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Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile

Frederick S. B. Kibenge[1,*], Oscar N. Gárate[2], Gerald Johnson[1], Roxana Arriagada[4], Molly J. T. Kibenge[2], Dorota Wadowska[3]

[1]Department of Pathology and Microbiology, [2]AVC Inc., and [3]EM Laboratory, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, Prince Edward Island C1A 4P3, Canada
[4]Aquatic Health Chile Ltda, Benavente 952, Puerto Montt, Chile

ABSTRACT: The isolation of infectious salmon anaemia virus (ISAV) from asymptomatic wild fish species including wild salmon, sea trout and eel established that wild fish can be a reservoir of ISAV for farmed Atlantic salmon. This report characterizes the biological properties of ISAV isolated from a disease outbreak in farmed Coho salmon in Chile and compares it with ISAV isolated from farmed Atlantic salmon in Canada and Europe. The virus that was isolated from Coho salmon tissues was initially detected with ISAV-specific RT-PCR (reverse transcription-polymerase chain reaction). The ability of the virus to grow in cell culture was poor, as cytopathology was not always conspicuous and isolation required passage in the presence of trypsin. Virus replication in cell culture was detected by RT-PCR and IFAT (indirect fluorescent antibody test), and the virus morphology was confirmed by positive staining electron microscopy. Further analysis of the Chilean virus revealed similarities to Canadian ISAV isolates in their ability to grow in the CHSE-214 cell line and in viral protein profile. Sequence analysis of genome segment 2, which encodes the viral RNA polymerase PB1, and segment 8, which encodes the nonstructural proteins NS1 and NS2, showed the Chilean virus to be very similar to Canadian strains of ISAV. This high sequence similarity of ISAV strains of geographically distinct origins illustrates the highly conserved nature of ISAV proteins PB1, NS1 and NS2 of ISAV. It is noteworthy that ISAV was associated with disease outbreaks in farmed Coho salmon in Chile without corresponding clinical disease in farmed Atlantic salmon. This outbreak, which produced high mortality in Coho salmon due to ISAV, is unique and may represent the introduction of the virus to a native wild fish population or a new strain of ISAV.

KEY WORDS: ISAV in Coho salmon · ISAV morphology · Immunoprecipitation of ISAV proteins

INTRODUCTION

Infectious salmon anaemia (ISA) virus (ISAV) is a new orthomyxovirus virus of fish (Dannevig et al. 1995, Mjaaland et al. 1997) that causes ISA in farmed Atlantic salmon *Salmo salar* (Thorud & Djupvik 1988). To date, ISAV has been isolated and shown to have caused high mortality in salt-water farmed Atlantic salmon in Norway since 1984 (Dannevig et al. 1995), in New Brunswick, Canada, since 1996 (Byrne et al. 1998, Mullins et al. 1998), and in Scotland, UK, since 1998 (Rodger et al. 1998). Most recently, ISA occurred in Nova Scotia, Canada, and in the Faroe Islands, Denmark, in 2000. In the latter, both Atlantic salmon and rainbow trout were affected but only Atlantic salmon showed clinical signs of ISA. The virus has not been known to cause clinical disease in fish species other than Atlantic salmon nor to occur outside of these geographical areas. Canadian isolates of ISAV have been shown to fall into 2 groups on the basis of their ability to replicate in the CHSE-214 cell line with production of cytopathic effect (CPE) (Kibenge et al. 2000). Isolates of the group which cause CPE in this cell line (i.e., the CHSE-positive phenotype) tend to remain cell-

*E-mail: kibenge@upei.ca

associated, resulting in focalized infection of the cell monolayer and slow development of CPE. ISAV isolates from Norway and Scotland, similar to the second Canadian group (the CHSE-negative phenotype), do not produce CPE in the CHSE-214 cell line. However, there are significant nucleotide and amino acid sequence differences between the European and Canadian isolates on RNA segments 2 and 8 (Blake et al. 1999), and the European isolates could be differentiated from Canadian isolates by reverse transcription-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) on RNA segment 2 (Kibenge et al. 2000).

