Disinfection of unfertilized salmonid eggs: a new method for prevention of vertical transmission of *Flavobacterium psychrophilum*

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Abstract

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS), inducing severe economic loss in aquaculture worldwide [1]. Brown et al. [2] and Cipriano [3] demonstrated that *F. psychrophilum* was transmitted within salmonid eggs by cultivating naturally infected eggs, indicating that iodophor treatment of eyed eggs, which has been previously recommended to prevent transmission of the pathogen on the egg surface, did not always prevent BCWD in subsequent fry. Thus, the vertical transmission of *F. psychrophilum* via intra-ovum infection appears to lead to the spread of BCWD among hatcheries with the delivery and transportation of contaminated eggs, resulting in serious adverse effects on the aquaculture of salmonids. The mode of intra-ovum infection of *F. psychrophilum* is still unknown. Since *F. psychrophilum* has been detected in the ovarian fluid of sexually mature salmonids [4], the surface of eggs obtained from ripe fish can be heavily contaminated with *F. psychrophilum*. Using rainbow trout eggs with surfaces contaminated with *F. psychrophilum*, the present study was undertaken to determine if the bacteria can enter eggs in the process of fertilization, to clarify the mode of intra-ovum infection of the bacterium. In addition, we evaluated the efficacy of povidone-iodine treatment of unfertilized eggs in preventing vertical transmission of *F. psychrophilum*.

In the first experiment, two mL of *F. psychrophilum* suspensions at five different concentrations (5.0×10^9, 5.0×10^8, 5.0×10^7, 5.0×10^5 and 5.0×10^3 CFU/mL and PBS as a control) were separately added to each newly spawned unfertilized egg group (400 eggs/ group) in conical flasks, and were gently agitated. Following the bacterial challenge to the egg surface, eggs were immediately dry-fertilized with milt and were water-hardened in water. Just after the water-hardening, all of the egg groups were disinfected with povidoneiodine (50 ppm, 15min). After the treatments, group of eggs was incubated in a tray in aquarium with flow-through water (8°C). The challenged eggs were examined for intra-ovum infection with *F. pychrophilum* 14 and 21 days after the challenge. Thirty live eggs were collected from each egg group, and were disinfected again with povidoneiodine (50 ppm, 15 min). Each egg was

homogenized with a sterile rod, and then the homogenate was spread on an enriched Anacker and Ordal's agar plate [5]. The plates were incubated at 15°C for 7 days.

When newly spawned unfertilized eggs were immersion-challenged with *F. psychrophilum* at concentrations of 10^7 CFU/mL or higher and subsequently inseminated and water-hardened, the bacteria were detected in the egg contents. The infection rate in eggs was related to the bacterial concentration in a dose-dependent manner. Immersion challenged eggs showed eyed-egg rates as high as that of unchallenged eggs (Table 1). In cases of natural infection, salmonid spawners such as coho salmon, rainbow trout and masu salmon have often ovarian fluid containing the bacteria at 10^7 CFU/mL and higher [6]. From these results, it is suggested that when female spawners are heavily infected with *F. psychrophilum*, the bacteria in the ovarian fluid are able to contaminate the egg surface and subsequently enter eggs during water-hardening [7].

Challenge dose (CFU/mL)	No. positive eggs/No. examined eggs			Eyed-egg rate (%)
	14*	21	23	
5.0×10^9	6/30	10/30		90
5.0×10^8	2/30	5/30		94
5.0×10^7	0/30	1/30		88
5.0×10^5	0/30	0/30		90
5.0×10^3	0/30	0/30		89
0			0/60	93

*Days after challenge

Table 1. Detection of *F. psychrophilum* from eggs challenged by the immersion method.

In the second experiment, newly spawned unfertilized eggs (N=400) were immersed in 2 mL of a suspension of *F. psychrophilum* (1.0×10^9CFU/mL), fertilized, and incubated as described above. Two hours and 1, 3, 5, 7, 14, 21, 28 and 42 days after incubation, ten live eggs were sampled at random and were disinfected with povidone-iodine (50 ppm, 15min). Live bacterial counts of contents of the disinfected eggs were examined by culturing method. After 28-day incubation, live eggs were sampled, and disinfected with povidone-iodine. Frozen sections of these eggs were made and stained with the IFAT.

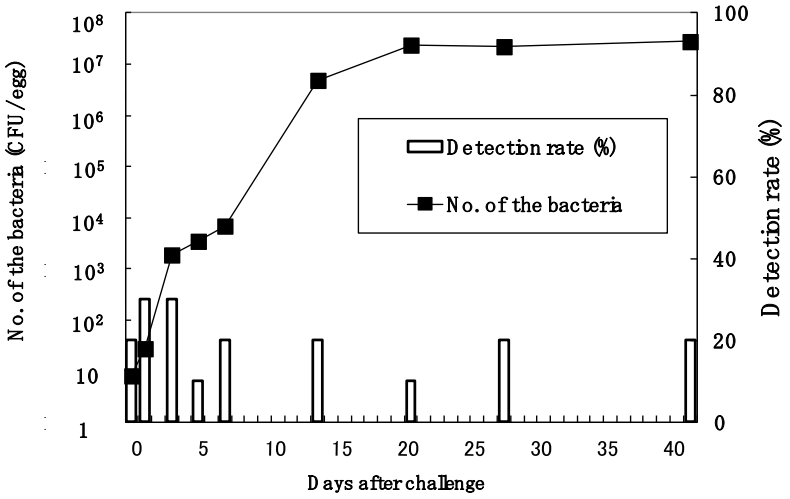

Figure 1. The number of live *F. psychrophilum* cells recovered from eggs challenged by immersion in bacterial suspension.

