Vol. 22: 25–32, 1995 | DISEASES OF AQUATIC ORGANISMS Dis aquat Org | Published May 4

Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease

R. T. Nelson, M. F. McLoughlin, H. M. Rowley, M. A. Platten, J. I. McCormick

Fish Diseases Unit, Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Stormont, Belfast BT4 3SD, Northern Ireland, UK

ABSTRACT: Pancreas disease (PD) of farmed Atlantic salmon *Salmo salar* has been recognised in Scotland, Ireland, Norway, the USA, France and Spain and can cause severe economic loss. This paper reports the isolation, from PD-affected fish, of a virus with physicochemical characteristics and morphology resembling members of the Togaviridae. When inoculated into Atlantic salmon post-smolts it causes pathological changes in pancreas, heart and muscle tissues which are indistinguishable from those present in field outbreaks of PD. It is proposed that the virus be named salmon pancreas disease virus (SPDV).

KEY WORDS: Pancreas disease · PD Atlantic salmon Toga-like SPDV

INTRODUCTION

Pancreas disease (PD) of farmed Atlantic salmon *Salmo salar* was first described in Scotland in 1984 (Munro et al. 1984) although it has been recognised there since 1976. It was subsequently reported in Ireland, Norway, the USA, France and Spain (Kent & Elston 1987, Poppe et al. 1989, Raynard et al. 1992). It causes major economic losses to the industry, with up to 50% mortalities reported by Wheatley (1994) in first year smolts in Ireland. Results of epidemiological studies and transmission experiments (McVicar 1987, 1990, Murphy et al. 1992, Raynard & Houghton 1993) suggested an infectious aetiology for the disease. However, previous attempts to isolate an infectious agent were not successful (Munro et al. 1984, McVicar 1987, Murphy et al. 1992). This paper describes the isolation, from an outbreak of PD in Ireland, of a spherical virus 65.5 (± 4.3) nm in size, which morphologically resembles members of the Togaviridae. When inoculated into Atlantic salmon post-smolts it produces pathological changes in the pancreas, heart and skeletal muscle tissues, which are indistinguishable from those observed in natural outbreaks of PD on commercial salmon farms (Munro et al. 1984, Ferguson et al. 1986, Murphy et al. 1992). Hereafter this isolate is referred to as salmon pancreas disease virus (SPDV).

MATERIALS AND METHODS

Cell cultures. The following cell lines were used: chinook salmon embryo (CHSE-214), *epithelioma papulosum cyprini* (EPC), fathead minnow (FHM), bluegill fry (BF-2), Atlantic salmon (AS), rainbow trout gonad (RTG-2) cells and a rainbow trout fibroblast cell line produced in this laboratory (RTF). Cells were maintained in Eagle's minimum essential medium (MEM) containing Earle's salts and sodium bicarbonate (2.2 g l^{-1}), supplemented with 200 mM L-glutamine, 1% non-essential amino acids, 0.01 M Hepes, 100 IU penicillin ml^{-1}, 100 µg ml^{-1} streptomycin and 10% foetal bovine serum, FBS (Gibco, Paisley).

Cells were propagated in either 150 cm^2 flasks or 24-well plates (Costar) at 20°C. Plates were incubated in closed containers in a 3% CO_2/97% air atmosphere. For maintenance medium (MEMM) used during virus isolation attempts, antibiotics were increased as follows: penicillin, 500 IU ml^{-1}; streptomycin sulphate, 500 µg ml^{-1}; amphotericin B, 0.625 µg ml^{-1}; and FBS was reduced to 2%.

Virus isolation. Samples of kidney, spleen, heart, liver, pancreas and gut were taken from 20 individual fish during the acute phase of an outbreak of PD in farmed Atlantic salmon on the west coast of Ireland. Samples from each fish were treated separately.

For isolation attempts by co-cultivation, half-portion aliquots of each kidney were fragmented by placing them in a 2 ml syringe and forcing them through a 16 gauge hypodermic needle into 10 ml of MEMM. The tissue pieces obtained were added to monolayers of CHSE-214 cells prepared 24 h previously. These were incubated at 15°C for 28 d or until a cytopathic effect (CPE) was observed. Further passage in CHSE-214 cells and incubation for 28 d was then carried out.

The remaining kidney portions and other tissues from each fish were pooled, ground with a pestle and mortar and 10% homogenates prepared in MEMM. They were centrifuged at 2500 × *g* for 15 min and 0.1 ml of the supernatants were inoculated at final dilutions of 1:20, 1:50 and 1:100 in MEMM, into 24-well plates containing CHSE-214 cells. These were incubated at 15°C for up to 28 d, then further passaged into CHSE-214 cells and incubated for 28 d at 15°C.