The present study characterizes an orthomyxo-like virus isolated from farmed Coho salmon *Oncorhynchus kisutch* in Chile, with clinical disease. While erythrocytic inclusion body syndrome virus (EIBSV) was detected in one fish sample, the orthomyxo-like virus was found in several fish tissue pools from the affected farms. The virus was biologically characterized by assessment of its ability to replicate in different cell lines, morphology, viral protein composition, and genetic heterogeneity by RT-PCR and DNA sequence analysis. The studies established that this orthomyxo-like virus was indeed an ISAV isolate.

MATERIALS AND METHODS

Clinical samples. Eleven samples were received at the Atlantic Veterinary College (AVC) in March-April 1999 from Aquatic Health Chile Ltda., Puerto Montt, Chile. The samples were homogenates of tissue pools consisting of kidney, spleen, and pyloric caeca collected from sick Coho salmon on fish farms in Chile.

Fish cell lines. Three fish cell lines (CHSE-214, SHK-1, and TO cell lines) were used in this study. The CHSE-214 cells (Fryer et al. 1965) were propagated using standard procedures for routine isolation of fish viruses. Growth medium was HMEM (Eagle's minimum essential medium containing Hanks' salts and sodium bicarbonate [0.2 g l^{-1}]; Canadian Life Technologies), supplemented with 292 mM L-glutamine, 100 units penicillin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B, with 10% foetal bovine serum (FBS). Cells were incubated at 16°C. The SHK-1 cells (Dannevig et al. 1995) were propagated at room temperature (22°C) in Leibovitz's L-15 medium supplemented with 10% FBS, 4 mM glutamine, 100 units penicillin, 100 µg streptomycin and 0.25 µg amphotericin B. The TO cell line was a gift from Dr Heidrun Wergeland (University of Bergen, Norway); it is a new salmonid cell line originating from Atlantic salmon head kidney leukocytes (Wergeland & Jakobsen 2001). TO cell monolayers were grown at room temperature (22°C) in HMEM (BioWhittaker Inc., Walkersville, MD, USA) supplemented with 292 mM L-glutamine (Sigma), 1% non essential amino acids (NEAA) (Sigma), 100 µg ml^{-1} gentamicin (Sigma) and 10% FBS. For all fish cell lines, FBS was reduced to 5% in the maintenance medium.

Virus isolation. SHK-1 and CHSE-214 cell monolayers in 24-well tissue culture plates were inoculated with 100 µl per well of tissue homogenate diluted 1:50 in serum-free medium and filtered through 0.45 µm syringe filters prior to use. Plates were sealed with plate sealers and incubated at 16°C for 1 h to allow for virus adsorption. Maintenance medium was then added and plates were further incubated at 16°C for 14 d with daily microscopic examination for CPE. All samples were passaged a minimum of 4 times on CHSE-214 or SHK-1 cell lines before they were recorded as negative for virus isolation. Cultures were also checked for virus by RT-PCR and/or electron microscopy (EM) and indirect fluorescent antibody technique (IFAT).

Virus adaptation to cell culture. Cultures were passaged up to 4 times in SHK-1 or CHSE-214 cell lines, in an attempt to adapt the virus to replicate in cell culture. The lysates at different passage levels were also passaged up to 4 times in TO cell line with or without added 2.5 µg ml^{-1} trypsin in the medium as previously described (Kibenge et al. 2000).

IFAT. IFAT was performed on fixed CHSE-214 or SHK-1 cell cultures in 48-well culture plates with rabbit antiserum to ISAV as described previously (Kibenge et al. 2000).

Electron microscopy. For EM of virus-infected and mock-infected TO cells, the washed cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at room temperature. Cells were collected by low-speed centrifugation (400 × *g*, 5 min), fixed with 1% osmium tetroxide, and processed for ultrathin sectioning on a Reichert-Jung Ultracut E microtome. Ultrathin sections were stained in 5% uranyl acetate prior to electron microscopic examination. The sections were examined in a Hitachi H600 electron microscope operated at 75 keV.

