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COMMUNICATIONS

Virulence and Pathogenicity of Infectious Salmon Anemia Virus Isolated from Farmed Salmon in Atlantic Canada

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Abstract.—Infectious salmon anemia virus (ISAV) was isolated from farmed Atlantic salmon *Salmo salar* associated with an outbreak of hemorrhagic kidney syndrome in the Bay of Fundy, Canada. The virus induced cytopathic effects in salmon head kidney cell line SHK-1 from Atlantic salmon and was positively confirmed as ISAV by an indirect fluorescent antibody test and by reverse transcriptase polymerase chain reaction. Atlantic salmon parr injected with ISAV from the SHK-1 line experienced significant reductions in hematocrits as early as 5 d postinfection (DPI). Mortality began 17 DPI and reached 76% by 24 DPI at a water temperature of 11°C. In a second trial, similarly high mortality occurred in salmon parr injected with 10-fold dilutions of supernatant from ISAV-infected SHK-1 cultures. The ISAV was reisolated from eight randomly selected salmon that died after experimental infection. Microscopic pathological changes among infected fish included congestion and necrosis, seen in the livers from 7 of 19 samples and in the kidney from 1 of 18 salmon examined. Other tissues affected included gill, intestine, and pyloric caeca. The absence of microscopic lesions in the remaining experimentally infected fish could not be explained. Further studies are therefore needed to better understand the factors contributing to pathological changes after natural or experimental infection.

In 1996, mortalities among farmed Atlantic salmon *Salmo salar* in the Bay of Fundy, Canada, that were associated originally with distinctive renal pathology led to the first description of hemorrhagic kidney syndrome (HKS; Byrne et al. 1998). Morbidity due to HKS in net-pen-reared salmon produced pathognomonic microscopic pathological changes characterized by renal interstitial hemorrhage and acute tubular necrosis with eosinophilic casting. Examinations of more recent cases (1997–1998) from affected farmed salmon populations in the Bay of Fundy have shown that hepatic congestion and necrosis frequently accompanies renal congestion and necrosis (Mullins et al. 1998). Fish with HKS also display numerous hematological abnormalities including anemia, hypoproteinemia, hyperosmolality, and increased serum concentrations of Na^+ and Cl^- (Byrne et al. 1998).

Initially, the etiology of HKS was unknown because bacterial, viral, or parasitic agents were not isolated from affected fish nor were significant levels of trace metals or pesticides detected (Byrne et al. 1998). However, a filterable agent with genetic and morphological properties and host-cell-susceptibility similar to infectious salmon anemia virus (ISAV) was subsequently recognized (Mullins et al. 1998) and isolated (Bouchard et al. 1999; Lovely et al. 1999) from moribund salmon collected from HKS-affected net-pens. The ISAV and infectious salmon anemia (ISA) had only been observed among farmed Atlantic salmon in Norway (Falk et al. 1997). Since the recognition of ISAV in Canada, the virus has also been reported among farmed Atlantic salmon in Scotland (Rodger et al. 1998). In Norway, ISA is associated with ascites, congestion and enlargement of the liver and spleen, congestion of the foregut, and petechiae in the visceral and subperitoneal fat (Thorud and Djupvik 1988). Microscopic examinations of infected fish commonly revealed hemorrhagic necrosis and congestion of the liver, spleen, and foregut (Evensen et al. 1991). Congestion of the renal interstitium with tubular necrosis and casting observed in salmon with HKS in Canada were infrequently associated with and not reported among salmon with ISA in Norway. The purpose of the present study was to examine the virulence and pathogenicity of the ISAV isolated from Atlantic salmon in Canada.

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Methods

Fish.—Atlantic salmon were obtained as eyed eggs or swim-up fry from a certifiable disease-free hatchery and maintained in flowing freshwater (11 ± 1°C). Fish were fed a maintenance ration of pelleted diet (Shur-Gain, Truro, Nova Scotia) at 1% body weight daily. At the time of the trials, fish weighed between 5 and 10 g.

Cell culture.—The Atlantic salmon head kidney cell line (SHK-1, donated by B. Dannevig, Veterinary Institute, Oslo) was maintained at 15°C in 25-, 75-, or 150-cm^2 polystyrene flasks (Corning, Cambridge, Massachusetts) in Leibovitz medium (L-15; Sigma, St. Louis, Missouri) supplemented with 5% fetal bovine serum (FBS; Cansera, Rexdale, Ontario), 4 mM L-glutamine, and 40 μM 2-mercaptoethanol (complete L-15) as previously described (Dannevig et al. 1997), except penicillin G (100 IU/mL) and streptomycin sulfate (100 μg/mL) were substituted for gentamycin.

