Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains

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Infectious salmon anaemia virus (ISAV) is a new orthomyxovirus-like virus. Thirteen isolates of ISAV (11 from Canada, one from Norway and one from Scotland) were studied for their replication in the CHSE-214 cell line compared with that in the SHK-1 cell line. All isolates replicated in SHK-1 cells, producing CPE between 3 and 12 days post-inoculation (p.i.). Six Canadian isolates also replicated in CHSE-214 cells, with production of CPE between 4 and 17 days p.i. Analysis of a one-step growth curve of ISAV in CHSE-214 cells showed that progeny virions remained predominantly cell-associated, accounting for the focalized nature of the CPE in the cell monolayer. One isolate (HKS 36) replicated in CHSE-214 cells, as shown by positive RT–PCR results of blind passages, but was non-cytopathic. All of the isolates were analysed for genetic heterogeneity by RT–PCR and RFLP with EcoRI and XhoI in a fraction of genome segment 2. The Canadian isolates showed a different RFLP profile to those of isolates Glesvaer/2/90 from Norway and 390/98 from Scotland. Structural proteins of four isolates, ‘Back Bay 98’, RPC/NB-877, RPC/NB-049 and Glesvaer/2/90, were examined further by SDS–PAGE. All viruses showed four major polypeptides, designated here as VP1–VP4, in Coomassie blue-stained gels. In isolates Glesvaer/2/90 and RPC/NB-877, these viral proteins had estimated molecular masses of 74, 53, 46 and 26–5 kDa, respectively. Viral proteins in isolates ‘Back Bay 98’ and RPC/NB-049 were of similar sizes, except that VP3 was 43 kDa. Taken together, these results show that there are phenotypic differences among strains of ISAV.

Introduction

Infectious salmon anaemia virus (ISAV), a new orthomyxovirus-like virus, is an important virus pathogen of fish. It causes ISA in salt water-farmed Atlantic salmon (Salmo salar), characterized grossly by exophthalmia, pale gills and ascites (Thorud & Djupvik, 1988) and microscopically by haemorrhagic liver necrosis (Evensen et al., 1991) and renal interstitial haemorrhage and tubular necrosis (Mullins et al., 1998). The virus was recently identified as being an orthomyxovirus-like virus because of its resemblance to influenza virus (Mjaaland et al., 1997), and it has been suggested that it probably belongs to a new genus in the family Orthomyxoviridae (Krossoy et al., 1999). The nature of strain variation among ISAV isolates is not known, although Blake et al. (1999) have reported genomic sequence data showing significant differences between Canadian and Norwegian ISAV isolates.

The difficulty, until recently, in finding cell lines that supported ISAV multiplication delayed isolation of the virus (Dannevig et al., 1993). Infection in the salmon head kidney (SHK-1) cell line is cytopathic (Dannevig et al., 1995), but only moderate infectivity titres of 10^6 TCID90/ml are obtained (Falk et al., 1997), which has hampered the characterization of this new virus. Moreover, SHK-1 is a very delicate cell line, requiring very low split ratios and a complex growth medium. The Chinook salmon embryo (CHSE-214) cell line (Fryer et al., 1965) is a commonly used fish cell line. It was recently used to recover ISAV directly from clinical specimens (Bouchard et al., 1999). In order to facilitate further biological characterization of ISAV, we have studied its replication in CHSE-214 cells. This has been compared with the replication of ISAV in the SHK-1 cell line.

Methods

Viruses and virus propagation. The viruses used in this study and their origins are listed in Table 1. Viruses were propagated in CHSE-214 and/or SHK-1 cells. The CHSE-214 cell line (1:4 split ratio) was grown at 16 °C in HMEM (Eagle’s minimum essential medium (Canadian...
Table 1. Infectivity virus titres of various ISAV isolates in SHK-1 and CHSE-214 cell lines

Virus titres were measured by end-point CPE of the last SHK-1 or CHSE-214 passage harvest, and are expressed as log_{10} TCID_{50}/ml.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>SHK-1</th>
<th>CHSE-214</th>
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<tr>
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<td>–</td>
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<tr>
<td>RPC/NB-280-2</td>
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<td>390/98</td>
<td>A. McVicar</td>
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* Sources of isolates were: RPC, Research and Productivity Council, Fredericton, NB, Canada; Micro Technologies Inc., Richmond, ME, USA; Aqua Health Ltd, Charlottetown, PEI, Canada; DFO, Department of Fisheries and Oceans, Moncton, NB, Canada; AVC, Atlantic Veterinary College Virology Research Laboratory; K. Falk, National Veterinary Institute, Oslo, Norway; A. McVicar, Fish Health Inspectorate, FRs, Marine Laboratories, Aberdeen, UK. –, No CPE was produced.

