No Evidence for the Presence of *Flavobacterium psychrophilum* within Ayu Eggs

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ABSTRACT—This study was made to clarify a possibility of vertical transmission of bacterial coldwater disease (BCWD) in ayu *Plecoglossus altivelis*. The presence of *Flavobacterium psychrophilum* on the surface and in the contents of unfertilized and fertilized eggs, which were obtained from ayu naturally carrying *F. psychrophilum*, was examined by a culture method. The bacterium was detected on the egg surface of 19 out of 65 unfertilized egg samples and of 5 out of 30 fertilized egg samples (300 eggs/sample). On the other hand, *F. psychrophilum* was not detected from the surface of eggs disinfected by povidone-iodine (5 ppm, 10 min) or hydrogen peroxide (150 ppm, 30 min). The bacterium was not detected in the contents of these surface-disinfected eggs. These results suggest that *F. psychrophilum* is not transmitted through intra-ovum infection in ayu eggs. Therefore, disinfection of eggs is useful to prevent the vertical transmission of BCWD in ayu hatcheries.

Key words: *Flavobacterium psychrophilum*, bacterial coldwater disease, intra-ovum infection, disinfection of eggs, *Plecoglossus altivelis*, BCWD

Bacterial cold water disease (BCWD) has been spreading throughout Japan and been causing serious damage to populations of ayu *Plecoglossus altivelis* in many farms and rivers since the middle of the 1990s. *Flavobacterium psychrophilum*, the causative agent of BCWD, has been detected from not only ayu but also other wild freshwater fish (Iida and Mizokami, 1996; Amita *et al.*, 2000) and cultured salmonid fish (Nematollahi *et al.*, 2003). Ayu is such a popular fish for angling in Japan that a large number of juvenile ayu produced in hatcheries or collected from Lake Biwa, local rivers and the sea coasts are annually released in rivers for the enhancement of ayu stocks. Outbreaks of BCWD occur mainly in rivers where the juveniles carrying the pathogen are released. Therefore, it is important to produce and release juveniles free from the pathogen.

Disinfection of eggs by chemical disinfectants before the introduction of eggs into hatcheries is com-

monly used in salmonid fish hatcheries in order to produce the pathogen free juveniles. Such disinfection is likely to be effective in ayu hatcheries. On the other hand, effectiveness of disinfection of the eggs depends on the location where *F. psychrophilum* exists. In the case that the bacteria have entered eggs, the perfect efficacy of disinfection cannot be expected (Evelyn *et al.*, 1984; Brown *et al.*, 1997; Kumagai *et al.*, 1998). Brown *et al.* (1997) showed evidence that *F. psychrophilum* was transmitted within salmonid eggs, and Kumagai *et al.* (2000) demonstrated by the experimental infection that *F. psychrophilum* entered salmonid eggs. However, it remains unclear whether *F. psychrophilum* is transmitted into ayu eggs. We conducted the present study to clarify whether *F. psychrophilum* is present within ayu eggs by cultivating the contents of unfertilized and fertilized eggs obtained from ayu carrying *F. psychrophilum*.

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Materials and methods

In vitro bactericidal effect of povidone-iodine and hydrogen peroxide on F. psychrophilum

F. psychrophilum strain FPC840, which was originally isolated from the kidney of ayu in Tokushima, Japan in 1987 (Wakabayashi *et al.*, 1994), was used in the experiment. The bacterium was grown in an enriched Ordal and Anacker's agar (AOAE) plates at 15°C for 5 days (Lorenzen and Karas, 1992). The bacterial cells collected from the colonies were suspended in phosphate-buffered saline (PBS) at densities of 2×10^7 , 2×10^6 , 2×10^5 CFU/mL.

Povidone-iodine (Isodine, Meiji Seika Kaisha, Japan) was serially diluted 2-fold with distilled water at available iodine concentrations between 1.25 and 40 ppm. A half milliliter of the bacterial suspensions was mixed with an equal volume of the serial 2-fold dilutions of povidone-iodine in a 24 well microtitration plate. Following 5, 10, 15 and 30 min of incubation at 15°C, the iodine was neutralized with 0.1 mL of N/10 sodium thiosulfate, and then a 0.1 mL aliquot of the mixture from each well was inoculated on two AOAE plates. After 7 days of incubation at 15°C, the growth of *F. psychrophilum* was recorded.

