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NOTE

Efficacy of oral vaccine against bacterial coldwater disease in ayu *Plecoglossus altivelis*

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ABSTRACT: The development of a practical vaccination method against bacterial coldwater disease (BCWD) in ayu *Plecoglossus altivelis* and the efficacy of oral administration of formalin-killed cells (FKCs) of *Flavobacterium psychrophilum* was investigated. The FKC was administrated at a dose of 0.1–0.2 g kg^{-1} body weight to juvenile ayu (0.5 g body weight) every day for 2 wk or on 5 days over 2 wk. Experimental immersion challenge at 3 and 7 wk after vaccination showed significantly higher survival rates than the controls. The results show the effectiveness of oral vaccination against BCWD in ayu.

KEY WORDS: Oral vaccine · Immunization · Ayu · *Flavobacterium psychrophilum* · Bacterial coldwater disease · BCWD

Flavobacterium psychrophilum, the causal agent of bacterial coldwater disease (BCWD), was first isolated from juvenile coho salmon *Oncorhynchus kisutch* in the USA in 1948 (Borg 1960). The isolation of *F. psychrophilum* from ayu *Plecoglossus altivelis* was reported from cultured and wild fish (Wakabayashi et al. 1994). The bacterium was also isolated from wild pale chub *Zacco platypus* (Iida & Mizokami 1996). The bacterium occurs in almost all freshwater areas in Japan, and drug-resistant *F. psychrophilum* causes serious management problems for fish farmers (Kondo et al. 2001a). To prevent the disease, vaccination by injection has been reported (Obach & Laurencin 1991, Rahman et al. 2000, LaFrentz et al. 2002). However, vaccinating fish by injection is not practicable because about 1000 t of juvenile ayu are released into Japanese rivers every year, and the fish are sensitive to handling treatment. In this study, the possibility of oral vaccination against *F. psychrophilum* in ayu is investigated.

Materials and methods. ***Preparation of vaccine:*** *Flavobacterium psychrophilum* strain G3724, isolated in 1998 from a diseased ayu in Tokushima Prefecture, Japan, was used. The bacterium was passed in ayu 3 times to increase the virulence and was stored in 10% skimmed milk at –75°C. The bacterium was cultured in 50 ml modified cytophaga (MCYT) broth (0.2% tryptone, 0.05% yeast extract, 0.02% beef extract, 0.02% CH_3COONa, 0.02% $CaCl_2$) at 15°C for 48 h on a rotary shaker at 100 rpm, and then 2.5 ml of the culture was inoculated into 1000 ml MCYT broth in a 2000 ml Sakaguchi-flask, which was incubated at 15°C and shaken at 100 rpm. The bacteria were cultured to the logarithmic phase, and inactivated at 15°C for 48 h by adding 0.3% formalin. The formalin-killed bacterial cells (FKC) were harvested by centrifugation at 8000 ¥ *g* for 40 min. The precipitate, including FKC, was resuspended at a concentration of 0.1 g FKC per ml of saline containing 0.3% formalin and was stored at 4°C.

Fish and vaccination: Test fish, juvenile ayu of an average weight 0.5 g and 75 d post-hatching, were provided by a hatchery of the Kochi Freshwater Fisheries Association. Approximately 2000 fish in each group were reared in 2 t tanks (3.0 ¥ 1.0 ¥ 0.7 m) with well-aerated flowing water at 16 to 18°C. Fish were fed 0.5 mm commercial dry pellets (Maruha) corresponding to 4% of the fish body weight d^{-1} for the entire experiment. Two groups were immunized and another group was used as a non-immunized control. The fish were immunized by feeding the dry pellets mixed with the vaccine at a rate of 0.1 to 0.2 g FKC per kg fish body weight per day (Fig. 1). Control fish were fed with the dry pellets mixed with sterile physiological saline equal to the volume of vaccine. Group 1 was fed vaccine every day for 2 wk (15 times); Group 2 was fed vaccine on 5 days over 2 wk (Table 1).