Effect of virus titre of inoculum. Serial 10-fold virus dilutions were inoculated onto CHSE-214 cell monolayers in 48-well plates in order to test whether larger doses of virus inoculum would generate defective interfering particles (DIPs) in CHSE-214 cells. The inoculated cell monolayers were monitored daily until 34 days p.i. or until maximum CPE was observed.

Effect of regular changes to fresh maintenance medium. To test the effect of changing maintenance medium on virus replication, the cell culture medium on infected cultures was changed at 14, 21 and 28 days p.i. and the virus titre in the medium removed was determined as described below.

Effect of pH of cell culture medium. The effect of the pH of the maintenance medium was examined as follows. MEM containing Hank’s salts was adjusted to pH 3, 4, 5, 6, 7 or 8. The medium was then filter-sterilized and used as diluent for virus inoculum. Virus was adsorbed onto cells for 1 h at 16 °C and cells were then incubated in the pH-adjusted MEM with 2% FBS. Cultures were frozen at 7 days p.i. and the virus titre was determined as described below.

Effect of trypsin. In preliminary experiments, different amounts of trypsin were added to the cell culture medium to test the susceptibility of CHSE-214 cells to trypsin. It was found that the large amounts of trypsin, 20–40 µg/ml, used by Falk et al. (1997) on SHK-1 cells caused detachment of a large proportion of CHSE-214 cells, resulting in a web-like appearance of the cell monolayer. Therefore, a lower trypsin concentration was chosen. For this purpose, the effect of trypsin on ISAV replication in CHSE-214 cells was tested by using 2.5 µg/ml trypsin in the medium as described by Ilbii et al. (1998).

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Virus growth curve. CHSE-214 cells in 48-well plates were infected with isolate ‘Back Bay 98’ at an m.o.i. of 1. After virus adsorption for 1 h at 16 °C, the cell monolayers were washed with PBS to remove residual unadsorbed virus. Fresh maintenance medium was then added and incubation was continued at 16 °C. At intervals after infection, the cell culture medium from two wells was harvested and pooled; 0.5 ml fresh medium was then added to each of the two wells to harvest the cell monolayers, which were pooled. Both the cell culture medium and cell monolayer harvests were stored at −80 °C until they were assayed for cell-free virus infectivity and cell-associated virus infectivity, respectively.

Virus purification. Infected cell culture harvests were clarified at 4000 x g for 20 min. The supernatant was ultracentrifuged at 100 000 x g for 2 h at 4 °C in an SW40 rotor (Beckman) to pellet the virus. Alternatively, virus was concentrated by precipitation with PEG 8000 and NaCl or with saturated ammonium sulphate solution. The precipitate was collected by ultracentrifugation at 17 000 g for 2 h. The virus pellet or precipitate was resuspended in 1 ml TNE (10 mM Tris–HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.5) and clarified by centrifugation at 15000 g for 10 min for the PEG precipitate or, in the case of the ammonium sulphate precipitate, it was dialysed against several changes of TNE. The virus suspension was layered on a Ficoll-400 (Amersham Pharmacia Biotech) step gradient consisting of 10% and 25% (w/v) Ficoll in TNE and ultracentrifuged at 95 000 g for 1 h at 4 °C in an SW40 rotor. The virus band was collected from the interface by side puncture of the tube and virus was pelleted by ultracentrifugation at 95 000 g for 45 min at 4 °C in an SW40 rotor. The virus pellet was resuspended in approximately 100 µl TNE prior to use.