SPDV growth in CHSE-214 cells. Virus growth in CHSE-214 cells was measured by inoculation of a suspension of SPDV onto CHSE-214 cells in a 24-well plate at a multiplicity of infection (MOI) of 1 and allowing virus to absorb for 1 h at 15°C. The inoculum was removed and the cells washed 3 times with MEMM, before replacing 1 ml MEMM. Samples were removed for assay at 0, 2, 4, 6, 8, 10, 12, 14, 21 and 28 d post-inoculation (dpi) as follows. Half of the culture medium was removed from each of 4 wells, pooled and centrifuged at 800 × *g* for 5 min to remove the cells. The supernatant contained extracellular virus. The remaining 0.5 ml of culture medium, along with the adherent cells removed by scraping, was pooled, and frozen and thawed once. This represented total virus present. Both samples were assayed separately for virus infectivity by titration in CHSE-214 cells incubated at 15°C for 14 d. The 50% end points in this and all subsequent tests were estimated by the method of Reed & Muench (1938).

Growth of SPDV in various cell cultures. Ten-fold dilutions of a virus pool containing 10^7 $TCID_{50}$ ml^{-1} were inoculated into AS, BF-2, FHM, EPC, RTG-2 and RTF cells incubated at 15°C for 14 d. Any CPE was noted and cultures were tested for virus growth by titration of culture fluids in CHSE-214 cells. All cultures were also given 1 further passage for 14 d in the same cells at 15°C, and checked again for virus growth in CHSE-214 cells before discarding.

Chloroform sensitivity. Sensitivity to chloroform was determined by adding 0.05 ml chloroform to 1 ml of SPDV. The mixture was shaken for 10 min at room temperature, then centrifuged at 400 × *g* for 5 min to remove the chloroform. Presence of infectious virus was detected by titration in CHSE-214 cells. One control consisted of 0.05 ml of MEMM added to 1 ml of virus. Infectious pancreatic necrosis virus (IPNV) and infectious haematopoietic necrosis virus (IHNV) were also included as non-sensitive and sensitive virus controls respectively.

Stability at pH 3.0. Stability at pH 3.0 was determined by adding 0.1 ml SPDV to 0.9 ml MEM adjusted to pH 3.0, holding for 4 h at 4°C, and then determining presence of infectious virus by titration in CHSE-214 cells. SPDV was added to MEM at pH 7.2 as a control, IPNV was included as a pH 3.0 stable virus control and IHNV as a pH 3.0 sensitive control.

Temperature stability. Aliquots of viral suspensions were heated for 30 min at 15, 25, 37, 45, 50, 55 or 60°C, and then cooled immediately by immersion in ice water. The concentration of infectious virus remaining was assayed by titration in CHSE-214 cells.

Nucleic acid inhibition test. The nucleic acid type of the virus was presumptively determined by growing it in the presence of the DNA inhibitor 5-bromo-2'-deoxyuridine (BUDR) with and without thymidine (THY). Groups of 4 wells in each of three 24-well plates containing CHSE-214 cells were inoculated with 0.1 ml of 10-fold dilutions of virus, which were allowed to absorb for 1 h at 15°C. To each plate, either 1 ml of MEMM alone, MEMM with 1 mM BUDR or MEMM with 1 mM BUDR and 1 mM THY was added. The plates were incubated at 15°C for 14 d and examined for CPE. An RNA virus (IPN) grown in CHSE-214 cells, and a DNA virus (lymphocystis) grown in BF-2 cells, were included as controls.

Negative contrast electron microscopic (EM) examination. Virus suspensions for EM examinations consisted of either SPDV-infected cell culture medium used without prior fixation or after the addition of glutaraldehyde (2% final conc.) for 1 h at 4°C and subsequent ultracentrifugation at 100 000 × *g* for 4 h, then resuspending the pellet in a few drops of distilled water. A carbon coated copper grid was placed on top of a drop of virus suspension and allowed to stand for 10 min. Excess fluid was drained off and the grid was stained with 2% phosphotungstic acid (PTA), pH 7.2, for 1 min. Virus was examined using a Hitachi H7000 transmission EM at × 50 000 magnification.