Isolation, culture, and identification of ISAV.—Groups of 5–10 salmon received intraperitoneal injections of a 0.22-μm filtered homogenate prepared in 10 mL sterile minimal essential medium (MEM; Gibco) from spleen, liver, and head kidney of a fish with HKS obtained directly from a farm. The infectious agent was serially passaged three times in salmon by intraperitoneal (IP) injection with filtered homogenates prepared from fish that recently died as above. Intact tissue samples from recently dead fish were also fixed in neutral-buffered 10% formalin for histological examination or in 2% glutaraldehyde for electron microscopy.

A filtered homogenate, obtained from the third experimental passage, was diluted 10^{-2} with L-15 medium and incubated on monolayers of SHK-1 cells for up to 18 h at 15°C, after which the inoculum was replaced with complete L-15. Cultures were monitored for cytopathic effect (CPE) and samples of medium were periodically taken and allowed to air-dry on glass slides. Dried samples were fixed with absolute methanol and examined by indirect fluorescent antibody test (IFAT) using the monoclonal antibody and the method described earlier (Falk et al. 1998). Infected supernatants were stored at 5°C or −20°C with or without a freeze–thaw cycle. Levels of ISAV in infected supernatants were estimated by calculating the TCID50 (tissue culture infective dose producing cytopathic effect in 50% of inoculated cultures) in SHK-1 cells using the method of Kärber (1931). Cultured ISAV was maintained by serial passage in SHK-1 cells.

Culture supernatants were also assayed for ISAV by using a reverse transcriptase polymerase chain reaction (RT-PCR) with primers obtained from cDNA sequences of Norwegian ISAV, using the method of Lovely et al. (1999). Supernatants were also screened by using RT-PCR for a Toga virus-like agent occasionally isolated from Atlantic salmon in the Bay of Fundy (F. Kibenge, Atlantic Veterinary College, personal communication).

Experimental design.—Trial 1 was designed to assess the virulence of cultured ISAV and included four groups each containing 25 Atlantic salmon parr. Fish in groups 1 and 2 received IP injections with 0.1 mL supernatant diluted 10^{-2} from infected SHK-1 cultures (approximately 10^2 TCID50/fish), and those in groups 3 and 4 received IP injections with culture medium alone. Fish were monitored for mortality twice daily, and blood samples were obtained from five fish in groups 1 and 3 on days 5, 10, 15, and 20 after injection. Hematocrit was measured for each blood sample after centrifugation of microhematocrit tubes for 4 min. Two-sample *t*-tests were used to calculate the statistical significance ($P < 0.05$).

Trial 2 was designed to assess microscopic pathological changes among Atlantic salmon parr after inoculation with different concentrations of ISAV. Four groups of 8–12 salmon were established in separate tanks. Culture medium from infected SHK-1 cells with an ISAV titer of approximately $10^{5.2}$ TCID50/mL was diluted 10^0, 10^{-1}, 10^{-2}, and 10^{-3} with L-15. Fish in each group received IP injections with 0.1 mL of either the undiluted or diluted culture media. Fish were monitored for mortality twice daily and samples of liver, kidney, gills, pyloric caeca, and spleen from moribund or freshly dead specimens were fixed in neutral-buffered 10% formalin, processed routinely for histological examination, and stained with hematoxylin and eosin. The presence of ISAV in dead and moribund fish was assessed by inoculating filtered tissue homogenates onto SHK-1 cells. When CPE was evident, the virus was identified by the IFAT as described above.

Results

Mortality, microscopic pathology typical of HKS, and viral particles were observed among salmon receiving injections with experimental filtered tissue homogenates. Fish that died after injection with tissue homogenates showed evidence of microscopic pathological changes consistent with HKS (see Byrne et al. 1998) in 20 of 45