RT-PCR. Samples were examined for presence of ISAV nucleic acids by RT-PCR as previously described (Kibenge et al. 2000). Briefly, viral RNA was extracted from 250 µl volumes of either tissue homogenates or cell culture lysates using TRIZOL LS Reagent (Canadian Life Technologies) following the manufacturer's protocol. The PCR primers targeting ISAV RNA segment 8 and consisting of 5′-GAA GAG TCA GGA TGC CAA GAC G-3′ (FA-3, sense) and 5′-GAA GTC GAT GAT CTG CAG CGA-3′ (RA-3, antisense), which yield a PCR product of 220 bp (Dr Are Nylund pers. comm.) or 5′-GGC TAT CTA CCA TGA ACG AAT C-3′ (OIE, sense) and 5′-GCC AAG TGT AAG TAG CAC TCC

(OIE, antisense), which yield a PCR product of 155 bp (Mjaaland et al. 1997), were used for detection of ISAV nucleic acids in fish samples and cell cultures. To obtain a larger segment 8 fragment, the following PCR primers 5′-GGC TAT CTA CCA TGA ACG AAT C-3′ (Cun8 F, sense) and 5′-TCT TTT GTA TAA TGA TCA AGT ACA C-3′ (Cun8 R, antisense), which yield a 878 bp DNA fragment (Cunningham & Snow 2000), were used. One-step RT-PCR was carried out using the Titan™ One Tube RT-PCR System kit (Roche Diagnostics). The RT-PCR was performed in a PTC-200 DNA Engine Peltier thermal cycler (MJ Research, Inc, Watertown, MA, USA). Cycling conditions consisted of 1 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 94ºC 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 a 2% agarose gel and visualized under 304 nm UV light after staining with ethidium bromide (Sambrook et al. 1989). The PCR products were then cloned into the pCRII vector using a TOPO TA cloning kit (Invitrogen) in preparation for sequencing.

Protein labeling and immunoprecipitation. ISAV-infected TO cell lysates were tested for ISAV proteins by metabolic labeling followed by immunoprecipitation, SDS-PAGE and autoradiography. The TO cells in 6-well tissue culture plates infected with different isolates of ISAV (Chilean isolate 7833-1, Canadian isolates Back Bay 98 and RPC/NB-970 877-2, Scottish isolate 390/98, and Norwegian isolate Glesvaer/2/90) at a high multiplicity of infection (moi, >1.0) were labeled as described previously (Kibenge et al. 1999), with minor modifications. At 24 h post-infection, the monolayers were washed twice with cold phosphate buffered saline (PBS), pH 7.2, and were incubated in 1 ml per well methionine-free HMEM supplemented with 5% dialysed FBS to deplete residual methionine. After 2 h, cells were radiolabeled for 20 h with 340 µCi of [^{35}S] methionine (specific activity approximately 1000 Ci mmol^{-1} at 10 µCi l^{-1}; Amersham Pharmacia Biotech) in 1 ml methionine-free HMEM containing 5% dialysed FBS. The media of virus-infected and mock-infected cultures were collected and the volume was reduced to approximately 500 µl by osmosis with dry polyethylene glycol (PEG 8000), prior to storage at –20°C. The cells were washed twice with PBS and then lysed with 500 µl RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% aprotinin, 1% Triton X-100, 1% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 7.2) prior to storage at –20°C.

ISAV proteins were analyzed by immunoprecipitation with rabbit antiserum to purified ISAV isolate RPC/NB-980 049-1 prepared as described previously (Kibenge et al. 2000). The immunoprecipitation was carried out as described by Sambrook et al. (1989). The 500 µl of media and 100 µl of cell lysates of virus-infected or mock-infected cells were initially precleared with pre-immune rabbit serum and Protein-A Sepharose (Sigma) to reduce nonspecific background. For the specific immunoprecipitations, the precleared supernatant of cell lysate or medium was brought to 500 µl total volume in NET-gel buffer (150 Mm NaCl, 0.1% Nonidet P-40, 1 mM EDTA, pH 8.0, 0.25% gelatin, 0.02% sodium azide, 50 mM Tris-HCl, pH 7.5) were each reacted with 20 µl of rabbit hyper immune antiserum at 0°C for 1 h. Each mixture was then reacted with Protein-A Sepharose at 4°C for 1 h. The Sepharose beads were washed with 10 mM Tris-HCl, pH 7.5, 0.1% NP-40, mixed with 30 µl of 2× SDS sample loading buffer, and heated at 100°C for 5 min prior to analysis on 12.5% SDS-PAGE and autoradiography for 48 h at room temperature.

