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ASSOCIATION OF *CYTOPHAGA PSYCHROPHILA* WITH MORTALITY AMONG EYED EGGS OF ATLANTIC SALMON (*SALMO SALAR*)

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ABSTRACT: Although *Pseudomonas fluorescens* was the predominant bacterium associated with Atlantic salmon (*Salmo salar*) eggs incubated at the White River National Fish Hatchery (Bethel, Vermont) during January 1992, the fish pathogen *Cytophaga psychrophila* was isolated only from specific lots of eggs that displayed poor survival (35% eye-up).

Key words: *Cytophaga psychrophila*, Atlantic salmon, *Salmo salar* eggs, bacterial flora, *Pseudomonas fluorescens*.

INTRODUCTION

The quantity of eggs from mature, sea-run Atlantic salmon (*Salmo salar*) is a major limiting factor for the eventual success of the Connecticut River Atlantic salmon restoration effort; therefore, insuring the quality of these eggs is essential. Most feral, sea-run brood salmon are collected at the Holyoke Dam, on the main stem of the Connecticut River (Holyoke, Massachusetts, USA). Sea-run salmon also are collected at the Leesville Dam on the Salmon River (East Haddam, Connecticut, USA) and at the Rainbow Dam on the Farmington River (Windsor, Connecticut). Egg sources are further supplemented by domestic brood stocks and kelts (reconditioned, sea-run, female brood fish) to provide a maximum number of progeny for the restoration effort.

Production of progeny is limited not only by total numbers of sexually mature, adult fish, but also by less than satisfactory eye-up among fertilized eggs; eye-up is a stage of the embryo during which eye pigmentation is visible through the shell. The White River National Fish Hatchery (Bethel, Vermont) produces fry, parr, and smolts for the Connecticut River. This hatchery is used to incubate fertilized eggs from brood fish spawned at Richard Cronin National Salmon Station (Sunderland, Massachusetts), Berkshire National Fish Hatchery (Great Barrington, Massachusetts), Green Lake National Fish Hatchery (Ellsworth, Maine), Roger Reed State Fish Hatchery (Palmer, Massachusetts), Whittemore State Fish Hatchery (Waterford, Connecticut) and Kensington State Fish Hatchery (Kensington, Connecticut).

Historically, poorest eye-up occurs among eggs from sea-run brood fish at the Richard Cronin National Salmon Station. During October and November 1991, eye-up averaged 35% per lot from this facility, whereas eye-up exceeded 70% from all other sources (T. Nelson, pers. comm.). The cause of poor survival of eggs from the Richard Cronin National Salmon Station is unknown. This study was conducted to determine the bacteria associated with different lots of eggs incubated at the White River National Fish Hatchery and to determine if certain bacteria were associated with the excessive mortality.

MATERIALS AND METHODS

Eggs were transported to White River National Fish Hatchery (43°48′N, 72°42′W) during October and November 1991. The eggs originated from brood fish at the Roger Reed (42°12′N, 72°30′W), Green Lake (44°36′N, 68°24′W), Richard Cronin (42°24′N, 72°36′W), Berkshire (42°16′N, 73°24′W), Kensington (41°36′N, 72°48′W) and Whittemore (41°18′N, 72°06′W) facilities. Eyed eggs were segregated by origin into separate banks of double-stacked, eight-tray, vertical flow incubators (Heath Techna Corp., Kent, Washington, USA). The incubators were loaded at a density of about 6,000 eggs per tray. For any given lot, a sample of eggs was obtained from eggs of the last tray in the vertical flow series. In January 1992, eggs were transported on ice by overnight express mail from the White River National Fish Hatch-

ery to the National Fish Health Research Laboratory (Kearneysville, West Virginia, USA; 39°24'N, 77°36'W), where 50 eggs from each lot were examined for total bacterial content. Dead eggs were not used for bacteriological examination.

