

Short communication

Low Possibility of Intra-Ovum Infection with *Flavobacterium psychrophilum* or *Renibacterium salmoninarum* in the Salmonid Coelomic Cavity

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ABSTRACT—In this study, rainbow trout *Oncorhynchus mykiss* and amago salmon *O. masou* were used to study intra-ovum infection with *Flavobacterium psychrophilum* and *Renibacterium salmoninarum*, respectively. To investigate the possibility of intra-ovum infection of eggs in the coelomic cavity, the contamination of coelomic fluid and egg contents from ripe females were examined. The ranges of viable counts of *F. psychrophilum* and *R. salmoninarum* in coelomic fluid were $10^{1.0–4.2}$ and $10^{1.6–9.9}$ CFU/mL, respectively. However, neither *F. psychrophilum* nor *R. salmoninarum* was isolated from the egg contents. It was concluded that there was little possibility of intra-ovum infection of salmonid eggs in the coelomic cavity.

Key words: intra-ovum infection, coelomic fluid, salmonid egg, *Flavobacterium psychrophilum, Renibacterium salmoninarum*

In salmon aquaculture, prevention of vertical transmission of bacterial kidney disease (BKD) and bacterial cold water disease (BCWD) by intra-ovum infection is important for the production of healthy fry. According to recent studies (Kumagai and Nawata, 2010a; Kohara *et al*., 2012), intra-ovum infection with *Flavobacterium psychrophilum* or *Renibacterium salmoninarum* may occur during the water-hardening period when the pathogens enter the perivitelline space through the micropyle.

Although intra-ovum infection with *R. salmoninarum* in the coelomic cavities of females with heavily contaminated coelomic fluid has been reported (Evelyn *et al*., 1984, 1986a, 1986b; Bruno and Munro, 1986; Lee and Evelyn, 1989), fundamental questions on the mechanism of infection remained unanswered. A study on unfertilized (ovulated) eggs of salmonids indicated that although *F. psychrophilum* was widely distributed in cultured salmonid broodfish throughout Japan (Kumagai and Nawata, 2011a), intra-ovum infection with *F. psychrophilum* was unlikely to occur in the coelomic cavity (Kumagai and Nawata, 2011b). Unfortunately, the study did not report the contamination levels of coelomic fluid.

This study investigated the possibility of intra-ovum infection with *F. psychrophilum* and *R. salmoninarum* in contaminated and non-contaminated coelomic cavities of ripe salmonid females.

Materials and Methods

Collection of coelomic fluid and ovulated eggs

To study *F. psychrophilum* intra-ovum infection, samples (167 batches) of coelomic fluid and ovulated eggs were collected from two groups of ripe rainbow trout *Oncorhynchus mykiss* females cultured in 2009 at Nagano Prefectural Experimental Station where outbreaks of BCWD had been recorded (Table 1). To study *R. salmoninarum* intra-ovum infection samples (253 batches) of coelomic fluid and ovulated eggs were collected from two groups of ripe amago salmon *O. masou ishikawae* cultured in 2009 and 2010 at a private fish farm where outbreaks of BKD had occurred among amago salmon every year.

Approximately 1 mL of coelomic fluid was collected using an automatic pipetter (Yoshimizu *et al*., 1985) from each female after disinfection with an alcohol (70%) swab. Subsequently, 200 eggs were expressed from the genital opening of a female into a sterile tube. The samples of coelomic fluid and eggs were transported on ice and stored at 5°C in the laboratory. The coelomic fluid was used on the day and eggs were used within 3 days.

Viable counts of R. salmoninarum *and* F. psychrophilum *in coelomic fluid*

Agar plate Conradii method was used to determine the viable counts of *F. psychrophilum*, while the Miles and Misra method (Miles *et al*., 1938) was used to determine the viable counts of *R. salmoninarum*. The coelomic fluid was serially diluted with sterilized isotonic solution (sodium chloride 9.04 g, potassium chloride 0.24 g, calcium chloride 0.26/L) (Kohara *et al*., 2010). One hundred microliters of each solution was spread on an agar plate for the case of Conradii method, and 50 μL was dropped on an agar plate for Miles and Misra method. Enriched Anecker and Ordal's agar plates (tryptone 5 g, yeast extract 0.5 g, beef extract 0.2 g, sodium acetate 0.2 g, fatal bovine serum 50 mL/L, pH

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7.2) were used for the culture of *F. psychrophilum*, while KDM-SMRs agar plates (Matsui *et al*., 2009) were used for the culture of *R. salmoninarum*. After isolating procedures, plates were incubated at 15°C for 7 days for *F. psychrophilum* and at 15°C for 30 days for *R. salmoninarum*. Viable counts were calculated from the number of colonies on the plates.