Viral genome cDNA cloning and screening of the cDNA library. To prepare viral RNA for cDNA cloning, virus was purified as described previously (Kibenge et al. 2000). The purified virus was digested with 2 mg ml^{-1} Proteinase K at 37°C for 2 h and then treated with TRIZOL LS Reagent to isolate viral RNA. After washing of the isolated RNA with 75% ethanol in diethyl pyrocarbonate-treated water (DEPC-H_2O), the pellet was dissolved in sterile RNase-free water and the concentration of RNA was estimated by measuring the optical density at 260 nm. One microgram of total RNA was used to synthesize cDNA using a cDNA synthesis kit (Stratagene) with random primers. An *Eco*RI/*Not*I adapter was ligated to cDNA with T4 DNA ligase at 16°C for 18 h after the second-strand synthesis. The double-stranded cDNA was ligated in the *Eco*RI site of plasmid pUC18 (Amersham Pharmacia Biotech) and was used to transform DH5α competent *Escherichia coli* cells (Canadian Life Technologies).

Individual bacterial colonies from the cDNA library were analyzed for the presence of recombinant plasmids using the alkaline lysis method of Birnboim & Doly (1979) for plasmid DNA isolation followed by *Eco*RI digestion and agarose gel electrophoresis. Fresh cultures with cDNA inserts were screened by colony blot hybridization. Briefly, the membranes were placed uppermost on a series of solution saturated 3MM paper filters: in 10% SDS for 3 min to lyse the bacteria, then in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 5 min to denature the DNA, and in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) twice for 3 min each. The membranes were briefly washed in 2 × SSC (1 × SSC is 0.015 M trisodium citrate, 0.15 M NaCl, pH 7.0) and were used immediately or stored wrapped in Saran Wrap desiccated at 4°C until used in colony blot hybridization. Radiolabeled probes were

prepared using ISAV RT-PCR products or plasmid cDNA inserts purified from low-melting temperature agarose gels (Sambrook et al. 1989). The DNA probes were radiolabelled using the Random Primer Labeling Kit (Canadian Life Technologies) and 50 µCi of [α-^{32}P]dCTP (specific activity approx. 3000 Ci mmol^{-1}) (Amersham Pharmacia Biotech) following the manufacturers' instructions, and were purified and denatured before use (Kibenge 1992). Hybridization at 54°C contained 9×10^6 cpm of probe/membrane. Membranes were then washed under high stringency prior to autoradiography at –70°C (Keller & Manak 1989).

DNA sequence analysis. Plasmid DNA for sequencing was prepared as described before (Kibenge et al. 1991). Denatured plasmid DNA was sequenced using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc) and the PCR Express (Hybaid) thermal cycler. Sequencing reactions were resolved on a model 377 ABI Prism Automated DNA Sequencer (Applied Biosystems, Inc) using 36 lanes on a 36 cm plate. Amersham's 'mobility file' (US81072) that comes with the dye terminator kit was used to correctly call the bases. The electropherograms were inspected and edited using 'Sequencing Analysis 3.3' software provided with the 377 Prism by ABI. Sequence analysis used the Lasergene Biocomputing software for Windows (DNASTAR Inc), the Sequence Manipulation suite (Stothard 2000), and the FASTA program package for microcomputers (Pearson & Lipman 1988).

RESULTS

Clinical history

From early February to late April 1999, unusually high mortality occurred in Coho salmon *Oncorhynchus kisutch* held in the same sea netpens with rainbow trout *O. mykiss* and Atlantic salmon *Salmo salar* on fish farms in Chile. The clinical signs consisted of sudden rise in mortality 2 mo after entering seawater. At necropsy, the affected fish were jaundiced with pale gills, severe anaemia (haematocrit <10%), pale liver, with a gall bladder which adopted the same colour as the liver, and mild splenomegaly. Externally, the base of the fins had a yellow colouration, with a yellowish belly. Bacterial, nutritional and toxic factors were ruled out as possible causes of the mortalities. Clinical samples were forwarded to the AVC Virology Research Laboratory for analysis. Prior to this outbreak, only infectious pancreatic necrosis virus (IPNV), a member of the virus family *Birnaviridae* (Dobos et al. 1995), had been isolated regularly from farmed fish species in Chile. However, tissues and cell culture harvests from this particular disease outbreak were negative for IPNV by RT-PCR performed at the laboratory facility of Aquatic Health Chile Ltda. In duplicate samples in a separate submission for electron microscopy, viral particles typical of EIBSV were observed in red blood cells in the spleen of 1 fish from Farm A (Dr Dave Groman pers. comm.).