Individual eggs were placed in pre-weighed, sterile test tubes. The weight of each egg was determined, and then the egg was smashed with a jagged-edge, sterile glass rod. The egg was emulsified by repeated pipetting in a 1:10 (weight/volume) suspension of sterile phosphate-buffered saline to evaluate the total bacterial flora associated with or inside the egg. Serial $\log_{10}$ dilutions were prepared from this initial suspension, and 0.01 ml aliquots from each dilution were dropped onto Coomassie Brilliant Blue (CBB) Agar (Cipriano and Bertolini, 1988) and tryptone-yeast-gelatin (TYG) agar which consisted of tryptone (2 g), yeast extract (0.5 g), and gelatin (2 g) in 1 l distilled water (Bullock et al., 1986). Drops were allowed to absorb into the agar, and plates were inverted. Inoculated agar plates were incubated at 20 C (CBB) or 15 C (TYG). Visible colonies were counted after incubation for 72 to 96 hr. Descriptive statistics, including mean colony counts per gram of egg (n = 50) and ranges, were calculated for each of the seven lots of eggs on both plating mediums using single-factor analysis of variance. The Newman-Keuls multiple range test was used to compare mean colony counts (Newman, 1939; Keuls, 1952). Each colony, from the dilution that was counted was further subcultured and then identified by the microbiological procedures and taxonomic schemes of MacFaddin (1980) and Bullock (1972). Descriptions of *Cytophaga psychrophila* were based on Holt (1987) and Bernadet and Keronault (1989).

Because *C. psychrophila* is inert in many biochemical tests, serological analysis was conducted to further confirm bacterial identity. Whole cell lysates were prepared from suspect isolates as described by Pyle and Cipriano (1986). Lysates were subjected to sodium-dodecyl-sulfate polyacrylamide gel electrophoresis as described by Laemmli (1970), using a 4% stacking gel and 12% resolving gel. Following electrophoresis, lysates were electroblotted onto nitrocellulose, and Western blot immunoassays were conducted, as described by Cipriano and Pyle (1985). Nitrocellulose was probed with rabbit antiserum against an isolate of *C. psychrophila* (No. 217) obtained from coho salmon (*Oncorhynchus kisutch*) at the Washington state fish hatchery on the Lewis River (Woodland, Washington). A whole-cell lysate of isolate 217 was included in electrophoresis and Western blot assays as a control.

RESULTS

Bacterial counts usually did not differ significantly for samples cultured on CBB or TYG agar. One group of eggs from Cronin (sample 1) had a mean count on TYG agar that was statistically higher ($P < 0.05$; Newman-Keuls test) than counts produced on TYG agar for all other hatchery groups (Table 1). There also was a significant difference ($P < 0.05$) between colony counts on CBB and TYG agars for eggs from Cronin (sample 1). All other intra-hatchery comparisons were not significantly different. *Pseudomonas fluorescens* was the predominant bacterium cultured on CBB agar and accounted for 1,028 (91%) of 1,126 isolates identified from all sources of eggs (Table 2). To a much lesser extent, *Acinetobacter* sp., *Staphylococcus* sp., *Aeromonas hydrophila*, *Pseudomonas diminuta*, and *Enterobacter agglomerans* also were isolated.

When eggs originally were cultured on TYG agar, a long, thin, gram-negative yellow-pigmented rod was isolated that was specific for egg lots from the Richard Cronin National Salmon Station that experienced poor survival. Those eggs with bacterial counts exceeding 10^6 colony forming units (cfu)/g had pure cultures of this bacterium on TYG agar and did not grow when subcultured onto tryptic soy agar (Difco Laboratories, Inc., Detroit, Michigan, USA).

These bacteria from TYG agar had weak gliding motility, produced catalase and gelatinase, and grew on nitrate agar but did not reduce nitrate to nitrite. The organisms had negative reactions for indole, malonate, ornithine, arginine, lysine, cellulose digestion, starch hydrolysis, xanthine degradation, and KOH string tests. They did not grow on phenylalanine, Simmons citrate, urease, tween hydrolysis, and triple sugar iron agars. The bacteria also were inert for all carbohydrates tested. Phenotypic characterization of these isolates was consistent with biochemical reactions for *C. psychrophila*. These bacteria

TABLE 1. Total bacterial counts (colony forming units/gram of sample) associated with lots of Atlantic salmon eggs (n = 50 per lot) from different hatchery sources.

Egg source	Coomassie Brilliant Blue agar (mean ± standard deviation)	Tryptone yeast gelatin agar (mean ± standard deviation)
Cronin (1)	$1.2 \times 10^4 \pm 2.6 \times 10^4$	$3.1 \times 10^{6.a} \pm 1.2 \times 10^7$
Cronin (2)	$6.0 \times 10^3 \pm 1.6 \times 10^4$	$5.8 \times 10^5 \pm 1.6 \times 10^6$
Whittemore	$1.7 \times 10^5 \pm 2.0 \times 10^5$	$3.0 \times 10^4 \pm 2.7 \times 10^4$
Kensington	$5.4 \times 10^2 \pm 1.1 \times 10^3$	$2.4 \times 10^4 \pm 5.3 \times 10^4$
Berkshire	$1.2 \times 10^4 \pm 2.9 \times 10^4$	$9.5 \times 10^3 \pm 1.4 \times 10^4$
Roger Read	$1.6 \times 10^4 \pm 2.7 \times 10^4$	$1.9 \times 10^4 \pm 3.9 \times 10^4$
Green Lake	$9.3 \times 10^4 \pm 1.1 \times 10^5$	$2.8 \times 10^4 \pm 5.1 \times 10^4$