Caesium chloride density gradient centrifugation. SPDV was inoculated into CHSE-214 cells at a MOI of 1. At 8 dpi, cells and medium (400 ml) were harvested after freezing and thawing and then centrifuged at 10 000 × *g* for 30 min in a Beckman Type 35 angle rotor to remove cell debris. The supernatant was subjected to ultracentrifugation at 100 000 × *g* for 4 h. The resultant pellet was resuspended in a total of 2 ml of phosphate-buffered saline (PBS), pH 7.2, layered over a discontinuous caesium chloride (CsCl) gradient comprised of 5 ml of 1.30 g ml^{-1} CsCl and 4.5 ml of 1.22 g ml^{-1} CsCl, and centrifuged at 100 000 × *g* for 19 h at 4°C. Twenty fractions were collected and tested

for infectivity in CHSE-214 cells. The density of the fractions containing infective virus was determined using a refractometer.

Haemagglutination. Tests for haemagglutination were carried out with chicken, guinea pig, rainbow trout, and Atlantic salmon erythrocytes in U-bottomed 96-well plates, by adding 0.1 ml of a 0.8% suspension of red blood cells in PBS, pH 7.2 to 0.1 ml of virus (10^7 $TCID_{50}$ ml^{-1}) and incubating at 4, 15, and 37°C. Tests were examined after 1, 3 and 18 h.

Serological tests. SPDV was tested for neutralisation by hyperimmune rabbit sera to the following fish viruses: VHSV, IHNV, IPNV strains Sp, Ab, VR-299. SPDV was also tested against antisera to 2 members of the togavirus family, equine arteritis virus (EAV) and rubella virus, as well as to 1 member of the flavivirus family, bovine viral diarrhoea virus (BVDV). Antisera to alphaviruses were not available. An equal volume of SPDV (200 $TCID_{50}$ per 0.1 ml) was added to 0.1 ml of 2-fold dilutions of antisera and incubated at 15°C for 1 h. The mixtures were then inoculated into CHSE-214 cells in 24-well plates, 0.1 ml per well, allowed to absorb for 1 h at 15°C, 1 ml MEMM added and incubated at 15°C for 14 d. The cultures were examined microscopically on alternate days for evidence of CPE.

Experimental transmission design. ***Fish:*** Atlantic salmon post-smolts of mean weight 87 g were supplied, maintained and sampled by Marine Harvest Ltd, Lochailort, Scotland. They were kept in 2 × 1.5 m tanks with sea water supplied as a flow-through system at 12 to 15°C. They were maintained for 2 wk to acclimatize prior to inoculation, and samples of tissues from 10 fish were cultured for IPNV and examined for histological evidence of PD.

Inoculum 1: A virus pool was prepared by inoculating SPDV into CHSE-214 cells at a MOI of 1 and harvesting after 8 d incubation at 15°C. The cells were disrupted by freezing and thawing once and the cell debris was removed by centrifugation at 10 000 × *g* for 30 min. The resultant virus pool, with a titre of 10^7 $TCID_{50}$ ml^{-1}, was filtered through a 0.22 µm porosity Millipore filter and 0.1 ml inoculated intraperitoneally into each of 100 fish. Fifty fin-clipped, uninoculated fish were added to each of the tanks as in-contact fish.

Inoculum 2: Controls consisted of 100 fish inoculated with a lysate from uninfected CHSE-214 cells prepared in exactly the same manner as the virus infected cells, and 50 additional in-contact control fish were added.

Sampling: On 7, 10, 15, 21, 28, 35 and 42 dpi, samples of heart, spleen, liver, caeca/pancreas and muscle were taken from 10 test and 10 control fish for histological examination. Heart, spleen, kidney and caeca/pancreas samples were also taken from the same fish for virus isolation. On 14, 21, 28, 35 and 42 dpi, 5 in-contact fish were removed and tissues sampled for histological examination. In-contact fish were sampled for virus isolation on Days 14 and 21 only.

Histology: Samples for histological examination were fixed in 10% formaldehyde in buffered saline, pH 7.0, embedded in paraffin wax and 5 µm sections cut on a Reichert Ultracut S microtome. They were stained with haematoxylin and eosin.

Virus isolation: Tissues were prepared as 10% homogenates in MEMM using mortars and pestles, centrifuged at 2500 × *g* for 15 min, then inoculated at final dilutions of 1:20 and 1:100 into each of 2 wells in a 24-well plate containing CHSE-214 cells and incubated at 15°C for 28 d. Samples showing no CPE were given 1 further passage into CHSE-214 cells before being considered negative.