Challenge test: Challenge tests were done by the immersion infection method at 3 wk (Challenge 1) and 7 wk (Challenge 2) after the immunization. The fish were immersed in aerated well water containing bac-

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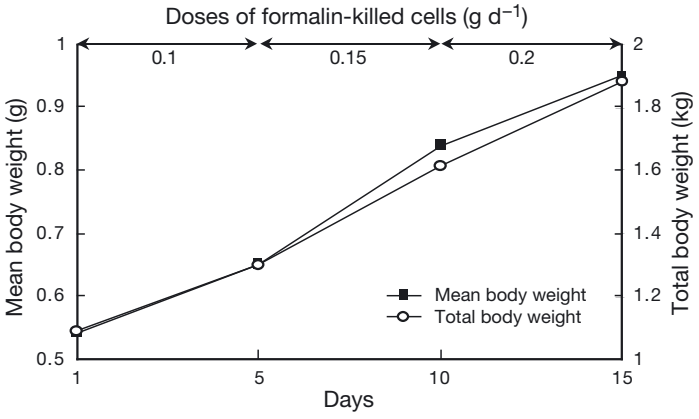

Fig. 1. Body weight of ayu *Plecoglossus altivelis* and doses of formalin-killed cells of *Flavobacterium psychrophilum* for administration periods

teria in a logarithmic culture phase at concentrations of 2.1×10^7 and 1.4×10^8 CFU ml^{-1} at 15 to 16°C for 1.0 h. After infection, the fish were reared in 200 l tanks with well-aerated flow water at 15 to 16°C, and the mortality was recorded. Dead fish were examined for external signs of BCWD. Affected body sites were smeared, and infection diagnosed using a fluorescent antibody technique with anti-*Flavobacterium psychrophilum* G3724 rabbit serum. The significance in the challenge was calculated by the chi-square test.

Results. Changes in the survival rate in the 2 experimental groups at 3 and 7 wk after the oral vaccination are shown in Fig. 2. Mortality began from 1 to 3 d after infection. The first challenge was 3 wk after vaccination. When infected at a dose of 4.4×10^7 CFU ml^{-1}, Groups 1, 2 and the control showed 94.1, 96.6 and 69.2% survival rates, respectively, after 10 d (Table 2). Groups 1, 2 and the control showed 53.5, 91.7 and 34.7% survival rates, respectively, at the infection dose of 1.2×10^8 CFU ml^{-1}. In the challenge at 7 wk after vaccination, Groups 1, 2 and the control showed 86.6, 88.1 and 76.4% survival rates, respectively, at the infection dose of 2.1×10^7 CFU ml^{-1}, and 76.5, 78.8 and 42.2% survival rates, respectively, at the infection dose of 1.4×10^8 CFU ml^{-1}. The survival rates in the immunized groups were significantly different ($p < 0.01$) by the chi-square test compared with the control groups. All the dead fish showed 1 or more typical signs of BCWD, including haemorrhage in the lower part of operculum, lack of lower jaw, partially eroded or lack of caudal fin edge, and lesion in the peduncle of the caudal fin (Fig. 3).

Discussion. In the challenge test, bacteria in the logarithmic culture phase were used because this phase showed higher virulence than other phases (Kondo et al. 2001b). The results showed that vaccination by either method gave significantly higher survival rates than the control in both challenge tests. However, in the challenge at 3 wk after vaccination, the group with daily administration showed an apparently lower survival rate than the other vaccinated fish. This group always showed lower rates than the group of administration 5 d for 2 wk. This may be caused by immunological tolerance as reported for carp and channel catfish (Joosten et al. 1995, Patrie-Hanson & Ainsworth 1996). Dunier & Siwicki (1993) cautioned about organic pollutants in aquaculture environments inducing immunotoxicity in fish. Immunological tolerance and stress due to toxic substances included in the vaccine as formalin are to be investigated further.

Protection mechanisms in ayu after oral vaccination against vibriosis have been investigated (Kawai et al. 1981). A challenge test by intramuscular injection of

Table 1. Profiles of oral vaccination, feeding *Flavobacterium psychrophilum* FKC (formalin-killed bacterial cells) to ayu *Plecoglossus altivelis*. Group 1 was fed vaccine every day for 2 wk; Group 2 was fed vaccine on 5 days over 2 wk

Group	Approx. no. of fish	Mean body weight (g)	Administration dose (FKC g^{-1} kg^{-1} d^{-1})	No. of administrations over 2 wk
1	2000	0.5	0.1–0.2	15
2	2000	0.5	0.1–0.2	5
Control	2000	0.5	–	–

