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Experimental infection of *Flavobacterium psychrophilum*, inducing typical signs of bacterial coldwater disease in the ayu *Plecoglossus altivelis*

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Abstract: An experimental infection system of bacterial coldwater disease (BCWD) is described for the ayu *Plecoglossus altivelis*. Three infection methods, immersion, intramuscular injection and oral administration, were investigated. With the immersion infection fish were immersed in $10^{6.5}$-$10^{8.5}$ CFU/ml bacterial suspensions resulting in cumulative mortalities of 27-67 %. Most of the dead fish had typical lesions at the caudal site or hemorrhaging in the lower part of the operculum. With the intramuscular injection at a dose of $10^{5.6}$ -$10^{7.6}$ CFU/fish, cumulative mortalities were 93-100 %. This method eroded markedly the injection site. With the oral administration of $10^{8.1}$-$10^{8.5}$ CFU/fish/day, mortalities were 50-92 %. However, marked external symptoms were not observed in this experimental infection. The results indicated that immersion infection is the most appropriate method to show infectious mechanisms or to develop preventions against BCWD epidemics.

Key words: ayu, *Flavobacterium psychrophilum*, bacterial coldwater disease (BCWD), experimental infection

Introduction

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD), was first isolated from juvenile rainbow trout *Oncorhynchus mykiss* in the USA, and was named "peduncle disease" (Davis, 1946) because the disease produced lesions on or near the peduncle. Later the bacterium was isolated from juvenile coho salmon *Oncorhynchus kitsch* (Borg, 1960), and from European eel *Anguilla anguilla* and carp *Cyprinus carpio* (Lehmann et al., 1991). Today the disease is a serious problem in North America and Europe in salmonid fish. In 1987, the bacterium was isolated from the ayu *Plecoglossus altivelis* in a hatchery in Japan (Wakabayashi et al., 1994). Before this, the bacterium was isolated from juvenile coho salmon in Japan (Wakabayashi et al., 1991), indicating that it was introduced from North America with coho salmon eggs. The bacterium was isolated also from wild ayu and pale chub *Zacco platypus* (Iida and Mizokami, 1996). Isolation from cultured ayu was also reported in Korea (Lee and Heo, 1998). The bacterium has been detected from wild fish in almost all areas in Japan. As drug resistant bacteria frequently appear in fish farms, a vaccine needs to be developed as a prevention method.

To study the vaccination and infection mechanism, establishing a method of experimental infection to obtain steady results in mortality and infection in challenge tests is important. An experimental infection system has been reported for the infection of rainbow trout with *F.*

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psychrophilum named rainbow trout fry syndrome (Holt et al., 1989; Madsen and Dalsgaard, 1999; Decostere et al., 2000; Garcia et al., 2000). However, the experimental infection of the ayu has not been examined, despite the ayu being one of the important species in the Japanese fisheries industry. This study established an adequate method to infect the ayu experimentally by comparing three methods: immersion infection, intramuscular injection, and oral infection that closely resembles natural infection.

Materials and Methods

Preparation of bacteria

F. psychrophilum strain G3724 isolated from a diseased ayu in Tokushima Prefecture, Japan, in 1998 was used. The bacterium was passed in ayu twice to increase the pathogenicity and was stored in 10 % skimmed milk at -60℃. The bacterium was pre-cultured in 4 ml modified cytophaga broth (MCYT ; 0.2 % trypton, 0.05 % yeast extract, 0.02 % beef extract, 0.02 % CH_3COONa, 0.02 % $CaCl_2$) at 15℃ for 2 days, then 0.5 ml of the culture was inoculated into 200 ml MCYT broth. The bacterium was cultured for 36 h with shaking (100 rpm), and then the concentration was adjusted by adding PBS at absorbances 0.50 to 0.60 at 600 nm corresponding to approximately $10^{8.0}$ Colony-forming units per milliliter (CFU/ml). The cultured bacteria was harvested to enrich the concentration by centrifugation (4,500 × *g*, 20 min).