Susceptibility of *F. psychrophilum* (2×10^7 CFU/mL) to hydrogen peroxide (Katayama Kagaku, Japan) at final concentrations of 30, 60, 90, 120, 150, 180 and 210 ppm, was examined in the same manner as above except the neutralization with sodium thiosulfate. In order to remove the effect of the hydrogen peroxide on cultivation, the mixture from each well was diluted 10-fold with distilled water before inoculation on the plates.

Fish and eggs

Four lots of mature ayu reared in two hatcheries in the central part of Japan (lot number A1, A2, B1 and B2) were used for the experiments. As the natural occurrence of BCWD had once been recorded in all the lots, many of the experimental fish were expected to carry *F. psychrophilum*. From each mature female (15–20 fish per lot and 65 in total), eggs were aseptically squeezed into individual sterile petri dishes and similarly milt from each male (10–20 fish per lot and 55 in total) was squeezed into individual sterile petri dishes. After taking the eggs and milt, all the parental fish were examined for the bacterium by the following method; the kidneys of the fish were streaked on AOAE medium and incubated at 15°C for 7 days.

Approximately 900 unfertilized eggs (0.3 g per fish) and 0.1 mL of milt were taken from each fish of the 4 lots. In 2 lots (A2 and B2, 15 fish for each), the rest of the eggs were inseminated for each individual fish using milt, which was pooled from the 10 or 20 males of the same lot and was diluted 20-fold with the artificial seminal plasma (NaCl 130 mM, KCl 40 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5

mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.5 mM, NaHCO_3 2.5 mM, pH 8.2). From the inseminated eggs, approximately 3000 fertilized eggs (1 g per fish, 15 fish per lot) were dispensed into 15 mL sterile tubes as the fertilized egg sample from each fish and were washed 5 times with 10 mL of sterile distilled water. In the washing process, the tubes were kept shaken to prevent aggregation of the eggs during water-hardening.

Thus, the milt (0.1 mL per sample, 55 samples in total), unfertilized eggs (900 eggs per sample, 65 samples in total) and fertilized eggs (3000 eggs per sample, 30 samples in total) were prepared and transported to the laboratory on ice.

Estimation of the number of F. psychrophilum cells in milt and on the surface of unfertilized eggs

Each milt sample (0.1 mL per sample) was directly inoculated on AOAE plates and incubated at 15°C for 7 days.

Each unfertilized egg sample was divided into three batches of 300 eggs each. One of the three batches was vigorously shaken for 30 s on a vortex stirrer with 0.2 mL of PBS to detach bacteria from the egg surface. The number of viable bacterial cells released into PBS was determined by AOAE plate count after incubation at 15°C for 7 days. Identification of the isolated *F. psychrophilum* was made by PCR (Toyama *et al.*, 1994) and indirect fluorescent antibody test (IFAT) using an antiserum against *F. psychrophilum* FPC840.

Detection of F. psychrophilum in the egg contents

The other two batches of the unfertilized eggs taken from each fish were used for the detection of *F. psychrophilum* within the egg contents following a modified protocol of Evelyn *et al.* (1984) and Brown *et al.* (1997) as described below. To produce surface-sterile eggs, one batch of unfertilized eggs was treated with 5 ppm povidone-iodine for the lots A1 and B1 and 150 ppm hydrogen peroxide for the lots A2 and B2. After the treatment, eggs were rinsed 3 times with 3 mL of sterile distilled water, inoculated in 10 mL of modified Ordal and Anacker broth (Wakabayashi and Egusa, 1974) and incubated at 15°C for 3 days. The remaining batch was inoculated and incubated in the broth without disinfection as a control.