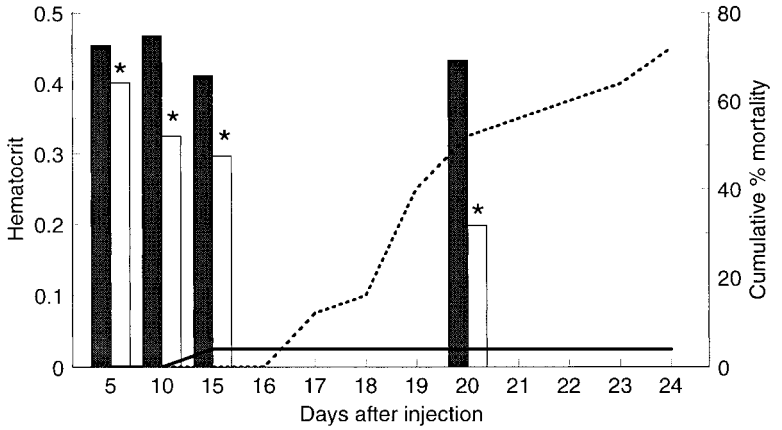


FIGURE 1.—Percent mortality (lines) and hematocrit (bars; packed cell volume/total blood volume) in Atlantic salmon parr after intraperitoneal injection with $10^{2.0}$ TCID50 of infectious salmon anemia (ISA) virus (NBISAV01) (right bar, dashed line) or L-15 culture medium (control; left bar, solid line). Bars represent means of five fish, and mortality curves are based on injected populations of 25 fish each. An asterisk (*) indicates value significantly different from control (*t*-test, $P < 0.05$).

(44.4%) liver samples and in 16 of 43 (37.2%) kidney samples. Inoculation of filtered homogenates onto SHK-1 cells resulted in CPE that was first observed between 7 and 14 d postinoculation (DPI). Although areas of cell detachment were occasionally observed in infected SHK-1 cultures, the monolayer remained partially intact throughout a 28-d observation period. Replicate 1-mL aliquots of medium from infected cultures at 35 d were stored at either 5°C (sample A) or −20°C (samples B and C) for 12 d. Sample C was subjected to two thaw–freeze cycles (−20°C to 17°C) before titration. After 12 d, virus titers in samples A, B, and C were calculated to be $10^{3.8}$ TCID50/mL, $10^{3.7}$ TCID50/mL, and $10^{4.2}$ TCID50/mL, respectively. Fluorescence was observed in IFAT preparations made from CPE-positive cultures but not in uninfected control preparations. The RT-PCR used to detect ISAV or Toga virus-like agent indicated that only ISAV was in the culture media. The isolate of ISAV obtained from these samples was designated NBISAV01 and used in all subsequent trials.

Compared with L-15-injected controls (trial 1, group 3), significantly reduced mean hematocrit values were measured among salmon in group 1 injected with supernatant from ISAV-infected SHK-1 culture at 5, 10, 15 and 20 DPI (Figure 1). Mortality among salmon in group 2 began 17 DPI and reached 76% (19 of 25) by 24 DPI. In contrast, one of 25 salmon in group 4 died of undetermined causes after injection with L-15 alone. Gross clinical signs associated with moribund fish in group 2 included darkening of the skin, abdominal distension with serosanguinous ascites, and bilateral exophthalmia with occasional intraocular hemorrhage. The presence of ISAV was confirmed by IFAT in SHK-1 cultures that had been inoculated with filtered tissue homogenates from eight fish that died.

In trial 2, mortality among salmon injected with undiluted ISA-infected SHK-1 culture medium began 16 DPI and reached 100% by 25 DPI (Figure 2). In contrast, onset of mortality ranged from 20 to 22 DPI among groups receiving diluted culture media and reached 67–75% (Figure 2). Clinical signs in trial 2 were similar to those observed in trial 1.

Samples obtained from fish in trial 2 showed significant morphological changes to gill, liver, pyloric caeca, mesenteric vasculature, and kidney. Seven of 19 (37%) samples examined had changes associated with the liver (1/5, 1/3, 4/5, and 1/6 samples injected with 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} diluted culture media, respectively), and 1 sample of 18 (6%) examined had changes associated with the kidney. Gill tissue was characterized by severe congestion of the filamental sinus (Figure 3). Multifocal regions of coagulative necrosis, sinusoidal congestion with areas of frank hemorrhage, or vasculitis with perivascular cuffing by a mixed-leukocyte population were observed in the liver (Figure 4). The intestine and pyloric caecae showed mild to marked congestion of the lamina propria and adjacent mesenteric vasculature (Figure 5). Trunk kidney lesions included mild to moderate

