

Horizontal transfer of *Renibacterium salmoninarum* among farmed salmonids via the fecal-oral route

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ABSTRACT: *Renibacterium salmoninarum* (Rs), the causative agent of bacterial kidney disease (BKD), can be transmitted vertically (i.e. from parent to progeny) and horizontally (i.e. from fish to fish). The purpose of this study was to investigate the role of the fecal-oral route of horizontal transmission among farmed salmon held in seawater. Horizontal transmission probably explained the significant increase in prevalence of Rs observed within a regularly sampled population of chinook salmon *Oncorhynchus tshawytscha* held in a seawater netpen. Viable Rs was detected in the seawater sampled from within a netpen of BKD-affected chinook, and once shed into seawater survives long enough to be ingested by neighbouring fish. The feces from these fish appeared to be the source of Rs in the seawater. Survival experiments revealed that Rs remained viable in seawater for up to 1 wk. A fecal-oral route of horizontal transmission was demonstrated by orally intubating Rs-laden feces into young coho salmon *O. kisutch*. The Rs-intubated group experienced significantly higher BKD-related mortality than the control group: 98 and 70%, respectively. This study indicates that the fecal-oral route of horizontal transmission may contribute significantly to the increasing prevalence of BKD in farmed salmon.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial kidney disease · Horizontal transmission · Aquaculture

INTRODUCTION

Bacterial kidney disease (BKD) is a systemic and usually chronic disease of wild and farmed salmonids. The causative organism is *Renibacterium salmoninarum* (Rs), a Gram-positive, rod-shaped bacterium (Sanders & Fryer 1980). BKD causes mortality in salmonids reared in fresh water and in seawater, resulting in a significant economic loss to the salmon farming industry in British Columbia (B.C.), Canada. Attempts to reduce BKD-related mortalities by chemotherapy and vaccination have met with minimal success (Evelyn 1988). The intracellular survival of Rs has been cited as the main reason for the difficulty associated with developing

effective control methods (Evelyn 1988). Rs can be transmitted horizontally between fish and vertically from the female parent to her progeny via the eggs (Evelyn 1988). However, the relative importance of each mode of transmission is unknown.

The salmon farming industry in B.C. routinely screens potential broodstock in an attempt to reduce the vertical transmission of the pathogen and subsequent BKD-related mortalities. Despite this, BKD is still prevalent among farmed salmon. Evelyn et al. (1984) reported that 15% of the eggs from heavily infected wild females can be infected with Rs. One is therefore left to explain why the prevalence of Rs in populations of farmed salmon can be as high as 80%. The most obvious explanation is that Rs is being transmitted horizontally between salmon reared in seawater netpens. Vertical transmission of Rs may be

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responsible for the initial low levels of Rs present in young fish. The process by which Rs is horizontally transmitted between fish is uncertain. Direct contact with infected salmon may not be the only way for healthy fish to be exposed to the pathogen. Rs infections may be acquired from water 'contaminated' by infected fish. The primary sites of entry into the fish are the gills, gut, eye, and external lesions or abrasions (Evenden et al. 1993).

The ingestion of Rs-laden fecal material from infected salmon may be a significant route for the horizontal transmission of BKD. Rs has been found in the lower gut (Bullock et al. 1978) and feces (Mitchum & Sherman 1981) of freshwater-infected salmonids. When farmed fish are being fed, they frequently defecate and can be seen mouthing and presumably ingesting this fecal material (pers. obs.). BKD can be produced by feeding Rs-laden food to salmon (Wood & Wallis 1955), therefore it is possible that farmed salmon consuming Rs-laden feces also become infected.

The experiments described here were designed to establish and document the presence of viable Rs in the feces of seawater-reared farmed salmon, and to test the hypothesis that Rs can be transmitted horizontally among salmon reared in seawater netpens by the fecal-oral route.

MATERIALS AND METHODS

Prevalence of Rs and BKD in farmed chinook salmon. Fish: A survey of the prevalence of BKD and Rs in seawater-farmed chinook salmon *Oncorhynchus tshawytscha* was carried out in cooperation with a commercial farm located on the northwest coast of Vancouver Island, B.C. A population of chinook (73 000) was sampled for the first time 9 mo after introduction to seawater, and then every 60 d, for a total of 6 samples over a 12 mo period.