Table 2. *Plecoglossus altivelis.* Survival rates in the challenge test by immersion infection. Challenges 1 and 2 took place 3 and 7 wk after vaccination. See 'Challenge test' for more details

Group	Mean body weight (g)	Challenge dose (CFU ml^{-1})	Dead fish/ challenged fish	Survival rate (%)
Challenge 1				
1	1.7		7/118	94.1[a]
2	1.8	4.4×10^7	4/119	96.6[a]
Control	1.8		36/117	69.2
1	1.9		53/114	53.5[a]
2	1.8	1.2×10^8	10/120	91.7[a]
Control	1.9		79/121	34.7
Challenge 2				
1	2.7		26/186	86.6[b]
2	2.9	2.1×10^7	20/168	88.1[b]
Control	2.7		41/174	76.4
1	2.7		40/170	76.5[a]
2	3.0	1.4×10^8	36/165	78.8[a]
Control	3.2		107/185	42.2

[a]Significant difference ($p < 0.01$) is shown compared with the value of control group
[b]Significant difference ($p < 0.05$) is shown compared with the value of control group

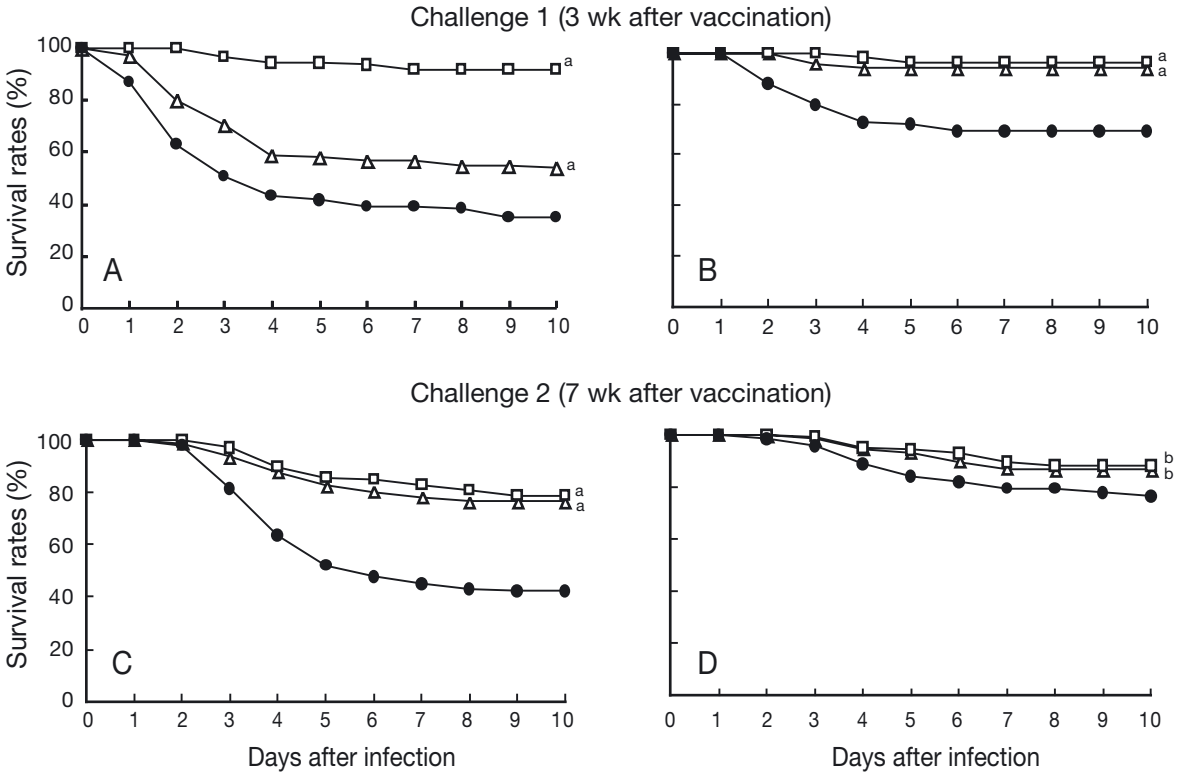

Fig. 2. *Plecoglossus altivelis.* Changes in the survival rate of ayu immunized by oral administration and challenged by immersion infection. (△) Administered daily; (□) administered 5 times over 2 wk; (●) control. Challenge doses by immersion were approximately 10^8 CFU ml^{-1} (A,C) and 10^7 CFU ml^{-1} (B,D). a: Significant difference ($p < 0.01$) is shown compared with the value of control group. b: Significant difference ($p < 0.05$) is shown compared with the value of control group