F. psychrophilum was identified by agglutination test with an antiserum against *F. psychrophilum* FPC-840 and PCR (Yoshiura *et al*., 2006). *R. salmoninarum* was identified by agglutination test or indirect immune-fluorescent antibody technique (IFAT) with an antiserum against *R. salmoninarum* ATCC33209. Both anti-sera were provided by the Japan Fisheries Resource Conservation Association.

Isolation of bacteria from egg contents

For studies on intra-ovum infection with *F. psychrophilum,* 15 batches of rainbow trout eggs were used. Of the 15 batches, nine were collected from females which had the coelomic fluids contaminated with *F. psychrophilum*, while six batches were from females with *F. psychrophilum*-free coelomic fluids. Similarly, for studies on intra-ovum infection with *R. salmoninarum,* 11 batches of amago salmon eggs were used. Of the 11 batches, five were collected from females which had the coelomic fluids contaminated with *R. salmoninarum*, while six batches were from females with *R. salmoninarum*-free coelomic fluids. For experiments related to *F. psychrophilum* preliminary confirmation of contamination status was performed by plate culture of coelomic fluid for 3 days. For experiments related to *R. salmoninarum* preliminary confirmation of contamination status was performed by IFAT of coelomic fluid. Results from the preliminary confirmation were checked against the results of plate culture for both bacteria at the end of the experimental period.

Prior to extracting egg contents for analysis, contamination with bacteria from the egg surfaces was avoided by egg-washing with sterilized isotonic solution. Thereafter, rainbow trout eggs were disinfected with 50 ppm of povidone-iodine for 15 min, while amago salmon eggs were disinfected with 100 ppm for 20 min. To ensure that disinfection treatment did not initiate water-hardening of soft eggs, the dilution of povidone-iodine was done with sterilized isotonic solution. The absence of bacteria on the egg surfaces was confirmed by rolling 60 eggs on agar plates followed by incubation under conditions similar to those described above for coelomic fluid analysis.

To analyze egg contents for intra-ovum infection, each egg was placed into a separate well of a microplate (96 wells). Egg content was extracted with a sterilized syringe (1 mL) and needle (23 G), and smeared on an agar plate. The plates were incubated under conditions similar to those described above for coelomic fluid analysis.

Results and Discussion

Analysis of the coelomic fluids from the two groups of rainbow trout used in this study revealed *F. psychrophilum* infection rates of 8.7 and 17.3%. On the other hand, *R. salmoninarum* infection rates for the coelomic fluids of the two groups of amago salmon were 1.2 and 3.7% (Table 1). The range of viable counts of *F. psychrophilum* was $10^{1.0–4.2}$ CFU/mL and that of *R. salmoninarum* was $10^{1.6–9.9}$ CFU /mL. These results are similar to those from studies on *F. psychrophilum* (Kumagai and Nawata, 2011a) and *R. salmoninarum* (Evelyn *et al*., 1984, 1986a). It is notable that the viable counts of *R. salmoninarum* were higher than those of *F. psychrophilum*. This result probably explains why intra-ovum infection with *R. salmoninarum* is more common problem than intra-ovum infection with *F. psychrophilum*. It has also been reported that higher concentrations of *F. psychrophilum* on egg surfaces are linked to higher intra-ovum infection rates (Kumagai and Nawata, 2010a; Kohara *et al*., 2012).