Netpen sampling and necropsy procedures: Samples of 57 fish were randomly dip-netted from a seined sample of fish, and placed in a bath containing a lethal concentration of the anesthetic (2-phenoxyethanol). The size of the sample permitted the detection (with a 95% confidence limit) of at least 1 carrier fish in a population where the prevalence of the disease was $\geq 5\%$ (Ossiander & Wedemeyer 1983). The fish were placed on ice, and examined by necropsy within 24 h post-mortem.

Each fish was examined for internal and external abnormalities, and those exhibiting white granulomatous lesions (typical of BKD) in the kidney were considered to be clinically infected with BKD. The prevalence of clinically infected fish in each sample, was expressed as the percentage of fish with clinical signs

of BKD. The kidneys from each fish were aseptically removed, weighed, homogenized, washed in iced peptone-saline (P-S; 0.1/0.85% peptone/NaCl, respectively), and a 50% homogenate (w/v) prepared using P-S (Evelyn et al. 1981). Ten-fold serial dilutions of the 50% homogenate were then drop-inoculated onto selective charcoal agar plates (SKDM-C) (Daly & Stevenson 1985, Austin et al. 1983). Inoculated plates were incubated at 15°C for 6 to 8 wk. Kidney material was also smeared onto microscope slides for the detection of Rs using the indirect fluorescent antibody technique (IFAT) (Bullock & Stuckey 1975).

Detection of Rs in seawater netpens. In the following experiment, seawater was sampled to determine the concentration of Rs present in the seawater of a netpen containing BKD-affected salmon.

Seawater sampling: Seawater samples were removed from within a netpen containing 14 000 chinook salmon that were experiencing high BKD-related mortalities (80% cumulative mortality). Some feed was sprinkled on the water surface to attract the fish and stimulate them to feed. When the fish were visible at the surface of the water, the seawater samples were taken using a 3 l Van Dorn water sampler from depths of 1, 2, 3, and 4 m. Seawater samples were placed in sterile containers, and transported to the laboratory at *in situ* temperature (10°C) to prevent possible changes in the natural microflora. Following the seawater sampling, the chinook population within the netpen was sampled for the prevalence of BKD and Rs using the procedures described above.

Detection of Rs in seawater and fish: Within 6 h of collection, a 1 l volume of water from each depth sampled was centrifuged (at 5°C and 5000 $\times g$ for 30 min). The resulting pellet was resuspended in 10 ml sterile, chilled P-S, mixed, and 0.1 ml portions spread onto SKDM-C agar plates in triplicate. Two 10-fold dilutions of the pellet suspension were similarly cultured in triplicate. The 'nurse-culture' technique (Evelyn et al. 1989) was used to accelerate the growth of Rs. After 6 wk incubation (at 15°C), growth was confirmed as Rs by IFAT. Immediately after the culture procedure, all the suspensions were fixed in formalin (2% v/v final concentration). The concentration of Rs in the suspensions was determined by the membrane filtration-IFAT (MF-IFAT) (Lee 1989), and the number of Rs cells ml⁻¹ of seawater was calculated. Kidney and feces were aseptically removed from the sampled chinook (n = 57) and examined for the presence of Rs using the IFAT and culture methods (as described above). The feces was obtained by gently squeezing the fish and collecting the expressed fecal material into a sterile test tube.

Survival of Rs in seawater. An experiment was designed to examine the ability of Rs to survive in seawater

Bacteria: Eighteen-day-old Rs (Pacific Biological Station, Nanaimo, B.C., isolate no. 384) cells were harvested from SKDM-C agar plates and transferred into sterile, ice-cold P-S. The suspension was aseptically homogenized with a teflon-tipped homogenizer to disrupt aggregates (microscopically confirmed). The concentration of Rs in the suspension was estimated from absorbance measurements made at 420 nm, and an appropriate volume of the suspension was added to each of the test solutions to produce a final estimated concentration of 10^6 colony-forming units (cfu) ml^{-1} . Actual cfu ml^{-1} values were determined from plate (SKDM-C) counts.

