Ineffectiveness of Iodophore Treatment in Disinfecting Salmonid Eggs Carrying *Cytophaga psychrophila*

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Eggs of coho salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*), and masu salmon (*O. masou*) were artificially inseminated and subsequently immersed in a modified Ordal and Anacker's broth culture of *Cytophaga psychrophila* (10^7 – 10^8 CFU/ml) for 30 min. The bacterium was recovered from the eggs at the eyed stage with viable cell counts ranging from 10^5 to 10^7 CFU/g. The fertilized eggs were disinfected with 50 ppm povidone-iodine for 15 min immediately after experimental infection. However, *C. psychrophila* was detected by a broth culture method from 60–80 percent of the surface-disinfected eggs. At the eyed stage, the eggs were disinfected again with povidone-iodine at concentrations of 50–1,000 ppm for 15–120 min. *C. psychrophila* was isolated from all of them at densities of 10^4 – 10^7 CFU/g. The uninfected control eggs were all negative for the bacterium. It is concluded that the povidone-iodine treatment is ineffective to eliminate *C. psychrophila* from salmonid eggs.

Key words: cold-water disease, povidone-iodine, salmonid egg, *Cytophaga psychrophila*, disinfection

The intensive marine farming of coho salmon (*Oncorhynchus kisutch*) in Japan began in November 1974 (Mahnken 1991). Subsequently, a large number of coho salmon eggs have been imported annually from the Pacific coast of North America, due to lack of the natural stock of this species. The imported eyed eggs are dipped in 50 ppm povidone-iodine for 15 min immediately after being received at hatcheries. Nevertheless, outbreaks of cold-water disease have often occurred in coho salmon hatcheries since the middle of 1980's (Wakabayashi *et al.*, 1991). Izumi and Wakabayashi (1997) reported that five of nine lots of coho salmon eggs imported from the USA in December 1995 were found to be positive for *Cytophaga psychrophila* by a polymerase chain reaction method and four of these five lots were also positive by cultivation on TYE agar plates. Outbreaks of cold-water disease occurred only among fry originating from the lots of eggs positive for *C. psychrophila* by the inspection. Therefore, it was concluded that the disease outbreaks in the hatcheries

were caused by the pathogen being accompanied by the imported eggs, even after the eggs were surface-disinfected with povidone-iodine (Kumagai and Takahashi, 1997). Although egg disinfection procedures using organic iodine compound had been recommended, it was noted that iodophore treatment of eggs did not always prevent cold-water disease in subsequent fry (Holt *et al.*, 1993). In a more recent study, Brown *et al.* (1997) showed the possibility that *Flavobacterium psychrophilum* (syn. *C. psychrophila*) might be transmitted within salmonid eggs. The purpose of the present study was to determine whether salmonid eggs were infected with *C. psychrophila* by immersion challenge and whether an iodophore treatment could effectively disinfect the experientially infected eggs.

Materials and Methods

Susceptibility of C. psychrophila to povidone-iodine
Povidone-iodine (Isodine, Meiji Seiyaku Co., Tokyo,

Japan) was serially diluted 2-fold with distilled water at available iodine concentrations between 1.25 and 80 ppm. The available iodine concentrations of the solutions were determined by titration with N/10 sodium thiosulfate.

C. psychrophila strain FPC828 was used in the experiments. This strain had been originally isolated from the kidney of coho salmon fry in Miyagi, Japan in 1990 (Wakabayashi *et al.*, 1991). The bacterium was grown in a modified Ordal and Anacker's agar (tryptone 0.3%, yeast extract 0.2%, beef extract 0.02%, sodium acetate 0.02%, agar 1.5%, pH7.2) at 15°C for 5 days. The bacterial cells collected from the colonies were suspended in phosphate-buffered saline (PBS) at a density of 7.5×10^7 CFU/ml. 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 0.5, 1, 2.5, 5, and 15 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 a TY agar plate (tryptone 0.3%, yeast extract 0.2%, pH7.5). After 7 days of incubation at 15°C, the growth of *C. psychrophila* was recorded.

Experimental infection of eggs with C. psychrophila

Eggs were collected from mature coho salmon, rainbow trout (*Oncorhynchus mykiss*), and masu salmon (*Oncorhynchus masou*) which had been kept in Miyagi Prefectural Freshwater Fisheries Experimental Station. The eggs of each fish were artificially inseminated in a glass bowl. Excess milt was rinsed off three times with PBS. Eggs were divided into four groups of 400 eggs.