Detection of ISAV in Coho salmon clinical samples

Two groups of clinical samples, designated here as Groups 1 and 2 were received from Chile. When tested in the AVC Virology Research Laboratory, 6 of 9 samples in Group 1 were RT-PCR positive with primer pair FA/RA amplifying ISAV RNA segment 8 sequences. These samples were also CPE positive in SHK-1 and CHSE-214 cell lines on the first passage. The CPE on SHK-1 and CHSE-214 cells developed very slowly and consisted of extensive vacuolation (beginning at 8 to 11 dpi) and rounding and detachment of single cells. The cell cultures were positive in RT-PCR, and some were also weakly positive on IFAT with rabbit antiserum to ISAV. Two of the group 2 samples were RT-PCR positive but no CPE was observed in the inoculated cell cultures.

The ISAV-specific RT-PCR products from all samples were molecularly cloned using the TOPO TA Cloning Kit (Invitrogen) and their nucleotide sequences were determined. The products amplified using primer pair FA/RA targeting ISAV RNA segment 8 had about 93 and 96 to 97% nucleotide sequence identity with published Norwegian and Canadian ISAV sequences, respectively.

Subsequently, duplicate samples from Group 1 that had been stored frozen at –80°C at the Aquatic Health Chile Ltda laboratory facility were tested at that facility by F.S.B.K.. The results of the RT-PCR test using the OIE primer pair (Mjaaland et al. 1997) are shown in Fig. 1. All samples were RT-PCR positive except clinical samples #7 (lane 7) and #11 (lane 11), and passage 2 (P2) of #5 in SHK-1 cell line (lane 13). Clinical sample #5 (lane 5) was also CPE positive in SHK-1 cells in the first passage, and the cell lysate was also RT-PCR positive (lane 12). Virus was subsquently isolated from this sample using SHK-1 cells (Dr Oscar Gárate pers. comm.).

Adaptation of ISAV isolated from Coho salmon in Chile to regularly produce CPE in cell culture

In all cases where samples were CPE positive in the first passage in cell culture, the CPE disappeared in subsequent passages. Cultures were passaged up to 4

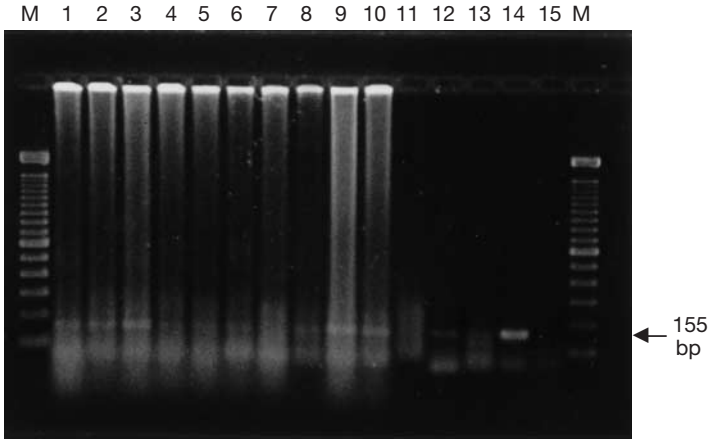

Fig. 1. Agarose gel electrophoresis of RT-PCR products from Coho salmon samples, with OIE primer pair (Mjaaland et al. 1997) targeting gene segment 8 of the ISAV genome. Lane M contains 100 bp DNA ladder (GIBCO BRL Life Technologies). Lanes 1 to 11 contain tissue samples #1, #2, #3, #4, #5, #6, #7, #8, #9, #10, and #11, respectively. Lanes 12 and 13 contain passages 1 and 2, respectively, of #5 in SHK-1 cell line. Lane 14 contains ISAV 'Back Bay 98' RNA used as positive control. Lane 15 contains a 'water only' negative control