Suspending media: The survival of Rs was examined in seawater (SW), filter-sterile seawater (FSW), and saline (SAL, 0.85% NaCl). The viability of Rs in sterile SKDM-2 broth (SKDM-2B; Evelyn 1977) was also determined as a control. Sea water (10°C, 22‰ salinity) was obtained from Burrard Inlet, B.C., using a sterile, 1 l Niskin sampler placed at a depth of 2 m. The seawater was stored in a sterile container and placed on ice for transport to the laboratory. The FSW solution was prepared from the sampled water by passing it through a 0.20 μm pore-size membrane filter (Nuclepore Corp., Pleasanton, CA, USA). Portions of test solution (75 ml each) were added to triplicate Erlenmeyer flasks.

Sampling protocol: Each flask was sampled at time 0 (T_0 , time immediately after Rs was added), and then at 8 h, 1, 2, 4, 7, 14, 22, and 28 d post-inoculation. The flasks were incubated in the dark at 10°C (*in situ* temperature of the seawater at the time of sampling) for 28 d. Each flask was gently shaken daily. Sampling involved aseptically removing 3 ml from each flask, preparing serial dilutions in iced, sterile P-S, and drop-inoculating 25 μl samples in triplicate, onto SKDM-C agar plates. The 'nurse-culture' technique was used to accelerate the growth of Rs (Evelyn et al. 1989). After incubation at 15°C for 8 wk, Rs colonies (IFAT confirmed) were counted and the average cfu ml^{-1} calculated for each test solution. In addition, to determine if Rs was adhering to the flasks, the inside walls were wiped with a sterile swab, which was then used to inoculate SKDM-C plates.

Fecal challenge experiment. Fish: Coho salmon *Oncorhynchus kisutch* (18 g average weight) reared at a commercial farm were used for this experiment. A pre-challenge sample of 57 fish was randomly taken from the coho population, to determine the background levels of Rs in the feces and kidney (using IFAT and culture methods described above). The remaining fish were then randomly divided into 2 groups (treated and control) of 300 fish each and placed into separate netpens located approximately 300 m apart from each other and the company netpens.

Preparation of inoculum: Fecal material was collected from rainbow trout *Oncorhynchus mykiss*, autoclaved, and aseptically homogenized to produce an easily injectable slurry. Feces was collected from locally available rainbow trout because it was not possible to obtain the required amount of fecal material from coho and chinook salmon. A 2 wk old culture of Rs (Pacific Biological Station, isolate no. 384) was harvested from SKDM-C plates into the iced, sterile fecal slurry. Two different concentrations of Rs were prepared, one for injection (2.5×10^6 cfu ml^{-1}) and the second for intubation (1.0×10^6 cfu ml^{-1}). The different concentrations ensured that despite the delivery of unequal challenge volumes (0.1 ml injected and 0.25 ml intubated) the overall challenge dose would be the same, i.e. 2.5×10^5 cfu fish $^{-1}$. The actual dose of Rs received by each fish was determined from plate (SKDM-C) counts performed at the time of challenge.

Challenge procedure. All fish were anesthetized (2-phenoxyethanol, 0.025% v/v) for the challenge procedure. Of the fish in the treated group, 150 were injected (ip) with 0.1 ml of Rs-laden fecal slurry, and 150 orally intubated with 0.25 ml of the Rs-laden fecal slurry. The injection challenge method was included as a positive control, to confirm the virulence of Rs in the fecal slurry. The fish in the negative control group were similarly challenged but with sterile fecal slurry. The adipose fins of the injected fish in each group were clipped to distinguish them from the intubated fish. Intubation was performed using an Eppendorf Repeater Pipetteman 4700, equipped with a sterile 'combitip' that was inserted into the esophagus of the anesthetized fish.

Data collection and analysis: The experiment was terminated at 73 and 75 d post-challenge for the treated and control groups, respectively. At that time, the fish that survived the challenges were killed with a lethal dose of anesthetic (2-phenoxyethanol). The kidney and fecal material of each fish was examined for Rs by the IFAT and culture (SKDM-C) methods described above. Differences in mortality and prevalence of Rs infection between the treated and control fish were analyzed using a binomial test (Zar 1974). This statistical test was also used to determine if the prevalence of Rs in surviving fish was significantly different from that in the pre-challenge sample.

