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NOTE

Shedding of *Renibacterium salmoninarum* by infected chinook salmon *Oncorhynchus tschawytscha*

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ABSTRACT: Laboratory studies of the transmission and pathogenesis of *Renibacterium salmoninarum* may describe more accurately what is occurring in the natural environment if test fish are infected by waterborne *R. salmoninarum* shed from infected fish. To quantify bacterial shedding by chinook salmon *Oncorhynchus tschawytscha* at 13°C in freshwater, groups of fish were injected intraperitoneally with *R. salmoninarum* at either 1.3×10^6 colony forming units (CFU) $fish^{-1}$ (high-dose injection group) or 1.5×10^3 CFU $fish^{-1}$ (low-dose injection group). *R. salmoninarum* infection levels were measured in the exposed fish by the enzyme-linked immunosorbent assay (BKD-ELISA). At regular intervals for 30 d, the numbers of *R. salmoninarum* shed by the injected fish were calculated on the basis of testing water samples by the membrane filtration-fluorescent antibody test (MF-FAT) and bacteriological culture. Mean BKD-ELISA optical densities (ODs) for fish in the low-dose injection group were not different from those of control fish ($p > 0.05$), and no *R. salmoninarum* were detected in water samples taken up to 30 d after injection of fish in the low-dose group. By 12 d after injection a proportion of the fish from the high-dose infection group had high (BKD-ELISA OD ≥ 1.000) to severe (BKD-ELISA OD ≥ 2.000) *R. salmoninarum* infection levels, and bacteria were detected in the water by both tests. However, measurable levels of *R. salmoninarum* were not consistently detected in the water until a proportion of the fish maintained high to severe infection levels for an additional 8 d. The concentrations of *R. salmoninarum* in the water samples ranged from undetectable up to 994 cells ml^{-1} on the basis of the MF-FAT, and up to 1850 CFU ml^{-1} on the basis of bacteriological culture. The results suggest that chinook salmon infected with *R. salmoninarum* by injection of approximately 1×10^6 CFU $fish^{-1}$ can be used as the source of infection in cohabitation challenges beginning 20 d after injection.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial shedding · Cohabitation challenge

Bacterial kidney disease (BKD) of salmonids is a chronic, granulomatous infection caused by the Gram-positive bacterium *Renibacterium salmoninarum* (Fryer & Sanders 1981). Because of the slow growth of the organism, infected fish may remain free of clinical signs until the final stages of the disease. *R. salmoninarum* can be transmitted vertically (Evelyn et al. 1986), and horizontally from affected fish in freshwater (Mitchum & Sherman 1981, Bell et al. 1984) or seawater (Balfry et al. 1996). Several routes of infection by waterborne *R. salmoninarum* have been reported, including the skin (Hoffman et al. 1984), eye (Hendricks & Leek 1975, Hoffman et al. 1984), and external abrasions (Evenden et al. 1993), or the gut following ingestion of contaminated faeces (Balfry et al. 1996). The exact mechanisms by which *R. salmoninarum* colonizes tissues or cells of healthy fish, however, are poorly understood.

To elucidate mechanisms of the horizontal transmission and pathogenesis of *Renibacterium salmoninarum*, it will be necessary to develop a reproducible laboratory challenge. Many studies with *R. salmoninarum* have relied on the use of naturally infected fish (Young & Chapman 1978, Hoffmann et al. 1984, Moles 1997), or fish infected by injection with large numbers of bacteria (Bell et al. 1984, Bruno 1986, Bruno & Munro 1986, Sami et al. 1992). The use of naturally infected fish makes it difficult to study the early stages of the disease because the researcher has neither control over the route by which the infection began nor an accurate estimate of its duration at the time of sampling. Whereas fish can be infected reliably with *R. salmoninarum* by injection, this method is not suitable for studying horizontal transmission because it bypasses many of the fish's natural barriers. A more natural method of infection is to introduce the bacteria into the water by using either cultured *R. salmoninarum* or infected fish as the source of waterborne bacteria (Murray et al. 1992). In cohabitation challenges with fish infected with *R. salmoninarum* by injection, it is important to introduce healthy test fish at a time when the infected fish are shedding bacteria.

The purpose of this investigation was to quantify the shedding of *Renibacterium salmoninarum* by chinook

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salmon *Oncorhynchus tshawytscha* infected by an injection challenge. These data were used to identify factors that may be important for a successful cohabitation challenge.