Electron microscopy. The purified ISAV was absorbed to freshly glow-discharged carbon-coated grids and negatively stained with 2% phosphotungstic acid, pH 7.2 (Brenner & Horne, 1959). The samples were examined in a Hitachi H600 electron microscope operated at 75 kV.
Preparation of antiserum to purified ISAV. One 250 g rabbit (Charles River Canada, St-Constant, Quebec, Canada) was inoculated subcutaneously three times at 3-week intervals with 100 μg purified ISAV isolate 'Back Bay 98', once in Freund's complete adjuvant and twice in incomplete Freund's adjuvant. Pre-inoculation serum and sera collected every 3 weeks until after the last injection were tested for the presence of antibodies against the same virus by indirect fluorescent antibody test by using CHSE-214 cells in 48-well or 96-well culture plates and by Western blot analysis as described below.

Indirect fluorescent antibody test (IFAT). IFAT was performed on fixed CHSE-214 cell cultures in 48-well culture plates or on coverslips in 6-well culture plates as described previously (Falk et al., 1997). Cultures in 48-well plates at 4–9 days p.i. showing CPE were used to screen the rabbit antiserum to ISAV, whereas the coverslip cultures were used to monitor ISAV replication in CHSE-214 cells.

Virus infectivity titrations. The serial 10-fold dilutions of each virus sample were prepared in maintenance medium. One hundred μl of each dilution was inoculated into three wells of a 48-well tissue culture plate with confluent cell monolayers drained of cell culture medium. Inoculated plates were incubated at 16°C for 1 h and then 0.5 ml maintenance medium was added per well and incubation was continued at 16°C for 21 days or until complete CPE was manifested. The virus titre was determined from the CPE by using the procedure described by Reed & Muench (1938).

Gel electrophoresis of viral proteins and Western blotting. Purified virus was mixed with an equal volume of 2× SDS sample loading buffer and heated in a boiling water-bath for 5 min. Proteins were resolved by 12.5% discontinuous SDS–PAGE (Laemmli, 1970) and visualized with Coomassie blue R-250 (Bio-Rad) and, if necessary, by staining with a silver staining kit (Bio-Rad). Duplicate gels were used in Western blotting. For this, the gel and nitrocellulose membrane were briefly soaked in transfer buffer (50 mM Tris base, 380 mM glycine, 0.05% SDS, 20% methanol). Proteins were then transferred from the gel to the membrane in the same buffer at 4°C. The blot was washed in TTBS (20 mM Tris–HCl, 500 mM NaCl, 0.05% Tween 200, pH 7.5) followed by Tris buffer and was then reacted with a 1:30,000 dilution of goat anti-rabbit alkaline phosphatase (Bio-Rad) for 4 h at 60°C (65 mA/cm2). The membrane was rinsed several times with PBS (pH 7.2) and the proteins were fixed on the membrane by immersing it in isopropanol for 1 min. The membrane was blocked with PBS (pH 7.2) containing 3% BSA for 1 h at 37°C, washed with Tris buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5) and then reacted with a 1:200 dilution of rabbit antiserum to ISAV isolate 'Back Bay 98' for 1 h at 37°C. The blot was washed in TTBS (20 mM Tris–HCl, 500 mM NaCl, 0.05% Tween 200, pH 7.5) followed by Tris buffer and was then reacted with a 1:30,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma) at 37°C for 1 h. Colour development was with BCIP/NBT substrate (Bio-Rad) at room temperature. The reaction was stopped by rinsing the membrane in distilled, deionized water.

RT–PCR. Viral RNA was extracted from 250 μl cell culture lysate by using TRIZOL LS reagent (Canadian Life Technologies) following the manufacturer’s protocol. Two primer pairs, one targeting genome segment 2 and the other targeting genome segment 8 of the ISAV genome, were used independently of each other under the same conditions in RT–PCR. The primer pair on segment 2 was designed by using Primer Detective version 1.01 (Clontech) from the published sequence of ISAV isolate 'Bliss Harbor 8/97' (Blake et al., 1999) and consisted of primers PB1F (sense, 5’ GTCAATGGAAGAAGATTCCG 3’) and PB1R (antisense, 5’ TACATACCTCTGGCACATGC 3’). The primer pair on segment 8 (Mjaaland et al., 1997) consisted of primers FA-3 (sense, 5’ GAAGAGTCAAGATCCAAAGAC 3’) and RA-3 (antisense, 5’ GAGCTGATGATCTGCAGCGA 3’) (Are Nyland, personal communication). One-step RT–PCR was carried out by using the Titan One Tube RT–PCR system kit (Roche Molecular Biochemicals). RT–PCR was performed in a PTC-200 DNA Engine Peltier thermal cycler (MJ Research Inc.). Cycling conditions consisted of one cycle of cDNA synthesis and pre-denaturation at 55°C for 30 min and 94°C for 2 min, followed by 40 cycles each consisting of denaturation at 94°C for 30 s, annealing at 61°C for 45 s and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. PCR products were resolved by electrophoresis on either a 0.9 or 2% agarose gel and visualized under 304 nm UV light after staining with ethidium bromide (Sambrook et al., 1989). The PCR product from genome segment 2 was also digested with HindIII, XhoI and EcoRI to confirm the specificity of the reaction and to assess ISAV strain variability in the target region.