RESULTS

Virus isolation, morphology and growth characteristics

Virus was isolated from 2 of 20 kidney tissues submitted for examination. These had been co-cultivated with CHSE-214 cells for 28 d and then given further passages in CHSE-214 cells. No CPE was seen in the original co-cultivation cultures. On passage, however, small discrete groups of cells which were pyknotic, vacuolated and irregular in appearance could be observed after 10 d incubation. After further passages in CHSE-214 cells the CPE had become widespread (Fig. 1) and appeared as early as 4 dpi. Virus titres between $10^{7.0}$ and $10^{8.0}$ $TCID_{50}$ ml^{-1} were routinely attained. Most of the affected cells remained attached to the monolayers. No syncytia or inclusion bodies were observed in any of the cultures.

Virus was not isolated from any of the tissue homogenates incubated without co-cultivation. Virus yields in CHSE-214 cells are shown in Fig. 2. Highest titres were reached between 6 to 8 dpi and these remained high for up to 14 d.

No CPE was observed in AS, BF-2, FHM, EPC, or RTG-2 cells and there was no evidence of growth of SPDV in these cells. However, in the RTF cell line SPDV titres reached 10^6 $TCID_{50}$ ml^{-1} on both first and second passage in these cells, although no CPE was observed.

EM examination of the glutaraldehyde-fixed material revealed the presence of spherical particles measuring 65.5 (± 4.3) nm (Fig. 3). These possessed an inner core of indefinite structure and were surrounded by an outer fringe. Many partially disrupted particles were also present. In the unfixed preparations only a few complete particles were seen and our experience indicates that the free virion is fragile and easily disrupted during preparation for EM examination.

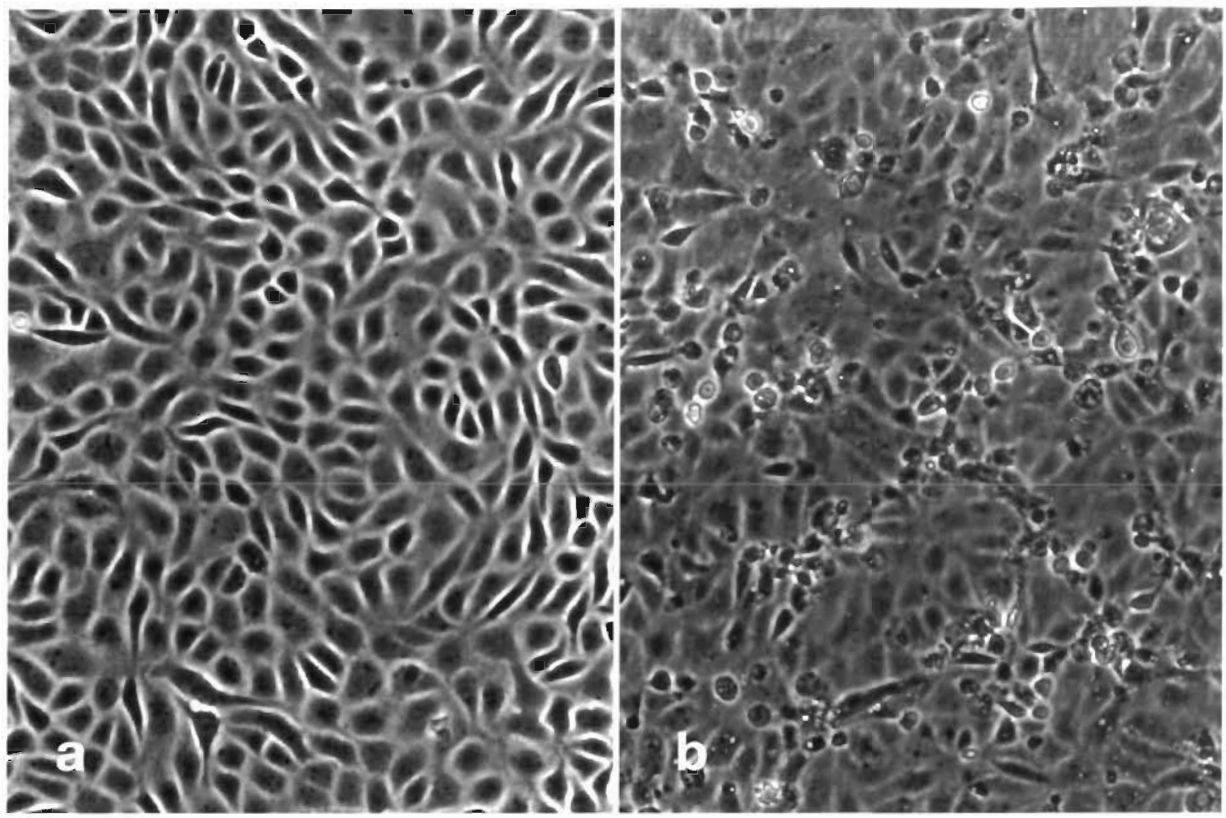

Fig. 1. Cytopathology of SPDV, isolated from *Salmo salar* in CHSE-214 cell cultures. (a) Uninfected cells. (b) Cells 8 d post-inoculation with SPDV

Physiochemical and serological characterisation

Growth of SPDV and IPNV was not affected by the presence of BUDR in the medium (Table 1), whereas the DNA control virus (lymphocystis) was inhibited.

