0.2 LD_{50}) of *E. tarda* FPC498. Kidney and peripheral blood were collected on various days after the experimental challenge. The peripheral blood was centrifuged at 500 × *g* for 10 min at 4 °C to obtain serum as a supernatant. Kidney leukocytes (KLs) were collected as previously described [25].

2.6. Real-time PCR

Total RNA was extracted from KLs (5×10^6 cells) using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First-strand cDNA was synthesized from purified total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Real-time PCR was performed using specific primer sets (Table 1) for the genes IFN-γ1 (*ifng1*), IFN-γ2 (*ifng2*), IL-4/13A (*il4/13a*), IL-4/13B (*il4/13b*), T-bet (*tbx21*), GATA-3 (*gata3*), IL-10 (*il10*), and for the housekeeping gene, EF1α (*eef1a*). Real-time PCR, using a Mini Opticon™ System (Biorad, Hercules, CA), was performed with 5 μl of 5-fold diluted cDNA, 10 μl of GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), and 200 nM primer sets for IFNγ1, IFNγ2, IL-4/13A, IL-4/13B, T-bet, GATA-3, IL-10 and EF1α, each, in 20 μl mixtures. The amplifications were performed as follows: 3 min at 95 °C followed by 50 cycles of 10 s at 95 °C and 10 s at 60 °C. A standard curve was generated by plotting the threshold cycle (ct) versus known copy number for each diluted plasmid template. A normalized target gene expression level was calculated by dividing the amount of target gene by the amount of EF1α as an endogenous control.

Table 1
PCR primers used for real-time PCR analysis. Q1

Gene	Primer	Sequence (5′ → 3′)	Accessions
eef1a	EF1α-F1	CGGCAGCTTCAATGCTCAGGTCATC	AB491676
	EF1α-R1	ACGACGGTCGATCTTCTCCTTGA	
ifng1	IFNγ1-F1	TGGGCGATCAAGGAAGATG	AB570431
	IFNγ1-R1	ACTTCAGATTTTTGGTGTTTTTGG	
ifng2	IFNγ2-F1	GCAGAAACCCTTTGGTCG	AB570432
	IFNγ2-R1	GTTCTTCAGGTTTCTCAGCTGTGT	
il4/13a	IL-4/13A-F1	GAGAAATGAAACCTTCGTGACAG	LC010113
	IL-4/13A-R1	ACAGCCCAGAAACCTCCTTT	
il4/13b	IL-4/13B-F1	CAGCATGCTACACAGACGAC	LC010114
	IL-4/13B-R1	CAAGGAAAACATCCATTCTGTG	
il10	IL-10-F1	TCAAAAACACCAATCAACTCCA	LC008369
	IL-10-R1	GGCGAACTCAAAGGGATTTA	
tbx21	T-bet-F1	CAGCTGAAAATAGATCATAACCCTTT	AB290187
	T-bet-R2	GGTTGGGGAAGGCGTAAG	
gata3	GATA-3-F1	ACAAGATGAACGGCCAGAAC	AB302073
	GATA-3-R1	GTGGTCGTTTGACAGTTTGC	

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