RESULTS

Prevalence of Rs and BKD in farmed chinook salmon

Results from the farm survey indicated an overall increasing trend in the prevalence of detectable Rs and clinical BKD with time (Fig. 1). The prevalence of Rs as

detected by IFAT and culture increased significantly ($p < 0.001$) from approximately 5% at the start of the survey, to 61 and 100%, respectively. Necropsy results also revealed a significant increase ($p < 0.001$) in the prevalence chinook salmon displaying clinical signs of BKD (white granulomatous kidney lesions).

Detection of Rs in seawater netpens

Attempts to isolate and quantify Rs from the different water depths met with minimal success because the culture plates (SKDM-C) were readily contaminated with faster growing seawater microflora. Only those plates inoculated with the 1 m seawater sample were found to contain a few small, white colonies that were confirmed as Rs using the IFAT. The MF-IFAT results provided an estimate of 254.6 ± 179.1 (SE) Rs cells ml^{-1} in the 1 m water sample. We failed to detect Rs in any other seawater sample by either method.

The population of chinook salmon from the netpen that served as the source of the seawater samples was found to be severely affected with BKD. Rs was detected by culture in 100% of the kidneys and in 86% of the fecal samples. Interestingly, Rs was cultured from the feces of 4 of the 7 fish that displayed no clinical signs of BKD.

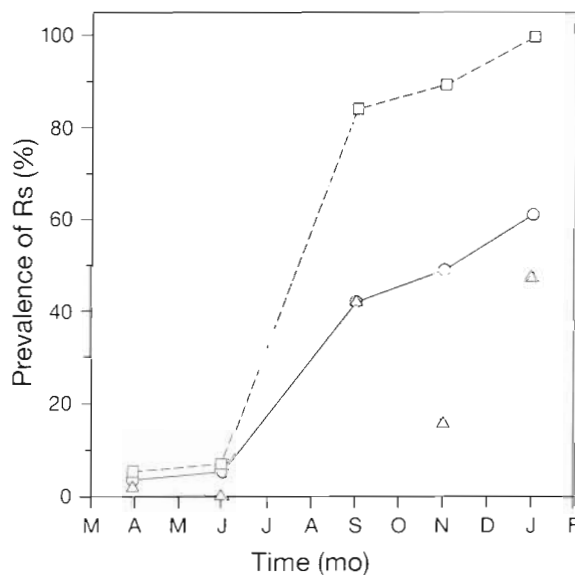


Fig. 1. Prevalence of BKD and *Renibacterium salmoninarum* (Rs) in a population of seawater-reared farmed chinook salmon *Oncorhynchus tshawytscha*. Prevalence of BKD is expressed as the percent of fish in the sample with clinical signs of BKD (dotted line). The prevalence of Rs in kidney homogenates was determined by the indirect fluorescent antibody technique (IFAT) (solid line) and by culture on SKDM-C agar plates (dashed line). The chinook population was sampled ($n = 57$) approximately every 60 d

The concentration of viable Rs in the feces could not be accurately determined, because faster growing bacteria present in the feces contaminated most of the plates. However, a few Rs were detected in the higher dilutions (10^{-6}) prepared from the fecal samples taken from heavily infected fish. Therefore, it was estimated that feces contained as many as 1×10^6 cfu Rs g^{-1} feces.

Survival of Rs in seawater

Rs demonstrated limited survival in the different suspending media (Fig. 2). The concentration of Rs in the SW, FSW, and SAL media all decreased significantly ($p < 0.05$) after the T_0 sample. Viable Rs was detected in SW for up to 1 wk; however, because the flasks became contaminated (presumably due to growth of the natural microflora), the concentration of Rs (cfu ml^{-1}) could not be calculated. In FSW, where the seawater microflora was filtered out, Rs was able to survive for up to 14 d. Saline was the least favorable of the suspending media, as Rs was cultured for only up to 2 d. The number of viable Rs cells suspended in SKDM-2B (control) increased steadily throughout the study period.