Results

Replication in SHK-1 cells

To check for viability of the viruses in the samples used in this study, as well as to confirm their identity as ISAV, we inoculated all ISAV isolates on SHK-1 cells and examined the cell monolayers daily for development of CPE. All ISAV isolates (Table 1) replicated in the SHK-1 cell line as acute infections. The viruses produced CPE between 3 and 12 days p.i. Virus titration of isolate RPC/NB-877 on SHK-1 cell monolayers revealed that low virus dilutions produced CPE by 3 days p.i., whereas at high dilutions, CPE did not appear until 12 days p.i. Once CPE appeared, it took 4–7 days for complete destruction of the cell monolayer. Virus dilutions that did not produce CPE by 14 days p.i. never developed CPE. All viruses isolates producing CPE on SHK-1 cells were confirmed as ISAV by RT–PCR and/or electron microscopy.

Replication in CHSE-214 cells

To study virus replication in the CHSE-214 cell line, SHK-1-propagated ISAV isolates were inoculated on CHSE-214 cell monolayers. Up to four blind passages at 14–21 day intervals were performed before a virus isolate was considered unable to replicate and cause CPE in CHSE-214 cells. Seven ISAV isolates, including isolate Glesvaer2/90 from Norway, isolate 390/98 from Scotland and isolates RPC/NB-877, RPC/NB-280–2, RPC/NB-033–4, RPC/NB-233 and HKS 36 from New Brunswick, Canada (Table 1), did not produce CPE by the fourth blind passage in CHSE-214 cells. The different blind passages of these viruses were examined by RT–PCR with primer pair FA-3/RA-3 to determine whether any of these virus isolates were non-cytopathic in CHSE-214 cells. Only isolate HKS 36 was positive in all four blind passages, indicating that this ISAV strain is non-cytopathic in CHSE-214 cells. The remaining six isolates were therefore considered to be unable to replicate in CHSE-214 cells.

Six of the 13 ISAV isolates studied replicated in the CHSE-214 cell line, producing CPE in the first passage, beginning 4 days p.i. The ISAV CPE in the CHSE-214 cell line involved only a limited number of cells in the monolayer, appearing as

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refractile clusters of cell rounding and lysis, as described previously (Bouchard et al., 1999). These clusters of CPE (Fig. 1) correlated with areas of immunofluorescence staining (Fig. 2) when cultures were examined by IFAT with anti-ISAV rabbit antiserum at dilutions of up to 1:28000. Examination of infected cell monolayers on coverslips harvested at different times after infection revealed weak stippled staining in the cytoplasm and nucleus at 2 days p.i. With progression of virus multiplication, the cytoplasmic staining became more prominent and involved scattered single cells and clusters of cells in the monolayer. However, nuclear fluorescence also persisted and was still present at 9 days p.i., although it did not increase significantly in intensity as the cytoplasmic staining did.

Virus titration of isolate ‘Back Bay 98’ on CHSE-214 cell monolayers revealed that an m.o.i. of 10 produced CPE by 4 days p.i., whereas at an m.o.i. of 0.001, CPE appeared by 17 days p.i. Thus, low virus dilutions did not seem to generate DIPS in CHSE-214 cells. Virus dilutions that did not produce CPE by 17 days p.i. never developed CPE.