Infectivity of SPDV was not inactivated by incubation at 4, 15 or 25°C but was reduced at 37 and 45°C while no infectious virus was detected after 30 min at 50°C. Infectivities of SPDV and IHNV were reduced following exposure to chloroform, indicating the presence of a lipid-containing envelope (Table 1). In contrast IPNV infectivity was not affected when treated in the same way.

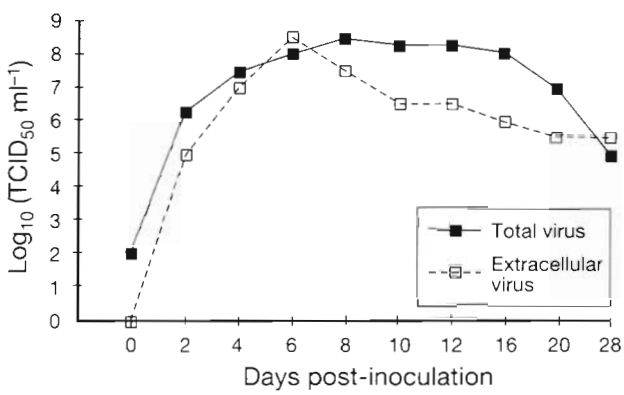

Fig. 2. Growth of SPDV, isolated from *Salmo salar*, in CHSE-214 cells

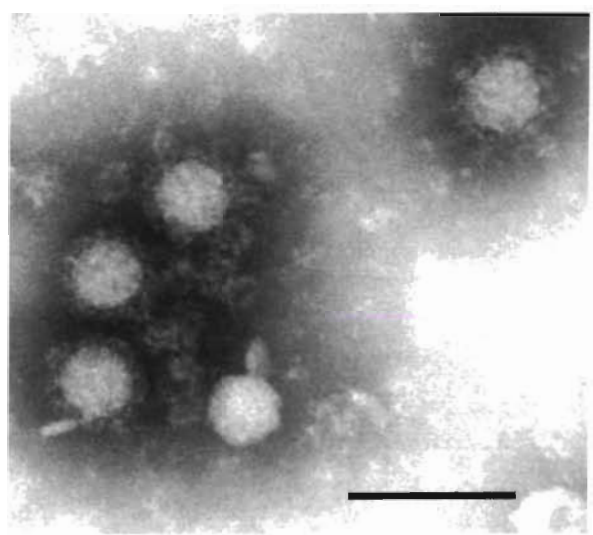

Fig. 3. Transmission electron micrograph of glutaraldehyde-fixed, SPDV-infected CHSE-214 cell culture fluid. Virus particles can be seen, most with surface projections but little internal structural detail. Scale bar = 100 nm

The infectivity of SPDV and IHNV was destroyed when they were exposed to pH 3.0, while IPNV was not affected (Table 1). Infectivity was detected in fractions from CsCl gradients with densities from 1.08 to 1.26 g ml^{-1}, with the maximum infectivity being located at a density of 1.20 g ml^{-1} This fraction also contained the greatest number of complete virus particles as assessed by EM examination. No haemagglutination was observed at any of the temperatures, or with any of the erythrocytes tested. In neutralisation tests SPDV was not neutralised by antisera to IHN, VHS, IPN, EA, BVD or rubella viruses.

Table 1. Growth of SPDV from *Salmo salar*, in the presence of BUDR [1 mM 5-bromo-2'-deoxyuridine or BUDR+THY (thymidine)], and effects of virus exposure to chloroform and pH 3.0. Values are $\log_{10}$ ($TCID_{50}$ ml^{-1}). nd: not done

Virus	Control	BUDR	BUDR+THY	Chloroform	pH 3.0
SPDV	7.0	7.2	7.0	<1.0	<1.0
IPNV	7.5	7.2	7.5	7.5	7.5
IHNV	6.0	nd	nd	<1.0	<1.0
Lymphocystis virus	7.2	5.0	7.0	nd	nd

Experimental transmission results

Clinical and pathological lesions

At 7 dpi, the fish inoculated with SPDV became anorexic and there was an increase in faecal casts in the tank. Focal to severe diffuse pancreatic acinar cell necrosis with concurrent multifocal cardiomyocytic necrosis was consistently observed from 7 dpi. Skeletal muscle fibre degeneration was detected at 15 dpi, affecting both red and white skeletal muscle fibres. These muscle lesions increased in frequency and severity at 35 and 42 dpi. Typical lesions observed at 21 dpi are illustrated in Figs. 4 to 7.