After incubation for 3 days, the turbidity of the broth was examined by the eye. Moreover, an aliquot of each broth sample was serially diluted 10-fold with PBS, and 0.1 mL of each diluted sample was dropped on AOAE plates and incubated at 15°C for 7 days. When bacterial colonies grew on the plates, identification of the isolated *F. psychrophilum* was made by PCR and IFAT. Eggs were considered to be surface-sterile when no turbidity in the broth and no subsequent bacterial growth of *F. psychrophilum* on AOAE plates were observed.

The unfertilized egg samples (300 eggs for each) disinfected as above were homogenized to obtain the egg contents. Each of the homogenates was inoculated in 10 mL of modified Ordal and Anacker broth, incubated at 15°C for an additional 3 days and examined for turbidity. Bacteriological examination using AOAE plate and identification of the isolated *F. psychrophilum* by IFAT and PCR was made in the same manner as above.

Two batches of 300 fertilized eggs each were taken from the two lots (A2 and B2). One batch of fertilized eggs each was disinfected with 150 ppm hydrogen peroxide for 30 min. *F. psychrophilum* in the egg contents of the surface-sterile eggs were examined in the same manner as used for the unfertilized eggs. The other batch of fertilized eggs each was examined without disinfection as a control.

The influence of disinfection on the survival of ayu eggs

Eggs were collected from 9 mature fish. The eggs of each fish were artificially inseminated in individual sterile petri dishes. Each of 9 inseminated egg samples was divided into two batches of 0.1 g (approximately 300 eggs). The inseminated egg samples were attached to artificial substances and water-hardened in the hatchery water according to standard hatchery practices. One of two batches (lot 1–5) was disinfected with povidone iodine (5 ppm, 10 min) and the other batch (lot 6–9) was disinfected with hydrogen peroxide (150 ppm, 30 min). Control batches were incubated without disinfection. After 10 days of incubation in flowing water at 15°C, the ratios of live eggs were examined.

Results

In vitro bactericidal effect of povidone-iodine and hydrogen peroxide on F. psychrophilum

Povidone-iodine solutions completely inactivated *F. psychrophilum* of 10^7 , 10^6 and 10^5 CFU/mL at the available iodine concentration of 20 ppm in 5 min, 2.5 ppm in 5 min and 0.63 ppm in 5 min, respectively. Hydrogen peroxide solutions also completely killed *F.*

psychrophilum (10^7 CFU/mL) at the concentration of 120 ppm in 30 min.

Detection of F. psychrophilum from spawning ayu

F. psychrophilum was isolated from the egg surface and milt as well as kidney (Table 1). The incidence of *F. psychrophilum* on the surface of unfertilized eggs sampled from the 4 lots of 2 hatcheries ranged from 5% to 47% (26% in total), while the incidence in milt was significantly lower than that on the egg surface (Fisher's exact probability test, $p < 0.05$), ranging from 0% to 20% (7% in total). The viable bacterial counts of *F. psychrophilum* in milt and eggs were lower than 10^3 CFU/mL-milt and 10^3 CFU/g-eggs, respectively. On the other hand, the incidence of *F. psychrophilum* in the kidney was higher than those on the egg surface and in the milt. There was no significant difference (Fisher's exact probability test, $p > 0.05$) in the incidence in the kidney between females and males. *F. psychrophilum* was isolated from the kidney of 11 out of 17 females and 2 out of 4 males whose egg surface or milt was positive for the bacterium.

Detection of F. psychrophilum from disinfected eggs

F. psychrophilum was detected on the egg surface of 19 unfertilized egg samples out of 65 samples (29% in total) and of 5 fertilized egg samples out of 30 samples (17% in total) (Table 2). By the disinfection with povidone-iodine (5 ppm, 10 min) or hydrogen peroxide (150 ppm, 30 min), *F. psychrophilum* on the surface of unfertilized and fertilized eggs was completely killed, although other bacteria remained in some samples. In the egg contents of the surface-sterile egg samples (51 unfertilized egg samples and 27 fertilized egg samples), *F. psychrophilum* was not detected at all (Table 2).