When cell culture medium removed prior to virus inoculation of the cell monolayer was put back on the cells after infection, development of CPE was delayed by 2 days and destruction of the cell monolayer was delayed by 9 days compared with using fresh maintenance medium. Moreover, the intensity of CPE and virus production in CHSE-214 cells increased significantly if the cell culture medium was replaced regularly with fresh maintenance medium. Under these conditions, once CPE appeared, it took 15–19 days for complete destruction of the cell monolayer and the amount of virus harvested by 35 days p.i. increased 100-fold. By using this modification, the six ISAV isolates that caused CPE in CHSE-214 cells had virus titres that were up to 100 times lower than those in SHK-1 cells (Table 1).

The addition of trypsin to the culture medium did not affect the speed with which CPE appeared, but caused a more uniform involvement of the cell monolayer and more intense CPE.

Adjustment of the pH of the maintenance medium had no beneficial effect on virus replication in CHSE-214 cells. Cell culture medium below pH 4.0 was toxic to the cells. The virus titres obtained with media adjusted to pH 5.0–8.0 were 10–100-fold lower than with the control medium with the unadjusted pH of 8.0. However, a decrease in the pH to 7.10 by 5 days p.i. and to 6.87 by 14 days p.i. was noted in the medium of the infected control flasks.

**ISAV growth curve in CHSE-214 cells**

The one-step growth of ‘Back Bay 98’ virus was examined in CHSE-214 cells. Cell monolayers of CHSE-214 cells were infected with ‘Back Bay 98’ at an m.o.i. of 1.0 and infectivity titres of the different fractions of infected cultures were assayed on various days p.i. As shown in Fig. 3, the cell-free virus titres increased after 24 h and reached a peak of 5.5 log_{10} TCID_{50}/ml at about 5 days and thereafter remained around 4.5 log_{10} TCID_{50}/ml. A second peak in the cell-free virus titres occurred at 12 days p.i., following the drop in cell-associated virus titres. The cell-associated virus titres had a rapid exponential rise after 24 h, reaching a titre of 5.5 log_{10} TCID_{50}/ml at about 6 days and a maximum titre of 6.5 log_{10} TCID_{50}/ml at about 10 days. Overall, the cell-associated virus titres were approximately 10-fold higher than the cell-free virus titres, indicating that progeny virions remained predominantly cell-associated.

**RFLP in segment 2 of ISAV genome**

In order to confirm that the virus samples that caused CPE in the different cell cultures were ISAV, RT–PCR was performed on the infected cell lysates with primer pair FA3/RA-3, targetting genome segment 8 of the ISAV genome. All the ISAV isolates reacted positively, yielding a 220 bp product that was visualized in ethidium bromide-stained agarose gels (data not shown). A second RT–PCR, with primer pair PB1F/PB1R targetting gene segment 2 of the ISAV genome, was also performed on all isolates and yielded a 600 bp product (Fig. 4), in accordance with published sequences of genome segment 2 (Blake et al., 1999). In order to determine whether this PCR product was conserved among different isolates of ISAV, RNA templates of all 13 ISAV isolates were used in RT–PCR and the products were digested with EcoRI, HindIII and XhoI and analysed. From the published nucleotide sequence of ‘Bliss Harbor 89/97’ (Blake et al., 1999), each of the restriction enzymes was predicted to yield two fragments; EcoRI, 153 and 447 bp; HindIII, 284 and 306 bp; XhoI, 248 and 352 bp. Restriction digestions with HindIII of the PCR products of all ISAV isolates tested yielded the expected number and sizes of fragments. The PCR products from the 11 Canadian isolates of ISAV also yielded the expected number and sizes of fragments with EcoRI and XhoI, whereas ISAV isolate Glesvaer/2/90 from Norway and isolate 390/98 from Scotland were resistant to digestion with these enzymes.
Phenotypic differences between ISAV strains

Fig. 2. Immunofluorescent antibody staining of ISAV-infected CHSE-214 cells using rabbit antiserum to ‘Back Bay 98’ virus. (a) Fluorescence of a localized area of ISAV CPE in the cell monolayer 5 days p.i. Magnification × 90. (b) Higher magnification showing both cytoplasmic and nuclear fluorescence. Magnification × 900. (c) Area of fluorescence involving only a limited number of cells in the monolayer at 5 days p.i. Magnification × 900.