C. psychrophila strain FPC828 was cultivated in a modified Ordal and Anacker's broth (tryptone 0.3%, yeast extract 0.2%, beef extract 0.02%, sodium acetate 0.02%, pH7.2) at 15°C for 3 days. Before water hardening, two of the four egg groups were immersed in 200 ml of the broth culture (1.4×10^7 – 7.1×10^8 CFU/ml) for 30 min. After the treatments, each batch of eggs was discretely incubated in flowing water at $8 \pm 1^\circ\text{C}$ at the rate of about 0.5 l/min. The other two control groups were bathed in PBS for 30 min before incubation.

Eggs were sampled immediately after experimental infection and at the eyed stage (28–42 days of incubation). At each sampling time, 10 eggs were removed at random from each group and homogenized with a sterile glass rod. The homogenate was then serially diluted 10-fold with PBS. Twenty-five microliter of

each diluted sample was dropped, in triplicate, onto an enriched Ordal and Anacker's agar plates (Lorenzen and Karas, 1992). The plate were incubated at 15°C for 7 days, after which colonies were counted. The detection limit by this plate counting method was estimated to be 4.0×10^3 CFU/g-egg. For coho salmon, 30 eggs were sampled again at 50 days of incubation, and examined individually for *C. psychrophila* in the same manner as above.

Disinfection of eggs with povidone-iodine just after bacterial infection

The eggs were disinfected with 50 ppm povidone-iodine for 15 min following the above-mentioned experimental infection. Duplicate sets of each group were separately incubated in flowing hatchery water. Ten eggs were sampled from each group at the beginning of incubation and at the eyed stage. The bacteriological examination was undertaken on the pooled 10 eggs.

An additional experiment using coho salmon eggs was performed in order to improve the detection method of *C. psychrophila*. The surface-disinfected eggs were divided into 5 groups of 5 eggs each. The eggs of each group were pooled into a sterile glass tube, washed with sterile PBS, and homogenized with a sterile glass rod. The homogenates were serially diluted ten-fold with PBS and 0.3 ml of each dilution was inoculated into a modified Ordal and Anacker's broth, which was incubated at 15°C for 7 days. The broth culture were smeared on glass slides. After being fixed with acetone, the specimens were examined for *C. psychrophila* by indirect fluorescent antibody technique (IFAT). This broth culture method raised the detection limit to 3.3×10 CFU/g-egg, comparing to the plate count.

Disinfection of eggs with povidone-iodine at the eyed stage

The fertilized eggs of coho salmon, rainbow trout, and masu salmon were experimentally infected with *C. psychrophila* (1.4×10^7 – 7.1×10^8 CFU/ml) as described above, incubated at $8 \pm 1^\circ\text{C}$, and disinfected with povidone-iodine at the eyed stage. The following 4 experiments were carried out: 1) Eight groups of 10 coho salmon eggs each were immersed in 10 ml of povidone-iodine solutions at concentrations of 0, 50, 100, 200, 400, 600, 800 and 1000 ppm, respectively, for 15 min. 2) Sixteen groups of 10 coho salmon eggs each were immersed in 10 ml of 0, 50, 100 and 200 ppm povidone-iodine, respectively, for 15, 30, 60 and 120 min, respec-

tively. 3) Three groups of 10 rainbow trout eggs each were immersed in 10 ml of 0, 100 and 200 ppm povidone-iodine, respectively, for 15 min. 4) Three groups of 10 masu salmon eggs each were immersed in 10 ml of 0, 100 and 200 ppm povidone-iodine, respectively, for 15 min. Each experiment was performed in duplicate. After iodophore treatment, the eggs were rinsed three times with 10 ml of PBS, and then the bacteriological examination was made on each sample in the same manner as above.

Results

Susceptibility of C. psychrophila to povidone-iodine

Povidone-iodine solutions killed completely *C. psychrophila* at the available iodine concentration of 20 ppm in 1.0 min or 40 ppm in 0.5 min. However, the bacterium was resistant to 10 ppm povidone-iodine for 15 min (Table 1).