Materials and methods. ***Experimental fish:*** Chinook salmon were obtained as eggs (brood year 1996) from Abernathy Salmon Culture Technology Center (U.S. Fish & Wildlife Service), Longview, Washington. Eggs from 5 adults that were categorized negative for *Renibacterium salmoninarum* antigen on the basis of testing kidney tissue by the enzyme-linked immunosorbent assay (BKD-ELISA described below) were pooled and transported to the Western Fisheries Research Center (U.S. Geological Survey), Seattle, Washington, for rearing in sand-filtered Lake Washington water sterilized with ultraviolet irradiation. At the time of this study the fish were 10 mo old with an average weight of 27 g.

Culture of Renibacterium salmoninarum for challenge: The ninth laboratory passage of *Renibacterium salmoninarum* isolate DWK90 originally cultured from a juvenile chinook salmon was grown at 15°C for 7 d in a stirred KDM2 broth medium (Evelyn 1977) as described by Pascho et al. (1997). The cells were separated from the growth medium by centrifugation at 5000 × *g* for 20 min at 4°C and resuspended in phosphate buffered saline (PBS) supplemented with 0.1% (w/v) peptone (PBS-peptone), then enumerated by the membrane filtration-fluorescent antibody test (MF-FAT) described below. Based on the MF-FAT results, 2 bacterial challenge doses were prepared in PBS-peptone. On the basis of bacteriological culture (Pascho et al. 1997), the concentrations of viable *R. salmoninarum* in the preparations were 7.5×10^3 colony forming units (CFU) ml^{-1} for the low-dose injection group and 6.5×10^6 CFU ml^{-1} for the high-dose injection group.

Injection challenge: Groups of 100 fish were injected intraperitoneally with either 1.3×10^6 CFU $fish^{-1}$ (high-dose injection group) or 1.5×10^3 CFU $fish^{-1}$ (low-dose injection group) in 200 µl. A third group of 100 fish was injected with 200 µl PBS-peptone and served as a control. Each group was held at a starting density of 13 g l^{-1} in a separate tank supplied with single-pass treated lake water averaging 13°C.

Monitoring of bacterial shedding: Water samples were collected 5, 12, 15, 18, 20, 22, 24, 26, 28, and 30 d after injection for the MF-FAT and bacteriological culture. On each sample day, 10 fish from each group were put into one of three 5 l aerated buckets supplied with the running treated 13°C lake water. The fish were held at a density of 54 g l^{-1} and a flow rate of about 0.67 l min^{-1} for 4 h. The water flow was then turned off and 2 water samples were taken immediately from each bucket. The first sample was used to quantify the total number of *Renibacterium salmoninarum* cells by the MF-FAT; 100 ml of water was preserved by the addition of 0.01 g thimerosal. Three 10 ml aliquots from each preserved water sample were filtered separately through syringe-mounted 0.2 µm pore-size polycarbonate membranes and stained with fluorescein isothiocyanate-conjugated polyclonal goat antiserum against *R. salmoninarum* (Kirkegaard & Perry Laboratories, Inc., USA). Each filter was examined at 1000× magnification and the numbers of fluorescing bacteria with the correct size and morphology were tabulated in 150 microscope fields. Bacterial counts as cells ml^{-1} were calculated as described by Elliott & Barila (1987).

For bacteriological culture, a second 5 ml water sample from each bucket was combined with 5 ml of 0.85% (w/v) saline containing 0.1% (w/v) peptone and the following antimicrobial compounds: cycloheximide at 10 mg ml^{-1}, D-cycloserine at 12.5 mg ml^{-1}, polymyxin B at 12.5 mg ml^{-1}, and oxolinic acid at 2.5 mg ml^{-1}. The treatment solution was intended to reduce the number of contaminating organisms while preserving the viability of *Renibacterium salmoninarum*. Treated water samples were plated undiluted at 200 µl per plate onto KDM 2 selective agar medium (Austin et al. 1983) supplemented with 1.5% (v/v) *R. salmoninarum* metabolite (Evelyn et al. 1990). Each sample was plated in triplicate and the plates were incubated at 15°C for 8 wk. Culture plates were examined at 4 wk and again at 8 wk for the presence of bacterial colonies. Colonies characteristic of *R. salmoninarum* were tested by the direct FAT (Pascho et al. 1991).