In this study, no pathogens were isolated from the egg surfaces. This indicates that the egg-washing and disinfection protocol used in this study was effective. Furthermore, analysis of the egg contents showed that there was no intra-ovum infection with *F. psychrophilum* or *R. salmoninarum* (Table 2). This was irrespective of whether the eggs were from females with contaminated coelomic fluid or not. This finding differs from previous

Table 1. Isolation of *F. psychrophilum* and *R. salmoninarum* from coelomic fluids of rainbow trout and amago salmon respectively

Year	Brood fish		Isolation of bacteria from coelomic fluid			
	Species	No. of cultured fish	Bacteria	No. of examined females	No. of infected females (%)	Range of viable counts (log_{10}CFU/mL)
2009	rainbow trout*	1,700	*F. psychrophilum*	75	13 (17.3)	1.0 – 2.7
		2,300		92	8 (8.7)	1.0 – 4.2
2009	amago salmon*	900	*R. salmoninarum*	81	3 (3.7)	8.1 – 9.9
2010		600		172	2 (1.2)	1.6 – 9.5

* Rainbow trout were cultured at Nagano Prefectural Fisheries Experimental Station, while amago salmon were cultured at a private fish farm in Nagano prefecture.

studies (Evelyn *et al*., 1984, 1986a, 1986b; Bruno and Munro, 1986; Lee and Evelyn, 1989) which relate the intra-ovum infection of soft eggs with *R. salmoninarum* to the contamination of coelomic fluid.

In those studies on *R. salmoninarum*, egg disinfection protocols involved rinsing with fresh water or disinfection with povidone-iodine and antibiotics dissolved in water. It is postulated that the use of water in disinfection protocols caused water-hardening which enabled *R. salmoninarum* to enter eggs through the micropyles by the mode we reported earlier (Kohara *et al*., 2012). In the present study, water-hardening of eggs was prevented by using isotonic solution for egg-washing and the dilution of povidone-iodine. As a result, intra-ovum infection with *F. psychrophilum* or *R. salmoninarum* did not occur even when coelomic fluid was contaminated. Therefore, it is reasonable to conclude that there is little possibility of intra-ovum infection in ovulated eggs before water-hardening. This conclusion agrees with the results of investigation by Kumagai and Nawata (2011b) on unfertilized (ovulated) eggs from domestic salmonid species in Japan.

Table 2. Viable counts of *F. psychrophilum* and *R. salmoninarum* in coelomic fluid as well as intra-ovum infection rates of ovulated eggs

Bacteria	Viable counts in coelomic fluid[*1] (log_{10}CFU/mL)	Rate of intra-ovum infection (positive eggs/examined eggs)
F. psychrophilum	4.2	0/60
	3.4	0/60
	2.7	0/60
	2.2	0/60
	2.2	0/60
	2.0	0/60
	1.8	0/60
	1.8	0/60
	1.5	0/60
	–[*2]	0/60
	–	0/60
	–	0/60
	–	0/60
	–	0/60
	–	0/60
R. salmoninarum	9.9	0/60
	9.5	0/60
	8.1	0/60
	8.1	0/60
	1.6	0/60
	–[*2]	0/60
	–	0/60
	–	0/60
	–	0/60
	–	0/60
	–	0/60

[*1] *F. psychrophilum* and *R. salmoninarum* counts were determined from rainbow trout and amago salmon respectively.
[*2] The detection limit for *F. psychrophilum* by Conradi method was 1.0 log_{10}CFU/mL, while that for *R. salmoninarum* by Miles and Misra method was 1.3 log_{10}CFU/mL.

Evelyn *et al*. (1984) has reported that *R. salmoninarum* cells were observed in the yolk of coho salmon *O. kisutch* eggs collected from severely infected females. Although in earlier study we concluded that bacteria enter the perivitelline space through the micropyle, we cannot explain the reason why the cells were in the yolk of eggs. It is, however, notable that the coho salmon eggs used in the above study had been incubated in KDM-2 broth for 38 days. Such extended storage in broth would definitely cause egg death, thus affecting the results. Other studies of Lee and Evelyn (1989) and Lee and Gordon (1987) allude to an alternative mechanism for intra-ovum infection in the body of the female. Further research using improved techniques is needed to clarify such mechanisms of intra-ovum infection.

The results of this study show that the contamination of coelomic fluid does not cause intra-ovum infection of ovulated eggs in the coelomic cavity. However, fertilizing and water-hardening of such eggs without prior elimination of bacteria may result in intra-ovum infection. High concentration of *R. salmoninarum* in coelomic fluid may also increase the risk of intra-ovum infection. The best way to prevent intra-ovum infection during artificial fertilization of salmonid eggs is to emphasize egg-washing (Kohara *et al*., 2010) and disinfection with povidone-iodine (Kumagai and Nawata, 2010b) in isotonic solution.

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