The influence of disinfection on the survival of ayu eggs

The ratios of live eggs in the disinfected groups with povidone-iodine (5 ppm, 10 min) or hydrogen peroxide (150 ppm, 30 min) were not significantly lower (χ^2 test, $p > 0.05$) than those of the control groups (Table 3).

Table 1. Detection rates of *F. psychrophilum* from spawning ayu

Lot	Female			Male		
	Kidney %	Egg surface %		Kidney %	Milt %	
A1	53 (8/15)* ¹	47 (7/15)	(2×10^2)* ²	47 (7/15)	7 (1/15)	(2×10)* ³
A2	87 (13/15)	40 (6/15)	(6×10^2)	80 (8/10)	1 (1/10)	(2×10^2)
B1	10 (2/20)	5 (1/20)	(2×10^2)	0 (0/20)	0 (0/20)	
B2	40 (6/15)	20 (3/15)	(2×10)	80 (8/10)	20 (2/10)	(1×10^2)
Total	45 (29/65)	26 (17/65)	(3×10^2)	42 (23/55)	7 (4/55)	(1×10^2)

*¹ No. of fish positive for *F. psychrophilum* / No. of fish examined.

*² Mean no. of live *F. psychrophilum* (CFU/g) on the egg surface.

*³ Mean no. of live *F. psychrophilum* (CFU/mL) in the milt.

Table 2. Detection of *F. psychrophilum* from disinfected ayu eggs

Eggs	Lot	No. of samples* ¹	Egg surface						Egg content			
			Without disinfection			Disinfection* ²			No. of samples* ⁴	<i>F. psychrophilum</i>	Other bacteria	No bacteria
			<i>F. psychrophilum</i>	Other bacteria	No bacteria	<i>F. psychrophilum</i>	Other bacteria	No bacteria				
Unfertilized eggs	A1	15	8 (5)* ³	4	3	0	3	12	12	0	1	11
	A2	15	6 (4)	6	3	0	2	13	13	0	0	13
	B1	20	2 (0)	12	6	0	9	11	11	0	1	10
	B2	15	3 (0)	0	12	0	0	15	15	0	0	15
	Total	65	19 (9)	22	24	0	14	51	51	0	2	49
Fertilized eggs	A2	15	3 (1)	3	9	0	2	13	13	0	2	11
	B2	15	2 (0)	1	12	0	1	14	14	0	3	11
	Total	30	5 (1)	4	21	0	3	27	27	0	5	22

*¹ Each sample consisted of 300 eggs pooled.

*² A1 and B1 were disinfected with 5 ppm povidone-iodine for 10 min. A2 and B2 were disinfected with 150 ppm hydrogen peroxide for 30 min.

*³ Number of the samples in which both *F. psychrophilum* and other bacteria were detected is given in parentheses.

*⁴ Egg samples, which had been considered to be surface-sterile by disinfection, were examined.

Table 3. Survival rates of fertilized ayu eggs after disinfection

Disinfectant	Lot* ¹	Ratios of live eggs (%)	
		Disinfected	Control
Povidone-iodine (5 ppm, 10 min)	1	75.7	69.8
	2	65.8	71.0
	3	57.7	64.6
	4	76.7	81.3
	5	80.0	78.3
	mean	71.2	73.0
Hydrogen peroxide (150 ppm, 30 min)	6	89.2	89.7
	7	87.6	84.2
	8	73.3	74.1
	9	68.6	64.1
	mean	79.7	78.0

*¹ Each sample consisted of 300 fertilized eggs.

Discussion

F. psychrophilum has been isolated from the reproductive fluid such as the ovarian fluid and/or milt of sexually mature adult salmonids (Holt, 1993; Rangdale *et al.*, 1996; Brown *et al.*, 1997). The bacteria were also detected on the egg surface and in the milt of parental ayu in the present study. If the disinfection of ayu eggs by chemical disinfectants was not carried out, *F. psychrophilum* on the egg surface and in the milt might be transmitted from mature adult ayu to alevins.