All the cohabitant fish developed similar lesions approximately 2 wk after the inoculated fish. A full description of the induced lesions will be published elsewhere.

No clinical signs or lesions were detected in any of the control fish.

Virus isolation

Virus was isolated from fish inoculated with SPDV and in-contact fish from the same tank but not from any of the control fish (Table 2). No IPN virus was detected at any stage in the transmission study.

DISCUSSION

Although it has been suggested for some time that PD of farmed Atlantic salmon is caused by an infectious agent, we have found, in common with other workers, that inoculation of tissue homogenates into cell cultures did not lead to recovery of an agent. These previous failures to isolate an agent from tissue homogenates by standard methods may have been due to the presence of inhibitory substances, as reported for IPNV by Dixon (1987) and suggested for PD by Raynard & Houghton (1993). In contrast we found that when PD-infected kidney tissues were co-cultivated with monolayers of CHSE-214 cells, small foci of altered cells were observed. After further passages in CHSE-214 cells these cytopathic effects increased, until approximately 75% of the monolayer

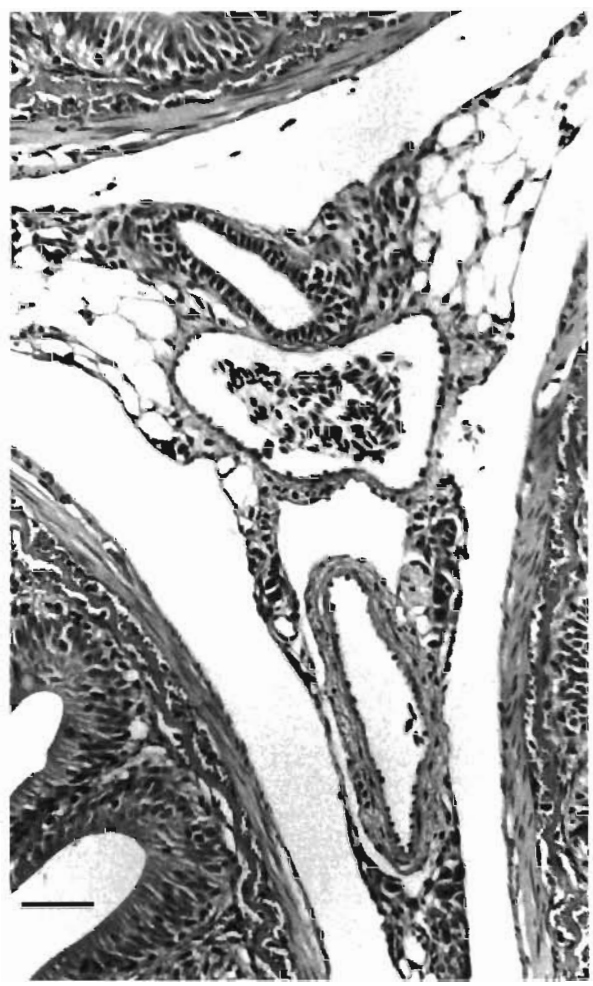

Fig. 4. SPDV infected *Salmo salar*. Significant pancreatic acinar cell loss typical of pancreatic lesions induced by SPDV in an experimentally infected fish, at 21 dpi. Scale bar = 50 µm