Live bacterial counts within experimentally infected eggs gradually increased until 14 days after the challenge, and reached to plateau at roughly 10^7 CFU/egg by 21 days after the challenge. Despite the gradual increase in the bacterial concentrations in the eggs, the detection rate of *F. psychrophilum* in the egg groups remained stable between 10% and 30% through the course of experimental period (Fig. 1). Many IFAT-positive bacteria were observed in experimentally challenged eggs 28 days after incubation. Slender rods with specific fluorescence were mainly located inside the chorion, namely the perivitelline space. Live bacterial counts within experimentally infected eggs and immunostaining observations on the eggs suggested that *F. psychrophilum* passively entered into the eggs (< 10 CFU/egg), and subsequently grew to over 10^7 CFU/egg in the perivitelline space by the eyed egg stage. Multiplication of the bacterium in the perivitelline space did not affect the survival of the eggs [7].

In the third experiment, we evaluated the efficacy of povidone-iodine treatment of unfertilized eggs in preventing vertical transmission of *F. psychrophilum*. Table 2 summarized manipulations in each experimental lot. In lot #1, the challenged eggs were immediately dry-fertilized and then water-hardened for 45 min in water. In lot #2, the challenged eggs were disinfected with povidone-iodine, inseminated and then water-hardened in water. In lots #3 and #4, after disinfection and insemination, the fertilized eggs were water-hardened in povidone-iodine at 50 ppm and 100 ppm, respectively. In lot #5, the challenged eggs were rinsed in PBS instead of povidone-iodine treatment, and then inseminated and water-hardened. In lots #6 and #7, the challenged eggs were immediately inseminated, and water-hardened in povidone-iodine at 50 ppm and 100 ppm, respectively. In lot #8, eggs were unchallenged, inseminated and water-hardened in water. Following water-hardening, all egg lots were disinfected with 50 ppm povidone-iodine. After this treatment, each lot of eggs was discretely incubated in flow-through water. The challenged eggs (60 eggs/lot) were examined for intra-ovum infection with *F. psychrophilum* by culturing method at 30–40 days post-challenge as described above.

When newly spawned eggs were immersion-challenged with *F. psychrophilum* (>10^9 CFU/mL) and subsequently disinfected with povidone-iodine (50 ppm in PBS, 15 min) prior to fertilization, the pathogen was not detected in the egg contents (lots #2, 3, 4). Without this prefertilization disinfection with povidone-iodine, however, procedures such as water-hardening of immersion-challenged eggs in povidone-iodine (lots #6, 7) and rinsing of the *F. psychrophilum* contaminated eggs with PBS (lots #5) were insufficient to control the intra-ovum infection (Table 3). All egg lots displayed eyed-egg rates as high as those of untreated control egg lots, indicating that the povidone-iodine treatments do not affect the survival of eggs (Table 3). These results indicate that surface disinfection of unfertilized eggs immediately after spawning is essential to prevent vertical transmission of *F. psychrophilum* [8].

Table 2. Summary of manipulations performed on coho salmon and rainbow trout eggs in experiment of the efficacy of povidone-iodine treatments in preventing intra-ovum infection of *Flavobacterium psychrophilum*.

Lot No.	Immersion challenge of eggs with *F. psychrophilum*	Egg treatment prior to fertilization with	Water-hardening of eggs (45 min) with	Disinfection of water-hardened egg with povidone-iodine (50ppm, 15min)
1	Yes	No	Water	Yes
2	Yes	Povidone-iodine (50ppm)	Water	Yes
3	Yes	Povidone-iodine (50ppm)	Povidone-iodine (50ppm)	Yes
4	Yes	Povidone-iodine (50ppm)	Povidone-iodine (100ppm)	Yes
5	Yes	PBS	Water	Yes
6	Yes	No	Povidone-iodine (50ppm)	Yes
7	Yes	No	Povidone-iodine (100ppm)	Yes
8	No	No	Water	Yes

Fish	Lot No.[*1]	Intra-ovum infection rate	Eyed-egg rate (%)
Coho salmon	1	10/60[*2]	69.9
	2	0/60	77.8
	3	0/60	75.2
	4	0/60	78.9
	5	8/60	76.7
	6	0/60	70.6
	7	1/60	75.0
	8	0/60	70.5
Rainbow trout	1	6/60	73.6
	2	0/60	79.4
	3	0/60	72.7
	4	0/60	69.8
	5	11/60	66.4
	6	1/60	68.9
	7	1/60	48.8
	8	0/60	57.2

[*1] See Table 2 for egg treatment protocol

[*2] No. positive/No. examined (%).

Table 3. Detection of *Flavobacterium psychrophilum* from the content of bacterial-challenged eggs of coho salmon and rainbow trout in different treatment with povidone-iodine.

References

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