In the experiment on the *in vitro* bactericidal effect of povidone-iodine, the higher concentration of povidone-iodine was needed to kill the higher density of *F. psychrophilum*. However, since the viable bacterial counts of *F. psychrophilum* on eggs and in milt were lower than 10^3 CFU/g and 10^3 CFU/mL, respectively, and 0.63 ppm was enough to kill 10^5 CFU/mL of the bacterium in 5 min *in vitro*, the treatment with povidone-

iodine at a concentration of 5 ppm for 10 min seemed to be enough to disinfect eggs. Treatment with hydrogen peroxide of 150 ppm for 30 min also exceeded the minimum lethal concentration (120 ppm) for the bacteria of 10^7 CFU/mL *in vitro*. Both the treatments with povidone-iodine (5 ppm, 10 min) and hydrogen peroxide (150 ppm, 30 min) showed complete efficacy to kill *F. psychrophilum* on the surface of ayu eggs. Furthermore, both the treatments did not affect the survival of the fertilized eggs of ayu. From these results, it is clear that the treatments with povidone-iodine and hydrogen peroxide can eliminate *F. psychrophilum* located on the surface of fertilized egg without affecting the survival of the eggs.

F. psychrophilum was not isolated from the contents of the unfertilized eggs, when the egg surface had been disinfected completely. There was no evidence that the bacteria had entered the eggs in the ovary of female ayu before spawning. Thus, it was suggested that intra-ovum vertical transmission is absent in BCWD in ayu. On the other hand, in salmonid fishes, *F. psychrophilum* has been shown to enter unfertilized eggs before spawning (Brown *et al.*, 1997). *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), was speculated to enter salmonid eggs via the vitelline membrane in the early oogenesis stage and/or via the micropyle in the late oogenesis stage (Evelyn *et al.*, 1984), but the mechanism responsible for the intra-ovum infection has not been fully clarified yet. The mechanism by which the pathogenic bacteria enter pre-spawned eggs is a rewarding area of investigation.

F. psychrophilum was not detected in the contents of the eggs disinfected with povidone-iodine or hydrogen peroxide after insemination with infected milt. The result shows that the bacterium present on the egg sur-

face and in the milt could not enter the eggs in the process of the fertilization and the water-hardening. Evelyn *et al.* (1984) reported that the chance of *R. salmoninarum* entering the egg would be favored by the presence of a large population (10^9 cells/mL) of the bacterium in the coelomic fluid of the parental fish. The lack of intra-ovum infection by *F. psychrophilum* may be due to the fact that amount of the bacteria on ayu eggs were lower (10^3 CFU/g) than that of *R. salmoninarum* in coho salmon *Oncorhynchus kisutch* investigated by Evelyn *et al.* (1984). Furthermore, even when fertilized ayu eggs were water-hardened in a suspension (10^8 CFU/mL) of *F. psychrophilum*, entrance of the bacterium into the eggs did not occur (Kumagai and Nakayasu, unpublished data), although the bacterium successfully entered into coho salmon eggs by the same treatment (Kumagai *et al.*, 2000). Ayu eggs respond to sperm penetration by forming a fertilization cone in the micropyle (Kudo, 1983) and fertilization envelope extract of ayu eggs has bactericidal and fungicidal effects (Kudo and Inoue, 1989; Kudo and Teshima, 1991). These characters of ayu eggs might have prevented *F. psychrophilum* from passing into ayu eggs via the micropyle while being fertilized and during water-hardening.

Holt (1993) and Kumagai and Takahashi (1997) reported that iodine treatment of salmonid eggs could not prevent BCWD in subsequent fry, because *F. psychrophilum* entered the eggs. On the other hand, disinfection of the egg surface by iodine or hydrogen peroxide will be effective for the prevention of BCWD in ayu, as indicated in the present study. However, ayu fertilized eggs are attached onto a fibrous substrate with organic materials during incubation in hatcheries. It is well known that the bactericidal activities of these disinfectants are reduced by the presence of organic materials. Furthermore, the presence of the fibrous substrate may prevent the disinfectants from reaching all the surface of the attached eggs. Further work is needed to establish the practical hatchery method for disinfection of ayu eggs.

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