Test fish

As test fish, 215 fishes, juvenile ayu of average weight 2.7 g, provided by the Foundation of Kochi Freshwater Fish Culture Center (Table 1), were stored in 600 l tanks with well aerated flowing water at 18-20℃. The fish were divided into 12 groups and were acclimatized at 15℃ for at least 5 days, and were fed 1.0 mm dry pellets (Maruha co. Ltd) corresponding to 2 % of the fish body weight for entire experiment.

Table 1. Experimental infection with *Flavobacterium psychrophilum* in the ayu *Plecoglossus altivelis*

Infection method	Average body weight (g)	Infection dose	Dead fish/ experimental fish
Immersion	2.1	[1] $10^{8.5}$	10/15
	1.7	$10^{7.5}$	9/15
	2.0	$10^{6.5}$	4/15
	2.3	Control	0/20
Intramuscular injection	2.9	[2] $10^{7.6}$	15/15
	2.7	$10^{6.6}$	15/15
	2.3	$10^{5.6}$	14/15
	2.7	Control	3/20
Oral infection	4.7	[3] $10^{8.5}$	23/25
	2.9	$10^{8.1}$	10/20
	2.9	$10^{6.1}$	0/20
	3.3	Control	1/20

[1]CFU/ml, [2]CFU/fish, [3]CFU/fish/day · 5 days.

Experimental infection

The immersion infection was done by immersing fish in aerated tap water having bacteria at a concentration range of $10^{6.5}$ - $10^{8.5}$ CFU/ml at 15℃ for 30 min. Each of three infection groups comprised 15 fishes. Twenty control fishes were immersed for 30 min in sterile MCYT broth

diluted 1 : 20 with tap water.

For the intramuscular injection, 15 fishes in each infected group were anaesthetized with 2-phenoxyethanol (Nakalai Tesque) and were injected intramuscularly below the dorsal fin with 0.05 ml bacterial suspension at doses of $10^{5.6}$ to $10^{7.6}$ CFU/fish. Control fish were injected with 0.05 ml sterile 0.85 % NaCl solution.

Oral infection was done by feeding freely commercial dry pellets mixed with bacteria for 5 days. Two percent mixed pellets of the body weight was incubated for 30 min at 15℃ after adding the bacteria. Infection doses were $10^{6.1}$, $10^{8.1}$ and $10^{8.5}$ CFU/fish/day on average. Control fish were fed dry pellets mixed with sterile MCYT.

After every infection experiment, fish were reared in 200 l circulated tanks at 15℃ and the mortality were recorded; this fish rearing was done until no mortality occurred. Dead and living fish were necropsied by external and internal examinations and the bacteria were isolated from the kidney.

Scanning electron microscopy (SEM)

Samples for SEM were fixed in 2.5% glutaraldehyde and then fixed again in 1% osmium tetroxide. The samples were dehydrated in alcohol and acetone, and then underwent critical point drying in liquid carbon, were coated with gold and were examined using a Hitachi S-2380N.

Results

Mortality curve

Table 1 shows experimental infection with ayu. With the immersion infection, mortality began from 3 to 5 days after infection. Groups infected at $10^{6.5}$, $10^{7.5}$ and $10^{8.5}$ CFU/ml showed 27, 60 and 67 % mortalities after 10 days, respectively. The mortalities of three infected groups and the control group were significantly different ($P<0.05$) using the chi-square test. The bacteria were isolated from the diseased parts, lower jaw, brain, operculum and caudal peduncle, in 89 %, 75 % and 80 % of the dead fishes of groups infected at $10^{6.5}$, $10^{7.5}$ and $10^{8.5}$ CFU/ml respectively, from the kidney in 50 %, 22 % and 10 % of the dead fishes of groups infected at $10^{6.5}$, $10^{7.5}$ and $10^{8.5}$ CFU/ml respectively. Bacteria were not isolated from living or control fishes.