Experimental infection of eggs with C. psychrophila

C. psychrophila was isolated not only from eggs just after being exposed to the bacterium but also from the eggs at the eyed stage. The numbers of live *C. psychrophila* cells recovered were always larger in the

Table 1. Bactericidal effect of povidone-iodine on *C. psychrophila*

Povidone-iodine concentration (ppm)	Exposure time (min)				
	0.5	1.0	2.5	5.0	15.0
0.63	—	—	—	—	—
1.25	—	—	—	—	—
2.5	—	—	—	—	—
5.0	—	—	±	±	±
10.0	±	±	±	±	±
20.0	±	+	+	+	+
40.0	+	+	+	+	+

+ Effective (No CFU after exposure)

± Incompletely effective (Reduction of CFU after exposure)

— Ineffective (Hardly reduction of CFU after exposure)

eyed eggs (Table 2). *C. psychrophila* was still isolated from 70% of coho salmon eggs (21/30) at 50 days of incubation and the viable bacterial counts ranged from 2.6×10^5 to 6.6×10^7 CFU/g (6.1×10^6 CFU/g in average) (Fig. 1).

Disinfection of eggs with povidone-iodine just after bacterial infection

No *C. psychrophila* was detected from the eggs of

Table 2. The numbers of live *C. psychrophila* cells recovered from salmonid eggs exposed to the bacterial suspension

Fish	Group	Number of live <i>C. psychrophila</i> cells	
		Eggs at the beginning of incubation	Eggs at the eyed stage* ¹
Coho salmon	Experimentally infected	1	5.9×10^5 * ²
		2	5.8×10^5
	Uninfected-control	1	— * ³
		2	—
Rainbow trout	Experimentally infected	1	2.0×10^6
		2	2.8×10^6
	Uninfected-control	1	—
		2	—
Masu salmon	Experimentally infected	1	1.2×10^5
		2	1.6×10^5
	Uninfected-control	1	—
		2	—

*¹ The eggs of coho salmon, rainbow trout and masu salmon were examined at 42, 28 and 35 days of incubation, respectively.

*² Bacterial numbers were expressed in CFU/g.

*³ Not detected. The lower limit of detection was 4.0×10^3 CFU/g.

Fig. 1. Viable bacterial counts in coho salmon eyed eggs which had been exposed to *C. psychrophila* just after fertilization.

* Not detected.

coho salmon, rainbow trout, and masu salmon, which were disinfected with povidon-iodine (50 ppm, 15 min) immediately after bacterial infection. However, the

bacterium was isolated from the eyed eggs of all these fishes at a considerable density. *C. psychrophila* was not detected from all the uninfected control groups (Table 3).

The broth culture method was capable of detecting *C. psychrophila* from the fertilized eggs which were negative for the bacterium in the plate count method. Sixty to eighty percent of the surface-disinfected eggs were positive for *C. psychrophila* at the beginning of incubation. Uninfected control eggs were all negative for the bacterium (Table 4).

Disinfection of eggs with povidone-iodine at the eyed stage

C. psychrophila was isolated from all the eyed eggs which had been surface-disinfected at various concentrations of povidone-iodine just before examined. Even if the coho salmon eggs were disinfected with 1,000 ppm povidone-iodine, the bacterium persisted at the density of 4.0×10^4 or 8.0×10^4 CFU/g-egg. There was no significant difference in the number of recovered bacterial cells between iodophore concentrations. Moreover, the viable bacterial counts of the surface-disinfected eggs were almost same as that of non-disinfected control eggs (Table 5). The exposure time to povidone-

Table 3. Detection of *C. psychrophila* from salmonid eggs disinfected with povidone-iodine just after bacterial infection

Fish	Group	Number of live <i>C. psychrophila</i> cells		
			Eggs at the beginning of incubation	Eggs at the eyed stage* ¹
Coho salmon	Experimentally infected	1	—* ³	4.4×10^5 * ²
		2	—	4.4×10^5
	Uninfected-control	1	—	—
		2	—	—
Rainbow trout	Experimentally infected	1	—	4.0×10^6
		2	—	4.0×10^7
	Uninfected-control	1	—	—
		2	—	—
Masu salmon	Experimentally infected	1	—	8.0×10^6
		2	—	4.4×10^6
	Uninfected-control	1	—	—
		2	—	—

*¹ The eggs of coho salmon, rainbow trout and masu salmon were examined at 42, 28 and 35 days of incubation, respectively.

*² The bacterial numbers were expressed in CFU/g.

*³ The detection limit was 4.0×10^3 CFU/g.

iodine had also nothing to do with the number of live *C. psychrophila* cells recovered from the surface-disinfected eggs (Table 6).

Discussion

In the experimental infection of fertilized eggs with *C. psychrophila*, no bacteria could be isolated from the

non-infected control groups, whereas a large number of the bacteria was recovered from the infected groups at the eyed stage as well as at the beginning of incubation. This result indicated that experimental infection of eggs with *C. psychrophila* was successful by immersing the fertilized eggs (before water-hardening) in broth culture.