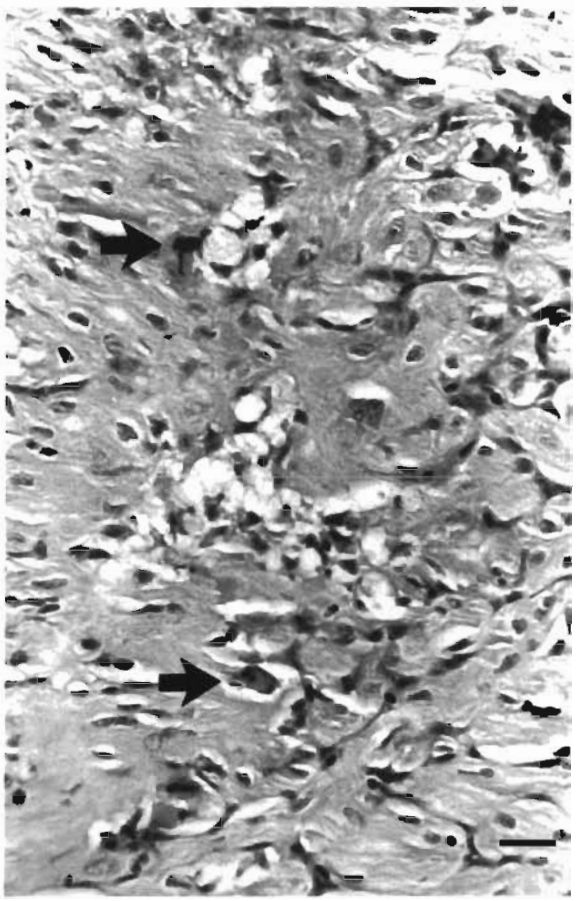

Fig. 5. SPDV infected *Salmo salar*. Multifocal cardiomyocytic necrosis which occurred concurrently with the pancreatic (arrows) lesions seen at 21 dpi in an experimentally infected fish. Scale bar = 20 µm

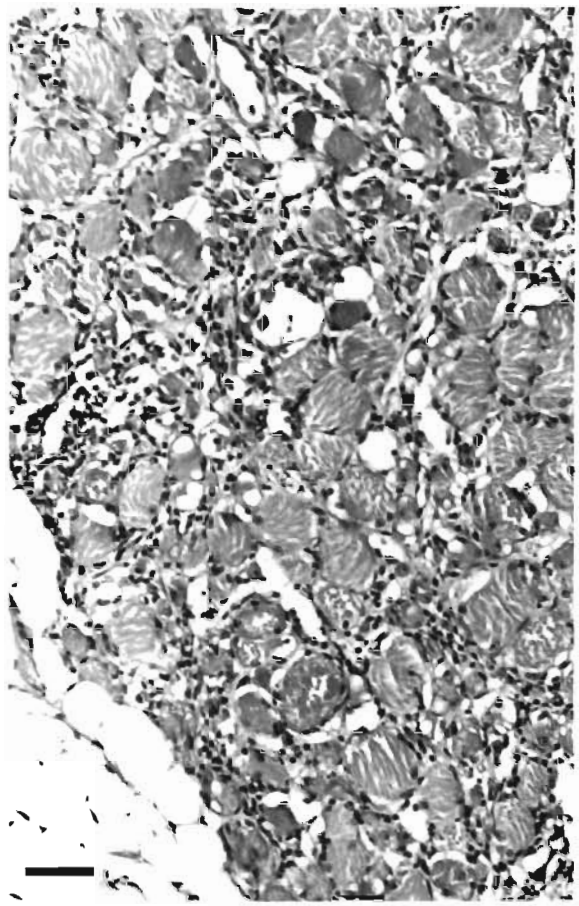

Fig. 6. SPDV infected *Salmo salar*. Degeneration of aerobic (red) skeletal muscle showing increased endomysial connective tissue, proliferation of sarcolemmal cells and hyaline degeneration of muscle fibres (21 dpi). Scale bar = 40 µm

was affected, although most of the cells still remained attached to the monolayer. It was found that the CHSE-214 cell adapted virus, when used to reinfect fish, could thus readily be isolated from infected tissue homogenates. EM examination of glutaraldehyde-fixed culture fluids from infected cells revealed the presence of virus-like particles approximately 65 nm in diameter, showing surface projections. Many partially disrupted particles could also be seen. The fact that virus preparations not fixed in glutaraldehyde before EM examination contained mostly disrupted particles indicated that prefixation was required to preserve the intact virion.

Table 2. Virus isolations from Atlantic salmon *Salmo salar* used in transmission experiments. Values presented as no. of positive fish/no. examined. nd: not done

Fish group	Days post-inoculation				
	7	10	15	21	28
Controls[a]	0/10	0/10	0/10	0/10	0/10
Virus-inoculated	7/10	8/10	10/10	3/10	nd
In-tank contacts	nd	nd	5/5	5/5	nd

[a] Inoculated with CHSE-214 cell lysate

The virus was sensitive to pH 3.0 and to chloroform, did not haemagglutinate a variety of erythrocyte species tested, was not neutralised by antisera used, and from its resistance to inhibition by BUDR appears to be an RNA virus. In CsCl density gradients, infectivity of the virus was detected across a broad density range with peak infectivity and greatest number of intact virus particles present at 1.20 g ml^{-1}.