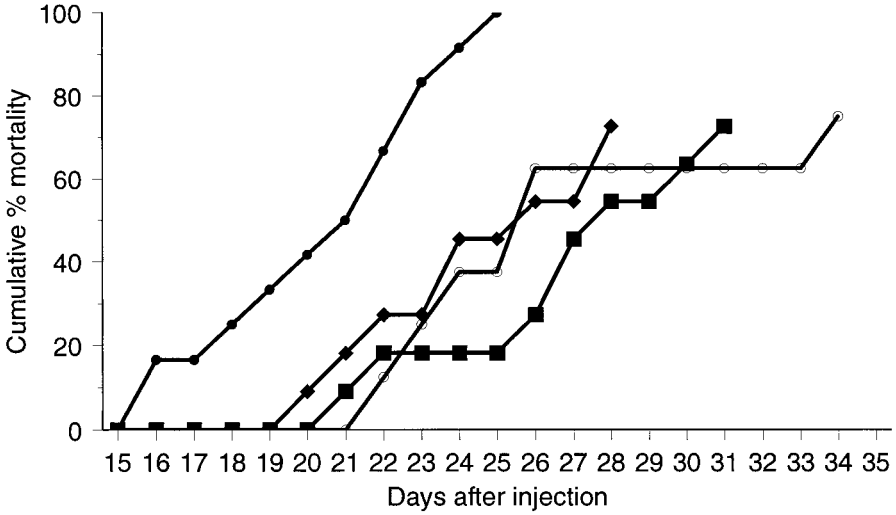
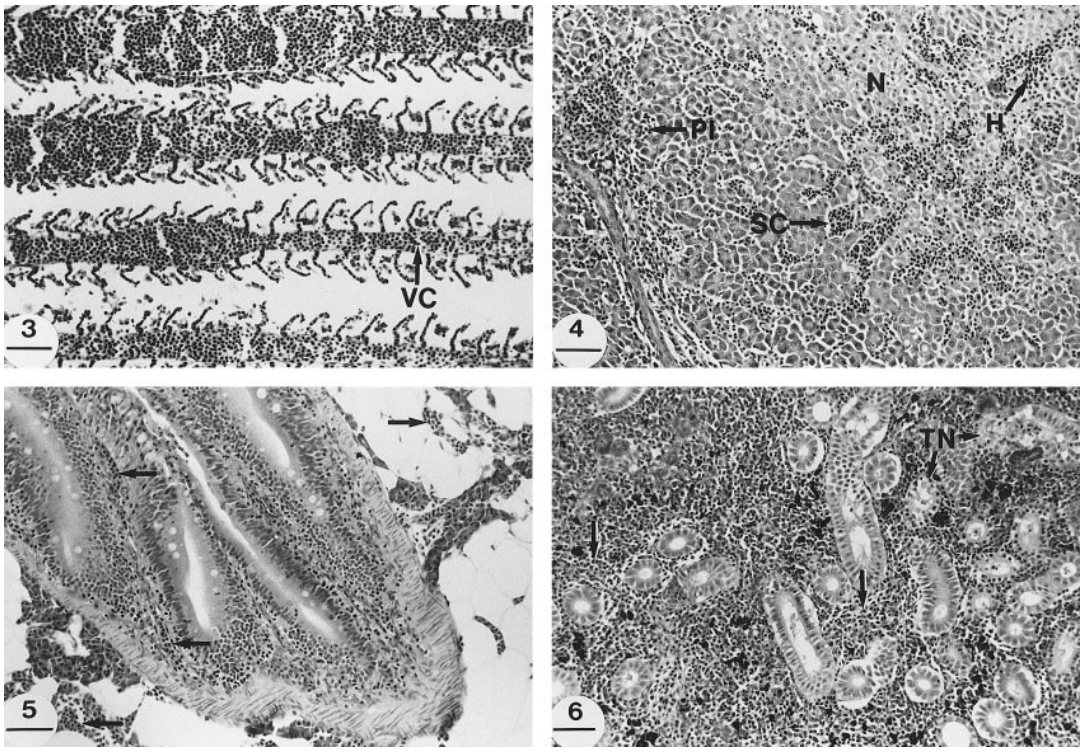


FIGURE 2.—Mortality in freshwater-reared Atlantic salmon parr after intraperitoneal injection with $10^{1.2}$ (♦), $10^{2.2}$ (■), $10^{3.2}$ (○) or $10^{4.2}$ (●) TCID50 ISA virus (NBISAV01).



FIGURES 3–6.—Tissues from Atlantic salmon parr after receiving an intraperitoneal inoculation with tissue culture supernatant containing infectious salmon anemia virus. Bar = 76 μm in all figures; all tissues stained with hematoxylin and eosin. **Figure 3.** Gill filaments. VC = vascular congestion. **Figure 4.** Liver. H = hemorrhage, N = hepatocellular necrosis, PI = perivascular leukocyte cuffing, and SC = sinusoidal congestion. **Figure 5.** Pyloric caeca, peritoneal mesenteries, and exocrine pancreas. Arrows indicate areas of vascular congestion. **Figure 6.** Trunk kidney. Arrows indicate regions of sinusoidal congestion and interstitial hemorrhage; TN = tubular epithelial degeneration.

sinusoidal congestion with multifocal regions of marked interstitial hemorrhage and occasional acute tubular epithelial necrosis (Figure 6). Frank eosinophilic casting of the tubular epithelium, however, was not present.