[a] Denotes statistically significant difference ($P < 0.05$), Newman-Keuls, multiple range analysis between this mean and all other means from tryptone yeast gelatin agar. This mean also was significantly different ($P < 0.05$) from the mean of Cronin (1) samples plated on Coomassie Brilliant Blue agar.

were isolated from both lots of eggs derived from Richard Cronin fish (Table 3). The frequency of distribution of other bacteria isolated on TYG agar was consistent among egg lots and was not associated with egg survival.

Based on Western blot immunoassays, rabbit antiserum to *C. psychrophila* produced intense reactions with whole-cell lysates from each of the isolates that had been phenotypically identified as *C. psychrophila* (Fig. 1). Specific reactivity differed slightly, however, from the reaction of antiserum with the homologous (No. 217) lysate. Normal rabbit serum did not react with electroblotted lysates. Results of serological and biochemical tests indicated, therefore, that the organism isolated specifically from Richard Cronin eggs was *C. psychrophila*.

DISCUSSION

The prevalence of bacteria associated with eggs of sea-run, adult Atlantic salmon was consistent with previous reports in which *Pseudomonas fluorescens* and *Cytophaga* sp. were predominant species (Bell et al., 1971; Trust, 1972; Barker et al., 1989). Unfortunately, the previous workers did not identify the *Cytophaga* species that were isolated. Barker et al. (1991) reported that *P. fluorescens* and *Cytophaga* sp. could colonize the surface of eggs from rainbow trout (*Oncorhynchus mykiss*). Although Barker et al. (1989) did not find a correlation between prevalence of these bacteria and egg survival, they suggested that high numbers of *P. fluorescens* may be deleterious to the egg. Trust (1972), however, reported a range in bacterial

TABLE 2. Percent distribution of bacterial species associated with different lots of Atlantic salmon eggs (n = 50 per lot) that were originally cultured on Coomassie Brilliant Blue agar.

Bacterium	Egg lots[a]							
	A	B	C	D	E	F	G	Total
Pseudomonas fluorescens	84	59	99.0	100	97	92	97	91
Staphylococcus sp.	3.5	18	0.7	0.0	0.0	0.9	1.4	3.1
Pseudomonas diminuta	4.3	0.0	0.0	0.0	0.0	0.0	0.0	0.4
Aeromonas hydrophila	0.9	0.8	0.0	0.0	0.0	2.6	1.0	0.7
Enterobacter agglomerans	0.9	0.0	0.3	0.0	0.0	0.9	0.7	0.4
Acinetobacter sp.	6.9	23	0.0	0.0	3.0	3.4	0.0	4.1

[a] Designation of egg lots: (A) Cronin No. 1, (B) Cronin No. 2, (C) Whittemore, (D) Kensington, (E) Berkshire, (F) Roger Reed, (G) Green Lake.

TABLE 3. Percent distribution of bacterial species associated with different lots (n = 50 per lot) of Atlantic salmon eggs that were originally cultured on TYG agar.

Bacterium	Designation of egg lots[a]							
	A	B	C	D	E	F	G	Total
Cytophaga psychrophila	17	5.7	0.0	0.0	0.0	0.0	0.0	3.5
Staphylococcus sp.	38	44	8.1	44	15	38	3.8	30
Pseudomonas diminuta	2.5	1.1	2.0	0.8	1.1	0.9	0.0	1.2
Corynebacterium aquaticum	6.9	21	7.7	21	17	19	10	16
Flavobacterium sp.	8.9	5.4	1.2	2.0	1.1	0.9	0.0	3.2
Acinetobacter sp.	3.4	2.5	4.4	7.9	4.4	1.4	4.3	3.9
Bacillus sp.	3.4	7.7	1.2	3.9	3.9	4.5	6.5	4.9
Chromobacterium violacium	2.8	1.6	0.0	2.8	6.7	3.2	0.0	2.2
Moraxella sp.	17	10	75	17	51	32	75	35

[a] Designation of egg lots: (A) Cronin No. 1, (B) Cronin No. 2, (C) Whittemore, (D) Kensington, (E) Berkshire, (F) Roger Reed, (G) Green Lake.

numbers from 4.2×10^3 to 10^7 cfu/cm^2 of the surface, which is consistent with the numbers observed in our study. Although *P. fluorescens* was the predominant organism in our study, its prevalence was consistent among the different lots of eggs and could not be associated with egg mortality.