Disinfection of fertilized eggs with povidone-iodine following experimental infection with *C. psychrophila* revealed that the iodophore treatment reduced the live bacterial number but the killing effect was not complete. Sixty or eighty percent of the surface-disinfected eggs were positive for *C. psychrophila*, and the number of viable bacterial cells was estimated to be 3.3×10^4 – 10^7 CFU/g-egg. The number of bacteria reached 10^4 – 10^7 CFU/g-egg at the eyed stage. Lorenzen (1994) reported that disinfection with Aktomar K30 (22 and 66 ppm I₂) just after infection could not remove *C. psychrophila* from experimentally infected eggs.

Disinfection of the experimentally infected eggs at the eyed stage with povidone-iodine resulted in no reduction in the number of live *C. psychrophila*, even if they were exposed high iodophore concentrations. These results indicate that *C. psychrophila* may be located at the inner part of the eggs where povidone-iodine cannot

Table 4. Comparison of methods in the detection of *C. psychrophila* from coho salmon eggs disinfected with povidone-iodine just after bacterial infection

Group	Detection of <i>C. psychrophila</i> by		
	Plate count * ¹	Broth culture * ²	
Experimentally infected	1	0/5* ³	4/5
	2	0/5	3/5
Uninfected - control	1	0/5	0/5
	2	0/5	0/5

*¹ The detection limit was 4.0×10^3 CFU/g.

*² The detection limit was 3.3×10 CFU/g.

*³ Number of eggs positive for *C. psychrophila*/number of eggs examined.

Table 5. Detection of *C. psychrophila* from salmonid eggs disinfected with povidone-iodine at the eyed stage

Povidone-iodine concentration (ppm)	Group	Number of <i>C. psychrophila</i> recovered from eyed eggs		
		Coho salmon	Rainbow trout	Masu salmon
0	1	4.0×10^4 * ¹	4.0×10^7	8.0×10^6
	2	8.0×10^5	4.4×10^7	4.0×10^6
50	1	8.0×10^5	NT* ²	NT
	2	4.0×10^5	NT	NT
100	1	4.0×10^5	1.7×10^7	1.6×10^6
	2	8.0×10^4	4.0×10^6	8.0×10^6
200	1	4.0×10^5	1.4×10^7	1.2×10^6
	2	3.6×10^5	8.0×10^5	1.6×10^7
400	1	2.4×10^5	NT	NT
	2	4.8×10^5	NT	NT
600	1	4.0×10^5	NT	NT
	2	8.0×10^5	NT	NT
800	1	8.0×10^3	NT	NT
	2	4.4×10^5	NT	NT
1,000	1	4.0×10^4	NT	NT
	2	8.0×10^4	NT	NT

*¹ Bacterial counts were expressed in CFU/g. The detection limit was 4.0×10^3 CFU/g.

*² Not tested

Table 6. Detection of *C. psychrophila* from coho salmon eggs disinfected with povidone-iodine at the eyed stage*¹

Povidone-iodine concentration (ppm)	Group	Exposure time (min)			
		15	30	60	120
0	1	3.6 × 10 ⁵ * ²	NT* ³	NT	NT
	2	8.0 × 10 ⁶	NT	NT	NT
50	1	1.6 × 10 ⁶	9.7 × 10 ⁵	1.2 × 10 ⁶	2.9 × 10 ⁶
	2	3.2 × 10 ⁶	1.5 × 10 ⁶	3.8 × 10 ⁶	6.0 × 10 ⁶
100	1	5.0 × 10 ⁵	3.6 × 10 ⁶	3.4 × 10 ⁵	1.1 × 10 ⁶
	2	1.5 × 10 ⁶	4.0 × 10 ⁵	2.7 × 10 ⁷	1.1 × 10 ⁶
200	1	9.7 × 10 ⁵	1.6 × 10 ⁶	1.2 × 10 ⁶	8.0 × 10 ⁴
	2	1.5 × 10 ⁶	4.9 × 10 ⁵	9.2 × 10 ⁴	1.6 × 10 ⁵

*¹ At 41 days of incubation
*² Bacterial numbers were expressed in CFU/g. The detection limit was 4.0 × 10³ CFU/g.
*³ Not tested

reach. Brown et al (1997) showed the evidence that *C. psychrophila* was transmitted within salmonid eggs. They speculated that the bacterium might be located within the peri-vetellin space of the eggs, rather than the yolk itself. Further work to find *C. psychrophila*'s niche within salmonid eggs is needed.

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