After the water samples were taken, the fish were killed with a lethal dose of MS-222 and frozen at −70°C for later processing for the BKD-ELISA.

BKD-ELISA: The BKD-ELISA of Pascho & Mulcahy (1987) was used with the modifications described by Pascho et al. (1991) to measure the prevalence and levels of *Renibacterium salmoninarum* antigen in both parents in mating pairs that provided eggs for the study, and in fish from the subgroups used to monitor bacterial shedding. Kidney tissue from adult fish was prepared as described by Pascho et al. (1991).

A pooled tissue homogenate consisting of the entire kidney and spleen was prepared for each fish in the monitoring subgroups as described by Pascho et al. (1997). For the BKD-ELISA, each adult fish kidney homogenate was diluted 1:4 (w/v) and each tissue pool homogenate was diluted 1:8 (w/v) in PBS pH 7.4 supplemented with 0.05% (v/v) Tween 20, then heated at 100°C for 15 min. The homogenates were then centrifuged at 8800 × *g* for 15 min at 22°C and the supernatant was used for testing in the BKD-ELISA. Samples with a mean BKD-ELISA absorbance (OD) ≥0.097 were considered positive for *Renibacterium salmoninarum* antigen.

Statistical analyses: Analysis of variance was used to compare the mean BKD-ELISA OD values of fish injected with *Renibacterium salmoninarum* with those of control fish. To determine if a correlation existed between the bacterial counts obtained by culture and the MF-FAT, a correlation coefficient (r) was calculated using all the sample dates.

Results and discussion. Under the conditions of this study, chinook salmon infected with *Renibacterium salmoninarum* by injection began shedding the bacterium in as few as 12 d. To consistantly attain measurable levels of *R. salmoninarum* in the water, however, several factors were important: the injection dose, the mean level of *R. salmoninarum* infection for a group of injected fish, and the number of days after injection. A satisfactory correlation (r = 0.98) was observed betweeen bacterial concentrations calculated from culture plates and those obtained from MF-FAT counts, and either method would be acceptable for monitoring bacterial shedding during a cohabitation challenge. Unfortunately, we found it difficult to calculate the proportion of viable bacteria in the various samples because the number of CFU often exceeded the total number of bacteria estimated on the basis of the MF-FAT. The hydrophobicity of *R. salmoninarum* results in autoaggregration, which can be reversed by suspending the bacteria in a low ionic strength buffer (Daly & Stevenson 1987). The higher bacterial concentrations calculated on the basis of bacteriological culture may have been a result of *R. salmoninarum* aggregates being disrupted during the preliminary incubation in saline pretreatment medium.

Mean infection levels as measured by the BKD-ELISA for the control and low-dose injection groups were similar on each sample date (data not shown; $p > 0.05$). Mean BKD-ELISA ODs for fish in the control group ranged from 0.095 to 0.200 and for the low-dose injection group from 0.114 to 0.293. In contrast, fish sampled from the high-dose injection group produced mean BKD-ELISA ODs ranging from 0.547 to 1.843 (Table 1). These findings suggested that the low dosage of *Renibacterium salmoninarum* was probably insufficient to cause infection levels high enough for bacterial shedding within the duration of this study. In a separate study, we observed similar results when 10 g chinook salmon were injected intraperitoneally with 2×10^4 *R. salmoninarum*. After 14 d, the mean BKD-ELISA OD for 81 fish was 0.270, and no *R. salmoninarum* cells could be detected in the water by the MF-FAT at this time (Elliott & Pascho 1993). Analysis by the MF-FAT and bacteriological culture of samples from the control and low-dose injection groups in the current study indicated that *R. salmoninarum* was not detectable in the water up to 30 d after injection of the fish.

Whereas fish with high (OD ≥ 1.000) to severe (OD ≥ 2.000) infection levels could be found in the high-dose injection group by Day 12, bacteria were not detected consistently in the water until the mean infection level remained elevated for an additional 8 d (Table 1). The Day 5 sample in the high-dose injection group contained 2 highly infected fish (OD ≥ 1.000). From 12 d post-injection until the end of the experiment, at least 1 severely infected fish (OD ≥ 2.000) was detected in

Table 1. Levels of *Renibacterium salmoninarum* in the tissues of chinook salmon infected at 13°C in freshwater by intraperitoneal injection of 1.3×10^6 CFU (high-dose injection group), and in the water at different times after injection of the fish. On each sample day, 10 fish were held in a 5 l flow-through bucket for 4 h, then the water was sampled for analysis by culture and the MF-FAT. Tissues from the 10 fish were then analyzed individually by the BKD-ELISA