Discussion

Mortality in laboratory-reared Atlantic salmon parr was associated with each of three serial passages of tissue homogenates originating from naturally HKS-affected salmon. Infectious salmon anemia virus, confirmed by IFAT and RT-PCR, was isolated in cell cultures from tissue associated with these passages. As with ISAV previously described from Norwegian salmon, the Canadian isolate replicated and caused CPE in the salmon head kidney cell line SHK-1 (Dannevig et al. 1995). These observations confirm those of other workers (Mullins et al. 1998; Lovely et al. 1999) who had previously recognized ISAV in farmed salmon in New Brunswick and shown it to replicate in SHK-1 cells, respectively. The present results add to earlier observations of the virus isolated from Canada by demonstrating the virulence and pathogenicity of the agent as isolated in cell culture. In addition, the reisolation of ISAV from experimentally infected fish is compelling evidence supporting the primary role of this agent in the disease. To our knowledge, this study and that of Lovely et al. (1999) represent the only documented evidence for the formal satisfaction of Koch's Postulates for ISAV, whether in Norway or Canada. In other studies, inoculation of 25–40-g Atlantic salmon parr with cultured Norwegian ISAV caused greater than 90% mortality when the virus was grown in either SHK-1 or AS (Atlantic salmon) cells (Dannevig et al. 1995; Sommer and Mennen 1997). Times to onset of mortality and to peak mortality in the present trials were similar to those seen in the Norwegian studies (Dannevig et al. 1995; Sommer and Mennen 1997). In addition to mortality, virulence of the Canadian ISAV was demonstrated by the anemia observed in experimentally infected fish. This was particularly evident among fish sampled 20 DPI when hematocrit values ranged from 9.6% to 29.2%. Evensen et al. (1991) characterized ISA in Norwegian Atlantic salmon by a progressive anemia and found that severity of gross lesions increased as hematocrit dropped below 30%. Interestingly, in another study, significantly reduced hematocrit levels were not observed in salmon injected with ISA-associated tissue homogenates until 18 DPI, coincident with the onset of mortality (Dannevig et al. 1994). In the present trials, hematocrit reduction was significant as early as 5 DPI, preceding the onset of mortality by 12 d.

The renal pathology observed in 1 of 18 fish after inoculation with cultured ISAV was similar to that reported for market-sized Atlantic salmon suffering from HKS in the autumn of 1996 (Byrne et al. 1998), differing only in the severity of tubular necrosis and the absence of eosinophilic casting. More significantly, the systemic pattern of hepatic, enteric, and branchial pathology was consistent with previous descriptions of morphological changes identified in Norway for fish infected with the ISAV (Evensen et al. 1991) and nearly identical to those noted for 1977 seawater entry postsmolts in New Brunswick (Mullins et al. 1998). Our observations are consistent with those of Evensen et al. (1991) who clearly identified liver as the primary site of lesions. In contrast, Dannevig et al. (1994) used infectivity in fish to show that at 14 and 18 d postinjection, viral replication was more significant in head kidney than in liver. Histopathological changes were not reported in the latter study.

Factors controlling the pathogenesis of ISAV and therefore the development of gross and microscopic lesions, and whether these differ between natural and experimental infection, are not fully understood. Thus, it is not clear from the present trials (salmon parr injected with tissue homogenates or culture medium) why a considerable proportion of tissue samples examined showed no evidence of microscopic pathological changes despite the high mortality. The reduced frequency of renal histological changes observed in culture-medium-injected salmon compared with homogenate-injected salmon was similar to that reported by Lovely et al. (1999). In the latter study, microscopic pathology consistent with HKS was observed in all Atlantic salmon that died after injection with filtered tissue homogenates obtained from farmed salmon showing clinical signs of HKS. However, histological changes in samples from the four fish that died after injection with infected SHK-1 culture medium were inconclusive for HKS (Lovely et al. 1999).

The differential capacity of tissue homogenates and cultured ISAV to induce histological lesions, despite their similarity in causing high mortalities, suggests either that tissue homogenates contain an unrecognized factor(s), possibly infectious, that affects the development of pathology or that in vitro culture of ISAV selects for a variant that is less capable of eliciting tissue damage. Other variables, including age (Dannevig et al. 1994) and

physiological status (freshwater or seawater adapted) of the fish (Mullins et al. 1998), probably affect outcomes of natural or experimental infections. Although the present results have demonstrated virulence and pathogenicity of a Canadian ISAV isolate, the low frequency of histopathological changes occurring in experimental infections emphasizes the need for a clearer understanding of the factors affecting viral pathogenesis.

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