Fecal challenge experiment

The fish farm was not accessible for the post-challenge period, and as a result dead fish were not collected and the cause of death could not be determined. The percent cumulative mortality in the group challenged by oral intubation was significantly higher ($p < 0.001$) in the treated group than that observed in the control group (98 and 70%, respectively) (Fig. 3). We assume that this difference in mortality was due to Rs-related deaths. The post-challenge cumulative mortality data demonstrated that the oral intubation method was as effective as the ip injection method for producing BKD-related mortalities. No significant difference was observed between the challenge methods.

Rs was not isolated from cultured kidney tissue or fecal material, obtained from the pre-challenge sample or from the sample of fish surviving the challenge. However in challenge survivors, significant differences in the prevalence of Rs in the control and treated groups were detected by the IFAT ($p < 0.05$). The prevalence of Rs in the kidney and fecal samples of the challenge survivors increased significantly over the pre-challenge levels (Table 1). There was a significant difference in the prevalence of Rs between the treated and control fish ($p < 0.05$) in almost all of the comparisons. The exception was there was no significant difference in the prevalence of Rs in the feces between the treated and control groups that were challenged by the ip injection route. The prevalence of Rs was higher in the kidney than in the feces in all groups.

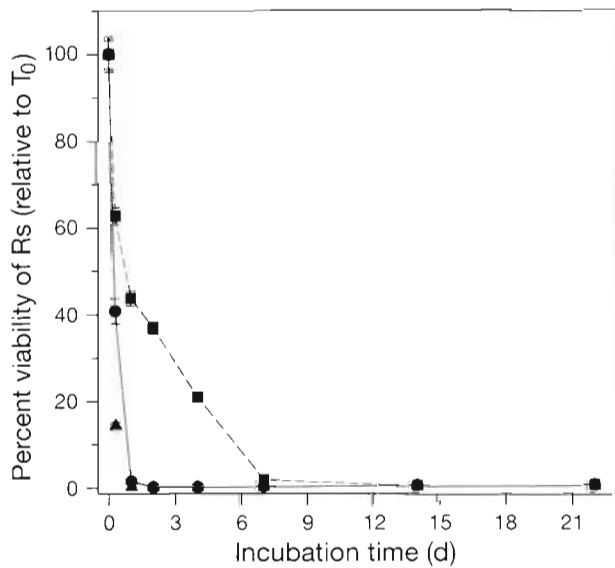


Fig. 2. Survival of *Renibacterium salmoninarum* (Rs) in seawater (solid line), filter sterile seawater (dashed line), and sterile saline (0.85% NaCl; dotted line). Growth of Rs on SKDM-C expressed as a percentage of growth at T_0 , time 0). The different suspending media were inoculated with 5×10^6 cfu ml, sampled immediately (T_0), and at 8 h, 1, 2, 4, 7, 14 and 22 d post-inoculation. Controls (Rs suspended in SKDM-2 broth) were also examined; growth increased steadily throughout the study period. Means \pm SE bars, $n = 3$

There was no significant difference ($p > 0.05$) in the prevalence of infection (of survivors) between the intubated and the injected challenge groups in either netpen (Table 1). There was also no significant difference between the prevalence of infection detected in the feces and kidney samples.

Table 1 Percent prevalence of *Renibacterium salmoninarum* (Rs) in the kidney and feces of coho salmon (*Oncorhynchus kisutch*) before and after fecal challenge (survivors sacrificed at 73 and 75 d post-challenge for the treated and control groups, respectively). The coho were challenged with sterile feces (control group) or Rs-laden feces (5×10^5 cfu Rs fish⁻¹) (treated group) by oral intubation and intra-peritoneal (ip) injection. Prevalence expressed as percent positives of total number examined. Rs was detected by IFAT (see text)

Challenge method Tissue sampled	Pre-challenge	Challenge survivors	
		Control group	Treated group
Oral intubation			
Kidney ^{ab}	5.3 (3/57)	24.4 (11/45)	100 (3/3)
Feces ^{ab}	0 (0/57)	11.1 (5/45)	100 (3/3)
Ip injection			
Kidney ^{ab}	5.3 (3/57)	26.5 (9/34)	100 (4/4)
Feces ^{ab}	0 (0/57)	29.4 (10/34)	75.0 (3/4)