Fig. 3. Growth curve of ISAV strain ‘Back Bay 98’ in CHSE-214 cell line. ○, Cell-free virus; ▲, cell-associated virus.

Fig. 4. RT–PCR and RFLP of PCR products. (a) RT–PCR products obtained with primer pair PB1F/PB1R targeting gene segment 2 of the ISAV genome. Lane M contains molecular size markers (1 kbp plus DNA ladder; Canadian Life Technologies). Lanes 1, 2 and 3 contain PCR products from ISAV strains Glesvaer/2/90, RPC/NB-877 and ‘Back Bay 98’, respectively. (b)–(c) PCR products shown in (a) digested with EcoRI (b) or XhoI (c). The 0.9% agarose gels were stained with ethidium bromide and the DNA was visualized under 304 nm UV light.

two enzymes (Fig. 4). Thus, the Canadian isolates of ISAV had an RFLP profile distinct from those of the Norwegian isolate Glesvaer/2/90 and the Scottish isolate 390/98 and both EcoRI and XhoI would appear to provide stable restriction-site markers for Canadian strains of ISAV.
proteins are indicated on the right (in kDa). Positions of the pre-stained molecular mass markers (New England Biolabs) are shown on the left (in kDa), while the positions of the viral proteins are numbered beginning with the largest protein (Bio-Rad), sizes of which are indicated to the left (in kDa). Lane 1 contains purified ‘Back Bay 98’ virus. Lanes 2 and 3 contain moderately purified and ultra-pure RPC/NB-877 virus, respectively. Estimates of the molecular masses (in kDa) of the ISAV proteins are indicated to the right. The 66 kDa protein shown is BSA. (a) SDS–12.5% polyacrylamide gel. Lane M contains the molecular mass marker proteins (New England Biolabs) sizes of which are indicated to the left (in kDa). Lane 1 contains purified ‘Back Bay 98’ virus. Lanes 2 and 3 contain moderately purified and ultra-pure RPC/NB-877 virus, respectively. Estimates of the molecular masses (in kDa) of the ISAV proteins are indicated to the right. The 66 kDa protein shown is BSA. (b) Western blot of structural proteins of ISAV as in (a), reacted with rabbit antiserum to strain ‘Back Bay 98’. Positions of the pre-stained molecular mass markers (New England Biolabs) are shown on the left (in kDa), while the positions of the viral proteins are indicated on the right (in kDa).

**Morphology**

Electron micrographs of negatively stained, purified ISAV isolate RPC/NB-877 grown in SHK-1 cells revealed pleomorphic virus particles with surface projections, as described previously by Dannevig et al. (1995). The comparison of isolate RPC/NB-877 with isolate ‘Back Bay 98’ grown in CHSE-214 cells showed no significant differences in morphology (data not shown).

**Identification of variation in the structural proteins among ISAV strains**

Since the above data indicated that there were differences between ISAV isolates with respect to their ability to replicate in CHSE-214 cells, we investigated whether there were additional phenotypic differences between these isolates. Two ISAV isolates from the group that did not grow in CHSE-214 cells (Glesvaer/2/90 and RPC/NB-877) and two isolates from the group that grew and produced CPE in CHSE-214 cells (‘Back Bay 98’ and RPC/NB-049) were examined. Preparations of purified ISAV and pellets of uninfected cell controls were resolved on 12.5% SDS–PAGE. The gels were either stained with Coomassie blue R-250 and/or silver staining kit or were blotted on nitrocellulose membrane followed by immunoblot staining. Both groups of ISAV isolates showed four major polypeptides in Coomassie blue-stained gels. In isolates RPC/NB-877 and Glesvaer/2/90, these viral proteins had estimated molecular masses of 74, 53, 46 and 26.5 kDa. In isolates ‘Back Bay 98’ and RPC/NB-877, the major viral proteins were 74, 53, 43 and 26.5 kDa; in these two isolates, the 46 kDa band appeared as a minor polypeptide. Fig. 5(a) shows a Coomassie blue-stained gel of isolates ‘Back Bay 98’ and RPC/NB-877. A 105 kDa protein and a 66 kDa protein and three smaller polypeptides (18.5, 16 and 14.5 kDa) were seen in some virus preparations (Fig. 5(a), lane 2) but are probably of non-viral origin, since they were also detected in preparations of uninfected cell controls and in preparations of other purified fish viruses (data not shown). In order to confirm which of the polypeptides were of viral origin, Western blot analysis was performed with rabbit antiserum to ISAV isolate ‘Back Bay 98’. As shown in Fig. 5(b), the blot of the homologous virus showed protein bands of 74, 46, 43 and 26.5 kDa, whereas the blot of the heterologous virus isolate RPC/NB-877 had protein bands of 74 and 46 kDa only. The 53 kDa protein band was not detected by Western blot analysis in any purified ISAV preparation. Western blots of the uninfected cell control showed protein band (data not only the 66 kDa shown). The structural proteins of the different ISAV isolates studied so far are summarized in Table 2.