With the intramuscular injection, mortality began from 1 and 2 days after injection and was 100 % from 4 - 6 days after injection in the groups injected with $10^{6.6}$ to $10^{7.6}$ CFU/fish doses. The group of $10^{5.6}$ CFU/fish dose showed 93% final mortality. Significant differences were detected between the mortalities of infected and control groups ($P<0.05$). The patterns of cumulative mortalities in the three dose groups were similar and parallel. The bacteria were isolated from the injection site in all dead fishes, and from the kidney in 7 %, 27 % and 47 % of the dead fishes of groups injected with $10^{5.6}$, $10^{6.6}$ and $10^{7.5}$ CFU/ml infection, respectively. Bacteria were not isolated from living and control fishes.

With the oral infection, mortality began from 2 - 3 days after infection in the $10^{8.5}$ and $10^{8.1}$ CFU/fish/day groups, resulting in 92 % and 50 % mortality, which were significantly different ($P<0.05$) from the control group. However, the group infected with $10^{6.1}$ CFU/fish/day did not have any mortalities. The bacteria was isolated from the kidney in 20 % and 4 % of the dead fishes of groups injected at $10^{8.1}$ and $10^{8.5}$ CFU/fish/day. Bacteria were not isolated from living and control fishes.

Symptoms

Disease symptoms in dead fish by immersion were hemorrhage in the lower part of operculum, lack of lower jaw (Fig. 1A) and partial lack of caudal fin edge (Fig. 1B). A few dead fish had

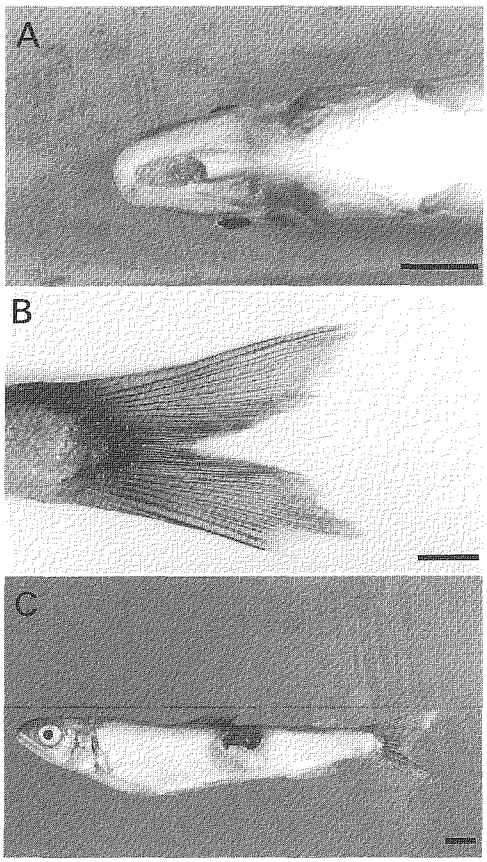

Fig. 1. Clinical signs in the ayu *Plecoglossus altivelis* experimentaly infected with *Flavobacterium psychrophilum* strain G3724. (A, B) Immersion. Partial lack of caudal fin edge, hemorrhage at the lower part of the operculum and lack of the lower side of the jaw were observed. (C) Intramuscular injection. Ulcer at the site of injection was observed. Scale Bar = 5 mm.

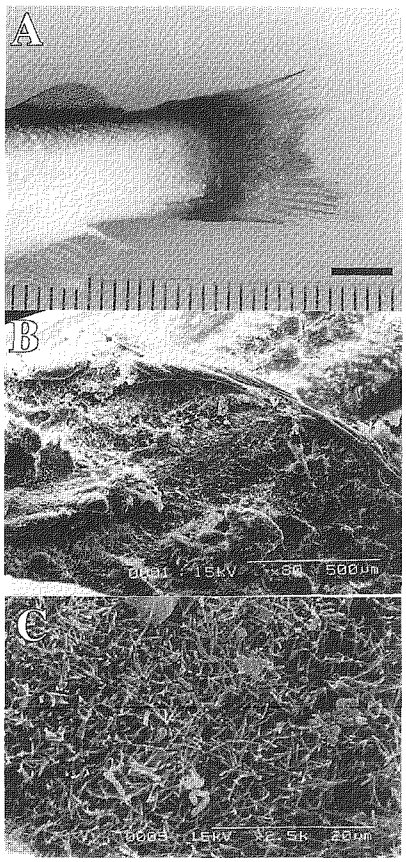

Fig. 2. (A) Caudal lesion of the ayu *Plecoglossus altivelis* infected by immersion. Scale Bar = 5 mm. (B, C) Scanning electron micrograph of the surface of the caudal lesion of fish infected by immersion. Scale Bar = 500 μm, 20 μm.