^a Significant difference ($p < 0.05$) between the treated and control groups
^b Significant difference ($p < 0.05$) between the pre-challenge sample and the treated and control groups

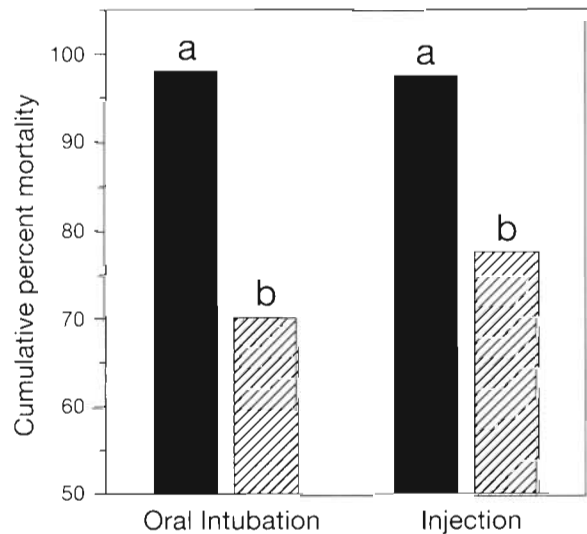


Fig. 3. Percent cumulative mortality in coho salmon *Oncorhynchus kisutch* challenged by oral intubation and by intra-peritoneal injection with Rs-laden feces (treated group; solid bars) and sterile feces (control group; hatched bars). Mortality results represent the number of deaths/total number of challenged fish ($n = 150$) at 75 and 73 d post-challenge in the treated and control groups, respectively. Identical letters over the bars indicate no significant differences between the challenge methods; different letters indicate a significant difference ($p < 0.05$) between the treated and control groups

DISCUSSION

The results support the conclusion that horizontal transmission of Rs occurs among salmonids reared in seawater netpens. Other researchers (see Evenden et al. 1993) have detected Rs in the fecal material of BKD-affected salmonids. We were also able to isolate viable Rs from the feces of clinically and subclinically seawater-reared BKD-affected salmon. The feces sampled from the chinook salmon collected during the seawater sampling experiment, revealed that perhaps as many as 1×10^6 cfu Rs g⁻¹ feces were present in fish severely affected with BKD. Embley (1983) also found high numbers of Rs (1×10^7 cfu g⁻¹) in the fecal material sampled from rainbow trout experimentally infected (10^7 cfu ip injected per fish) with Rs. A high percentage of chinook salmon yielded Rs-infected feces (86%) during the seawater sampling experiment, indicating that fecal material is a major source of Rs in netpen seawater. During feeding, fish could be seen to mouth and presum-

ably ingest (pers. obs.) feces. We did not attempt to quantify the amount of Rs ingested. However, the observation on the mouthing of feces, coupled with regular fecal sheddings, indicates the potential for a continuous oral exposure to Rs-infected feces.

The manner in which Rs contaminates fecal material in the gastrointestinal tract is uncertain. Rs in the gastrointestinal tract may be the result of ingesting Rs-infected feces (or other foodstuffs), or the pathogen may enter the gastrointestinal tract during systemic infections. For example, Rs may be released from the urinary tract and contaminate feces at the urogenital papillae. It is also possible that Rs may be released from the abdominal cavity to the feces via the abdominal pores that connect the intestine with the abdominal cavity (Goodrich 1958, as cited by Hoff 1989).

Sera & Ishida (1972) have found that few bacteria are able to survive the adverse conditions in the stomach but that they can grow vigorously once in the intestine. It is not known how the low pH and digestive enzymes present in the salmonid stomach affect the viability of Rs. However, the ability of some researchers (Wood & Wallis 1955) to produce BKD infections in salmonids by feeding Rs-contaminated food indicates that some of these cells are able to withstand such conditions. The results from the fecal challenge experiment conducted herein indicate that enough Rs cells survived the conditions in the stomach to establish systemic infections. In fact, we found the mortality and prevalence of Rs in the orally intubated fish was not significantly different from the fish challenged by ip injection (Fig. 3). This suggests that any loss in survival and virulence of the pathogen associated with the oral intubation challenge method was comparable to that observed when the Rs cells were ip injected.