**Discussion**

Virus replication in cell culture with production of CPE is an important property that allows not only recognition and isolation of the virus from clinical specimens but also the performance of biological studies on a virus. Although the growth of ISAV in the CHSE-214 cell line has been reported (Bouchard et al., 1999), others have found this cell line to be refractory to ISAV infection (Dannevig et al., 1995). In the

<table>
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<th>Protein</th>
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The viral proteins are numbered beginning with the largest protein seen on Coomassie blue-stained SDS–PAGE gels. Molecular masses are expressed in kDa.

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**Table 2. Comparison of estimated masses of major structural polypeptides of ISAV isolates**
present study, we used 13 different ISAV isolates to describe the nature of replication of ISAV in the CHSE-214 cell line and to characterize ISAV further. The data obtained reveal phenotypic differences between ISAV isolates.

Inoculation of SHK-1 cells with the ISAV isolates used in this study showed that all the isolates caused CPE in the SHK-1 cell line and were therefore viable. Electron microscopic examination of a negatively stained, purified virus preparation of one of the isolates revealed the characteristic morphology of long filamentous and spherical virions. RT–PCR was also used in the present study to confirm the identity of the virus isolates. Thus, all the virus isolates used meet the definition of ISAV (Dannevig et al., 1995; Falk et al., 1997; Mjaaland et al., 1997).

Only some of the ISAV isolates were found to replicate and cause CPE in the CHSE-214 cell line. The CPE developed slowly, was focal and involved only a limited number of cells in the monolayer. The fact that inoculation of CHSE cells with large virus doses did not result in a delay in the development of CPE indicated to us that the poor replication of ISAV in CHSE-214 cells was not due to generation of DIPs. Therefore, several attempts were made to increase the speed and intensity of CPE development including using re-used medium after virus inoculation, changing to fresh maintenance medium every 7 days after 14 days p.i., trypsin treatment of the medium and adjustment of the pH of the maintenance medium. Only the frequent change to fresh maintenance medium and trypsin treatment of the medium caused significant increases in the intensity of CPE and virus yield. Trypsin was previously reported to have a beneficial effect on ISAV replication in SHK-1 cells (Falk et al., 1997).

The fact that six of the ISAV isolates in the present study, as well as the ‘Bliss Harbor 8/97’ isolate reported previously (Bouchard et al., 1999), were able to replicate and cause CPE in the CHSE-214 cell line indicates that this cell line is susceptible to ISAV. It is well established that proteolytic cleavage of the haemagglutinin molecule on the viral envelope is a prerequisite for initiation of influenza virus infection (Lazarowitz et al., 1973; Lazarowitz & Choppin, 1975). It is possible that the regular changes to fresh maintenance medium in the present study had a similar effect on ISAV replication in CHSE-214 cells, since without it the infection remained focal, involving only a limited number of cells in the monolayer (Figs 1 and 2). The focalization (i.e. inability to spread in the liquid medium) of progeny virions released during replication of ISAV in CHSE-214 cells was confirmed by the demonstration of larger amounts of cell-associated virus compared with cell-free virus in the one-step growth curve of the virus (Fig. 3).