On the basis of virion morphology, size, nucleic acid composition and other physical characteristics the virus most closely resembles members of the Togaviridae (Strauss 1991). This family is presently comprised of 3 genera, *Alphavirus* (27 species), *Rubivirus* (1 species), and *Arterivirus* (1 species). The togaviruses are essentially isometric, enveloped particles with surface

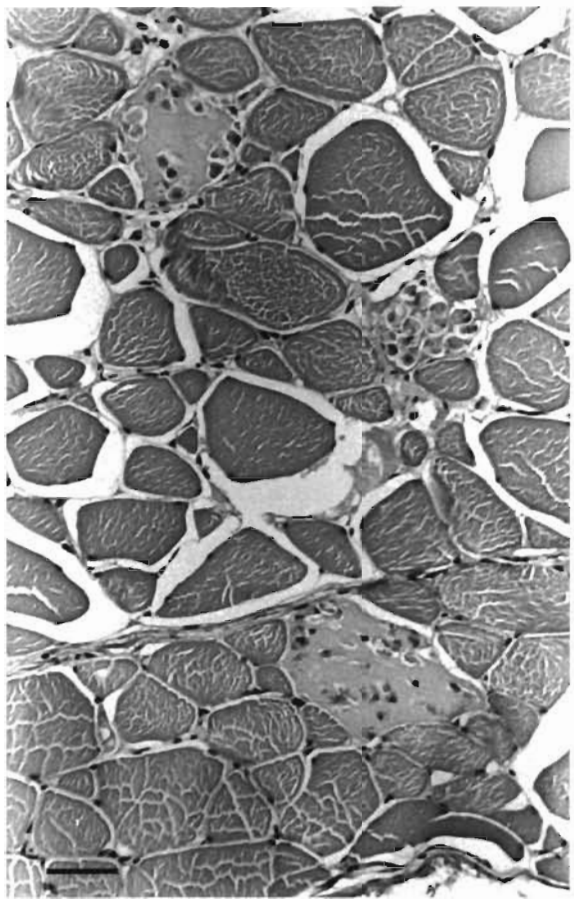

Fig. 7. SPDV infected *Salmo salar*. Hyaline degeneration of anaerobic (white) skeletal muscle fibres showing centralisation of muscle fibre nuclei and phagocytosis of fibre contents. Scale bar = 40 µm

projections, often with little internal structural detail on EM examination.

This is the first description of the isolation of a toga-like virus from PD-affected farmed Atlantic salmon. No other fish viruses have been convincingly shown to be members of the togavirus family at present, although it has been suggested that erythrocytic inclusion body syndrome virus (EIBSV) of salmon is probably a togavirus (Okamoto et al. 1992). This has not been isolated to date.

Our transmission study confirmed that SPDV could cause clinical signs and pathological lesions among experimental groups of salmon smolts in seawater and these signs and lesions were indistinguishable from published descriptions of field outbreaks of PD (Munro et al. 1984, Ferguson et al. 1986, McVicar 1987, 1990, Murphy et al. 1992). The fish became anorexic, and some had faecal casts and petechial haemorrhages in the pancreatic fat. While controversy exists as to the severity and tissue distribution of histopathological lesions associated with 'classical' PD (Wheatley 1994), our findings are in close agreement with the description of Ferguson et al. (1986) of an outbreak of PD in Scotland and Murphy et al. (1992), who described the sequential pathology of 2 PD outbreaks in Ireland. The lesions observed in our transmission studies represent the full range of acute and chronic pancreatic, cardiac and skeletal muscle lesions as observed by us in field outbreaks of PD and described by other workers. From this study we conclude that SPDV is the causative agent of PD in farmed Atlantic salmon. There is evidence that fish acquire a strong immunity to PD after field and experimental exposure (Raynard & Houghton 1993, Houghton 1994). It may therefore be possible to produce an effective SPDV vaccine. The isolation of this virus should enable the development of antigen and nucleic acid detection systems which would aid in the rapid diagnosis of PD and assist in more thorough investigations of the pathogenesis and epidemiology of this important disease of Atlantic salmon. Aspects of this work are subject to patent applications.

Acknowledgements. We thank A. Grant and D. Cox from Marine Harvest Ltd, P. Gallagher from Ocean Farm and D. Todd from Veterinary Sciences Division for their valued cooperation.

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Responsible Subject Editor: F. M. Hetrick, College Park, Maryland, USA

Manuscript first received: October 20, 1994
Revised version accepted: January 1, 1995