lesion in the peduncle of the caudal fin (Fig. 2A). The epithelial tissue of the caudal fin was collapsed (Fig. 2B). Conspicuous bacterial growth was on the surface of the caudal lesions by SEM (Fig. 2C). Internal symptoms of the dead fish were anemia in the gills and liver, swollen kidney and ascites accumulation. All dead fish injected with the bacteria hemorrhaged in the lower part of the operculum and had necrosis in the musculature of the injection site. The epidermal layer around the injection site had disappeared and the musculature was exposed (Fig. 1C). With the oral administration, dead fish showed poor external signs. Internal symptoms were hemorrhaging of the liver and accumulation of ascites.

Discussion

In this study, cumulative mortalities were 27-67 % by immersion at injection of $10^{6.5}$-$10^{8.5}$

CFU/ml, 93-100 % by intramuscular injection at injections of $10^{5.6}$-$10^{7.6}$ CFU/fish and 50-92 % by oral infection at injection of $10^{8.1}$-$10^{8.5}$ CFU/fish/day · 5days. The strength of infection by the three methods could not be compared strictly because the infection route, actual number of invaded bacteria and successive reactions of the host defense were different. However, intramuscular injections were the most effective, than immersion infection and oral infection. In other studies, with experimental infection of rainbow trout, mortalities by bath challenge using 10^7 CFU/ml was 27 - 31 % (Madsen and Dalsgaard, 1999) and no fish died by oral challenge (Madetoja et al., 2000). Our study results may disagree with these other studies because the difference of host fish. The sensitivity of the ayu was shown in the studies. All the ayu were dead by intramuscular injection of 6.2×10^2 CFU/fish of *Streptococcus* sp. and 8.2×10^0 CFU/fish of *Vibrio anguillarum* (Kusuda et al., 1981). The minimum lethal dose of a strain of *V. anguillarum* was 8.8 CFU/100 g body weight (Jo and Muroga, 1977). *F. psychrophilum* isolated from rainbow trout is not virulent in the ayu (Nakai, 1999), may be this bacterium in the ayu are more virulent than those from the rainbow trout.

We were not successful in isolating the bacteria from the kidney of dead fish in immersion infection and oral infection studies. Natural infections of *F. psychrophilum* are in the gills, rarely in dorsal muscles, but never in the internal organs of the chinook salmon *Oncorhynchus tshawytscha* (Wood and Yasutake, 1956). *F. psychrophilum* in the kidney or spleen of rainbow trout is difficult to isolate (Decostere et al., 2000; Wiklund et al., 2000). These findings indicate that *F. psychrophilum* grows mainly at sites contacting with environmental water. *F. psychrophilum* in the caudal lesions (Fig. 2) indicates that the bacterium grows at the affected site but does not appear to invade inside of the tissue. Another reason may be that *F. psychrophilum* on the body surface is slow growing and yields to the competition from other bacteria that grow faster on agar plates.

BCWD produces lesions on or near the peduncle site of the caudal fin (Davis, 1946). The lesions are formed at various sites on the body surface, but are often on or near the caudal fin in naturally infected ayu (Iida and Mizokami, 1996). Naturally infected ayu in hatcheries had caudal lesions, lacked a caudal fin, and hemorrhaged at the lower part of the operculum in our survey. Immersion infection and intramuscular injection of this study showed high mortality. However, caudal lesions were only on fish that had immersion infection. Oral infection produced no obvious external signs in the infected fish, but lethal doses by this infection were very high, suggesting that oral infection is not a major infection route.

In conclusion, immersion infection was the best method to study infection mechanisms of this disease in the ayu. Therefore, this method can be used in challenge tests to evaluate the effects of vaccines.

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