Some of the ISAV isolates were unable to grow and produce CPE by the fourth blind passage in CHSE-214 cells. This result suggests that caution should be observed when interpreting a negative virus isolation result of ISAV from an ISA disease outbreak when using CHSE-214 cells alone. Because the CHSE-214 cell line was previously reported not to support replication of ISAV isolates from Norway (Dannevig et al., 1995; Falk et al., 1997) and Canada (Byrne et al., 1998), it was necessary to rule out the possibility that the Canadian isolates of ISAV that did not grow in CHSE-214 cells were similar to the Norwegian isolates of ISAV. It was previously shown that genome segment 2 of the Canadian ‘Bliss Harbor 8/97’ isolate and the Norwegian Sotra 92/93 isolate of ISAV exhibited 82.9% nucleotide sequence identity, which suggested that the two could represent distinct genomic variants of ISAV (Blake et al., 1999). Therefore, all isolates used in the present study were examined by RT–PCR and RFLP of genome segment 2. All 11 Canadian isolates had the same RFLP profile, irrespective of their ability to grow and cause CPE in CHSE-214 cells, which was different from those of the Norwegian ISAV isolate Glesvaer/2/90 and the Scottish ISAV isolate 390/98.

The structural protein profile of ISAV has not been conclusively determined. Four major polypeptides with estimated molecular masses of 74, 53, 43 and 26.5 kDa were seen in Coomassie blue-stained gels of both ‘Back Bay 98’ and RPC/NB-049 viruses grown in CHSE-214 cells. The same number and molecular masses of proteins, except a 46 kDa instead of the 43 kDa polypeptide, were also detected in purified virions of isolates RPC/NB-877 and Glesvaer/2/90, which do not grow in CHSE-214 cells. This profile of major polypeptides is in good agreement with a previously published pattern of polypeptides of ISAV isolate Glesvaer/2/90 in silver-stained gels (Falk et al., 1997). In isolates ‘Back Bay 98’ and RPC/NB-049, the 46 kDa polypeptide appeared as a minor component, suggesting that in these isolates it may be processed further to the major 43 kDa polypeptide. Western blot analysis with antiserum to ‘Back Bay 98’ virus confirmed the viral origin of the 74, 46, 43 and 26.5 kDa polypeptides (Fig. 5 b). Failure to detect the major protein bands of 53 kDa in both ‘Back Bay 98’ and RPC/NB-877 viruses and 26.5 kDa in RPC/NB-877 virus in Western blots may be due to the conformational nature of epitopes in these proteins. Alternatively, the lack of reaction of the 53 and 26.5 kDa bands could be due to small amounts of antigen in the Western blots. From the data summarized in Table 2, we conclude that strains of ISAV show differences in the molecular masses of their polypeptides. Influenza viruses are also known to show strain variation in their polypeptide sizes (Kendal, 1975).

On the basis of the RFLP, the Canadian isolates of ISAV are distinctly different from the Norwegian and Scottish isolates of ISAV. Thus, our observations confirm the inference from the sequence data published by Blake et al. (1999) that the Canadian and Norwegian isolates are genetic variants of ISAV. However, it is interesting that there are apparent phenotypic differences among Canadian isolates, given that these isolates are from a very restricted area (Bay of Fundy, New Brunswick) and that the disease caused by this virus has only been recognized since July 1997. Orthomyxoviruses such as influenza A virus survive by circulating in more than one
animal species and this may also be the case for ISAV, since several fish species may serve as natural hosts for this virus (Nylund et al., 1994). It is possible that the virus had existed in the wild fishery in the Bay of Fundy for some time before the appearance of clinical disease in farmed Atlantic salmon. It is also possible that the ‘original’ ISAV that occurs in natural populations of salmonids may have a lower virulence compared with strains causing ISA on Atlantic salmon farms (Nylund et al., 1999). Thus, the virus may be evolving in the wild fishery and the strain variation we are detecting may be a reflection of the wide range of natural fish hosts for the virus in the Bay of Fundy.

In conclusion, we demonstrate that Canadian isolates of ISAV fall into two groups on the basis of their ability to grow and cause CPE in the CHSE-214 cell line. Isolates that cause CPE in this cell line tend to remain cell-associated, resulting in focalized infection of the cell monolayer and slow development of CPE. Further analysis of Canadian isolates of ISAV suggests the existence of variation in the molecular masses and composition of the structural viral proteins. Moreover, Canadian isolates of ISAV can be differentiated from Norwegian and Scottish isolates by RT–PCR and RFLP of genome segment 2.

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