Vibrio anguillarum showed that LD_{50} doses were the same for immunized and unimmunized fish, and oral vaccination did not increase the protective immune response against bacteria that had already infected the fish. No increase in the agglutination titer occurred in the serum of orally immunized fish, but skin mucus from this group agglutinated the bacteria and inhibited bacterial attachment. Oral vaccination therefore does

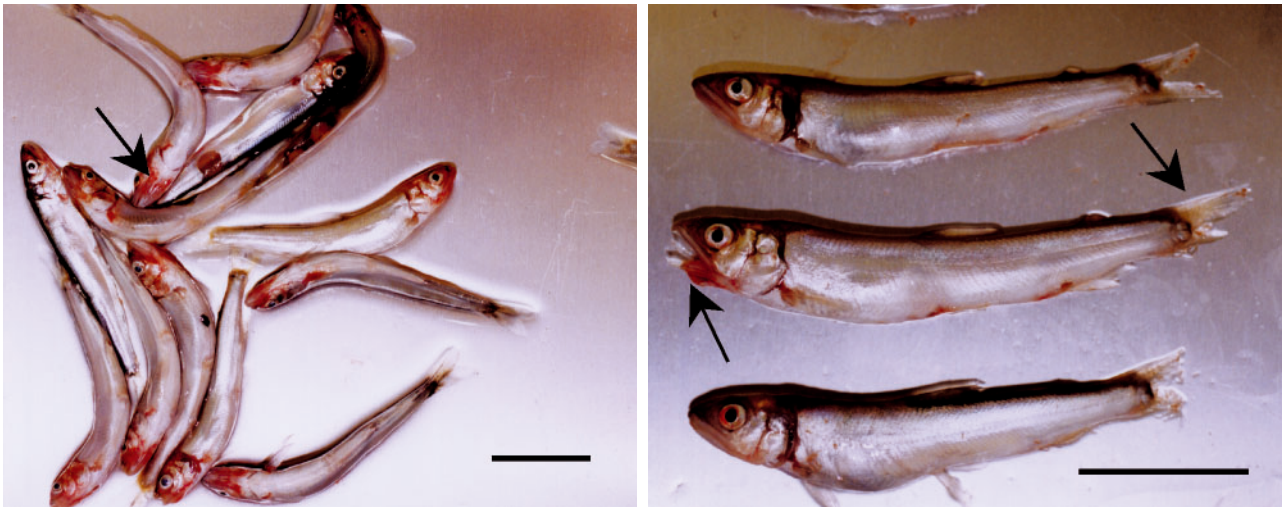

Fig. 3. *Plecoglossus altivelis.* Typical signs of bacterial coldwater disease (BCWD) in ayu challenged with *Flavobacterium psychrophilum* by immersion infection. Arrows indicate hemorrhage at the lower part of the operculum and jaw, and partially eroded or lack of caudal fin edge. Scale bar = 2.0 cm

not protect against infection, but prevents organisms entering the tissue, inhibiting the attachment and growth of organisms on the body surface of fish. In our experiment, it was unclear whether the protection factor existed in the body-surface mucus of ayu. However, Kondo et al. (2002) showed by electron microscopy that *Flavobacterium psychrophilum* adhered to infected ayu and invaded the skin at the beginning of infection. Therefore, immersion challenge was adapted for evaluation of the efficacy of oral vaccine in this study. These data indicate that developing an immnune response at the body surface of fish is important.

In this experiment, the logarithmic culture bacteria were used as the antigen for vaccination. Our previous study (Kondo et al. 2001b) showed that this bacterium possessed outer membrane components on the cell surface of the logarithmic phase culture. Rahman et al. (2002) reported the effectiveness of the outer membrane fraction of BCWD. Therefore, interaction between the immune response of the body surface of fish and the bacterial surface structure is important to consider when developing a BCWD vaccine.

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