Certain fish pathogens, such as *Aeromonas salmonicida* (Bullock and Stuckey, 1987) and *Renibacterium salmoninarum* (Bullock et al., 1978; Evelyn et al., 1984), have been associated with eggs. However, these studies were concerned more about the vertical transmission of disease by intra-ovum infection, than egg survival. Sauter et al. (1987) could not correlate an etiological bacterium with all egg lots experiencing high mortality rates, but they did observe high concentrations of *Vibrio* sp. in certain egg lots. In our study, we associate, but do not specifically confirm, the etiology of another fish pathogen, *C. psychrophila*, with poor egg survival.

The bacterial flora associated with eggs obtained from Atlantic salmon at the Richard Cronin National Salmon Station differed from flora associated with eggs originating at other hatcheries. Total bacterial counts were high in the Richard Cronin eggs, and *C. psychrophila* was specific for these groups. In fact, eggs with counts greater than or equal to 1×10^6 cfu/g of sample produced pure cultures of *C. psy-*

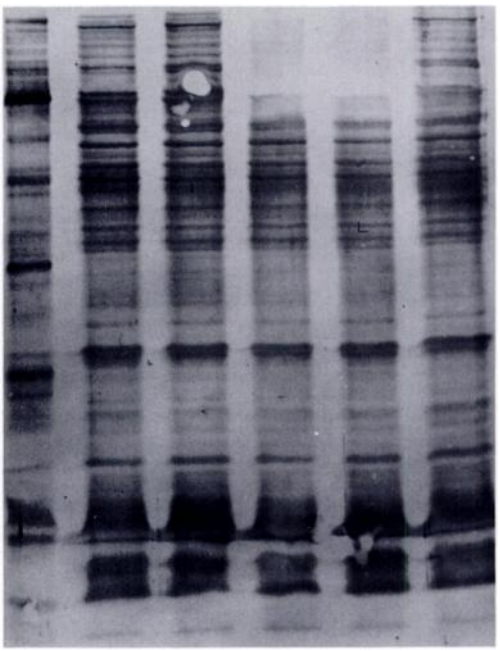

FIGURE 1. Results of Western blot immunoassay serologically confirming identity of *Cytophaga psychrophila* associated with Atlantic salmon eggs from the Richard Cronin National Salmon Station (Sunderland, Massachusetts, USA). Whole cell protein lysates from five suspect Cronin isolates (1 through 5) and a control isolate, derived from salmon undergoing clinical Coldwater disease (217), were subjected to electrophoresis, transferred onto nitrocellulose, and incubated with antiserum to *C. psychrophila* (isolate 217).

chrophila on TYG agar. *Cytophaga psychrophila* isolates from our study were phenotypically consistent with isolates of this species previously reported (Bernadet and Keronault, 1989); however, the banding patterns of these isolates differed from the isolate homologous to the antiserum used in Western blot immunoassays. Holt (1987) also observed somewhat similar antigenic differences between isolates of *C. psychrophila* obtained from fish. The consistency of the phenotypic characteristics and the intensity of reaction of the immunoassay are strong evidence for speciation of the *C. psychrophila* isolates identified in this study. Although this bacterium most commonly is associated with the etiology of coldwater disease (Pacha, 1968; Bernadet and Grimont, 1989), others report that *C. psychrophila* may be vertically transmitted from parent to offspring through the egg (Holt, 1987; Symula et al., 1990).

Water used to incubate eggs at the White River National Fish Hatchery originated from the White River and passed through ultraviolet irradiation before it was distributed to a head box that was a common supply for all of the vertical flow incubators. Furthermore, all eggs were disinfected at the point of origin and, once again, after arrival at White River hatchery. The specificity of *C. psychrophila* only in egg lots of Richard Cronin origin that experienced low percent survival supports the vertical transmission hypothesis of Symula et al. (1990) and is evidence that this bacterium may indeed induce egg mortality. Although many other factors can influence the viability of eggs (Springate et al., 1984; Thorpe et al., 1984), the presence of this pathogen could partially account for poor survival of Atlantic salmon eggs from the Richard Cronin National Salmon Station.

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