Day	BKD-ELISA tissue analysis			*R. salmoninarum* water analysis	
	Mean OD 492 (±SD)[a]	No. of fish with mean OD ≥ 1.000[b]	No. of fish with mean OD ≥ 2.000[c]	Bacteriological culture (CFU ml^{-1})	MF-FAT (cells ml^{-1})
5	0.547 (0.482)	2	0	0	0
12	1.484 (0.902)	7	5	3	20
15	1.280 (0.842)	5	3	20	39
18	1.158 (0.689)	5	1	0	1
20	1.248 (0.967)	5	4	110	250
22	1.736 (0.979)	7	6	1850	994
24	1.734 (0.667)	9	4	130	227
26	1.504 (0.987)	6	4	150	130
28	1.724 (0.977)	7	5	26	18
30	1.843 (0.862)	8	6	983	664

[a]BKD-ELISA ODs greater than 0.097 were considered positive for an *R. salmoninarum* infection

[b]Fish with mean BKD-ELISA OD between 1.000 and 1.999 were categorized as having a high level of *R. salmoninarum*

[c]Fish with mean BKD-ELISA OD ≥ 2.000 were categorized as having a severe level of *R. salmoninarum*, and the greatest likelihood of shedding the bacterium

each high group sample (Table 1). In contrast, only 1 fish in 1 sample (Day 20) from the entire low-dose injection group was highly infected (OD = 1.179).

The low number of bacteria detected in the water of the Day 12 sample, despite the presence of 5 fish in this sample with ODs >2.000, suggested that 12 d may not have been sufficient time for some of the severely infected fish to shed detectable numbers of bacteria under the conditions of this experiment. Interestingly, the sample for Day 18 showed no *Renibacterium salmoninarum* in the water sample culture. This sample contained only 1 fish with an OD level greater than 2.000, indicating that a severely infected fish is not necessarily shedding bacteria at any given time. These results demonstrated the practical necessity of injecting a sufficient number of fish for horizontal transmission experiments. We found that even though 100 fish were injected with the high dosage of *R. salmoninarum* under identical conditions, the end result was a group of fish with vastly different infection levels.

Evenden et al. (1993) emphasized that researchers need to know how many *Renibacterium salmoninarum* cells must be present in the water to initiate an infection. The effective bacterial concentration, however, may depend on the challenge conditions. The *R. salmoninarum* concentrations measured in the current study were several orders of magnitude lower than those used successfully for relatively short (30 min) immersion challenges in other studies (Murray et al. 1992, Jansson & Ljungberg 1998). Murray et al. (1992) recommended that the challenge suspension contain 1×10^6 or more *R. salmoninarum* if the evaluation is to be based on relative survival. However, survival estimates may not be necessary to evaluate the effects of cohabitation challenges. We have found that chinook salmon can become infected in freshwater when they are exposed to as few as 7×10^2 *R. salmoninarum* ml^{-1} for 24 h (Elliott & Pascho 1995). These results were based on detecting quantifiable levels of *R. salmoninarum* antigen in the tissues by the BKD-ELISA about 100 d after exposure. The goal of the current study was to determine when infected chinook salmon begin shedding *R. salmoninarum* near those levels so they could be used in relatively short-term (48 h) cohabitation challenges monitored by the BKD-ELISA. Under these experimental criteria, suitable levels of *R. salmoninarum* were measured in the water 20 d or longer after injection. An alternate approach is to continue the cohabitation challenge indefinitely. Murray et al. (1992) reported that the relative survival among groups of chinook salmon can be measured when test fish are challenged by constant cohabitation with infected fish when as few as 5% of the fish are the source of infection. The extended rearing periods necessary for significant mortality among the test fish may have allowed injected fish with slowly progressing infections to reach severe levels. However, these authors did not measure the levels of *R. salmoninarum* in the water during the cohabitation challenges. Either approach may be appropriate, depending on whether the experimental question requires healthy fish to have a transient or continuous exposure to other fish infected with *R. salmoninarum*. The present results confirm that chinook salmon infected with *R. salmoninarum* by injection will shed detectable levels of the bacterium, and could be used in short-term cohabitation challenges if healthy fish are introduced at the appropriate time after injection.

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