The coho that were orally intubated with Rs-laden feces suffered significantly higher mortalities than control fish (Fig. 3). In addition, the prevalence of Rs in the kidney and fecal samples of the fish orally intubated with Rs-laden feces were significantly higher than found for the control fish orally intubated with sterile feces (Table 1). The inability to culture Rs from the kidney tissue and fecal material of the pre-challenge and surviving fish is puzzling, but may be related to the fact that only low numbers of Rs cells were present (as detected by IFAT). It is possible that because the fish did not appear to be undergoing active infections, perhaps most of the cells were dead.

The seawater sampling experiment was successful in demonstrating that viable Rs cells are present in the surface layer of seawater within a netpen containing BKD-affected chinook salmon. Although we were not able to quantify the number of viable Rs cells by culture, the MF-IFAT procedure used on the same seawater sample detected 255 (\pm 179 SE) Rs cells ml⁻¹

seawater. This suggests that seawater within netpens containing BKD-affected salmonids may contain 'infectious doses' of Rs. A major source of Rs in the seawater sample was likely to be the infected fecal material shed from the infected chinook salmon held in the experimental pen. Rs was detected in fecal material from 86% of the chinook salmon sampled from the netpen.

The hydrophobic characteristic (Bruno 1988) of Rs may serve to prolong its survival in seawater. Gurijala & Alexander (1990) have demonstrated that hydrophobic bacteria are able to escape predation by protozoans because the bacteria tend to adsorb to surface microlayers and particulate materials too large to be ingested by the protozoans. These findings also suggest that most of the Rs in the feces shed from infected fish will likely remain adsorbed to the organic matter in the feces rather than disperse throughout the water column. Therefore the Rs cells present within the contaminated feces probably remain there until consumed by a fish (or another organism) or until the fecal material settles on the ocean bottom.

Gowen & Bradbury (1987) estimate that approximately 26% of the food eaten by a salmon is eventually released as feces. This may have important ramifications at fish farms that hold BKD-affected salmon. Water currents can exacerbate the spread of the potentially infectious feces, and probably affect the potential range for horizontal transmission. Based on the settling velocity of fecal material (0.017 to 0.06 m s⁻¹) (Warrer-Hansen 1979, cited by Gowen & Bradbury 1987), it has been calculated that on a typical salmon farm, feces may be distributed as far as 200 m from the source (Gowen & Bradbury 1987). This calculation considers the water depth and current as well as the settling velocity of fecal pellets. Because of the large amounts of fecal material that can be produced and the effects of water currents, salmonids in the general vicinity of sources of this material run the risk of becoming infected.

Results from the survival experiments indicate that Rs is capable of remaining viable in seawater for up to 1 wk at 10°C (Fig. 2). In the absence of competing seawater microflora, we found that Rs can survive for up to 2 wk at 10°C. Evelyn (1988) found that at 15°C, Rs remained viable for 12 d in filter-sterilized seawater. In contrast, in autoclaved seawater at 17.6°C, the survival of Rs is limited to less than 4 d (Paclibare 1989). The literature suggests that the survivability of Rs in seawater is variable and depends on many factors including temperature, nutrient availability, and the initial concentration of Rs. Regardless, the survival of Rs in seawater is limited. Therefore the constant shedding of Rs (via the feces of infected fish) into the seawater is probably most important for the horizontal transmission of Rs between neighbouring fish.

To summarize, the results indicate that Rs occurs at high prevalence and often in high numbers in the feces of BKD-affected salmon. Viable Rs was found in the seawater of net pens containing BKD-affected salmonids, and proved capable of surviving in seawater long enough for horizontal transmission to occur among neighbouring fish. Coho salmon orally challenged with Rs-laden feces had significantly higher mortality and prevalence of Rs than observed in sham-challenged control fish.

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