times in each cell line before declaring a sample negative or positive for virus. In an attempt to adapt the Chilean viruses to cell culture, passage 1 (P1) of sample 3, which was CPE positive in the TO cell line, was inoculated to fresh TO cell monolayers with or without trypsin. Only the passage in presence of 2.5 µg ml^{-1} trypsin resulted in CPE. The CPE in P2 was weak but the supernatant gave a strong positive RT-PCR result with primer pair FA/RA. Moreover, when this P2 CPE-positive material was inoculated on fresh TO, SHK-1 and CHSE-214 cell monolayers, it produced CPE in all 3 cell lines in the absence of trypsin. P4 of the SHK-1 viral harvest in SHK-1 cell line in the absence of trypsin also produced frank CPE at 4 dpi, which progressed to involve the whole cell monolayer by 7 dpi when the virus was harvested. The CPE produced by the Chilean virus, designated here as 7833-1, consisted of cell rounding, enlargement and detachment from the substrate as well as syncytia formation, with some cells unaffected, which is different from that produced by ISAV isolates from Canada and Europe (characterized by cell shrinkage and necrosis, and detachment from the substrate involving all cells in the monolayer [Mjaaland et al. 1997] with no evidence of syncytia formation).

Virus morphology

To obtain virus for electron microscopic examination, TO cell monolayers in 4-well tissue culture plates were inoculated with P4 of ISAV isolate 7883-1. Virus-infected and mock-infected TO wells were used at 4 dpi. The mock-infected cells appeared normal when examined by electron microscopy (data not shown). Fig. 2 shows electron micrographs of positively stained cell pellets from virus-infected preparations. The cytoplasm of these infected cells showed extended endoplasmic reticulum (Fig. 2A). Numerous virus particles consistent with ISAV morphology (Mullins et al. 1998) were seen adhering to the outside of several cells and within the intercellular spaces, and a few virus particles were also found in intracytoplasmic vacuoles (Fig. 2A). At higher magnification, the virus particles appeared pleomorphic and enveloped, each with a uniform fringe surrounding several electron-dense nucleocapsids (Fig. 2B).

Immunoprecipitation of ISAV proteins

Pilot experiments demonstrated to us that there were no viral proteins present in the cell culture medium after 20 h of radiolabeling virus-infected cultures at 24 h post-infection. The radiolabeled proteins in the cell lysates with or without immunoprecipation using rabbit antiserum to ISAV are shown in Fig. 3. Several proteins appear in cell lysates without immunoprecipitation (Fig. 3, lanes 1 to 5). The 3 main ones, which also appeared in the control uninfected cell lysate (data not shown), were the 63, 39 and 25 kDa proteins, of which the 63 kDa is most likely bovine serum albumin. No protein was immunoprecipitated by the ISAV antiserum in the control uninfected cell lysate (Fig. 3, lane 6), indicating that the immunoprecipitated proteins seen in the infected cell lysates (lanes 7 to 11) were viral proteins. A total of 12 proteins (Table 1) were immunoprecipitated in the different infected cell lysates, and they resolved in 12.5% SDS-PAGE in 7 clusters as 80–94, 69, 38–41.5, 33.5–36, 30, 25, and 19–20 kDa bands (Fig. 3, lanes 7 to 11). The immunoprecipitate of the Chilean 7833-1 virus (lane 11) had a similar protein profile to ISAV isolate 'Back Bay 98' (lane 10), and both differed from ISAV isolates Glesvaer 2/90, and 390/98 in the 33.5–36 kDa cluster of proteins (lanes 7 and 8).

Nucleotide sequence of RNA segments 2 and 8 of ISAV isolate 7833-1

To identify RNA segment 2 clones in the ISAV 7833-1 cDNA library, RT-PCR was performed using the PB1F and PB1R primer pair (Kibenge et al. 2000) and RNA extracted from 7833-1. The 600 bp RT-PCR product

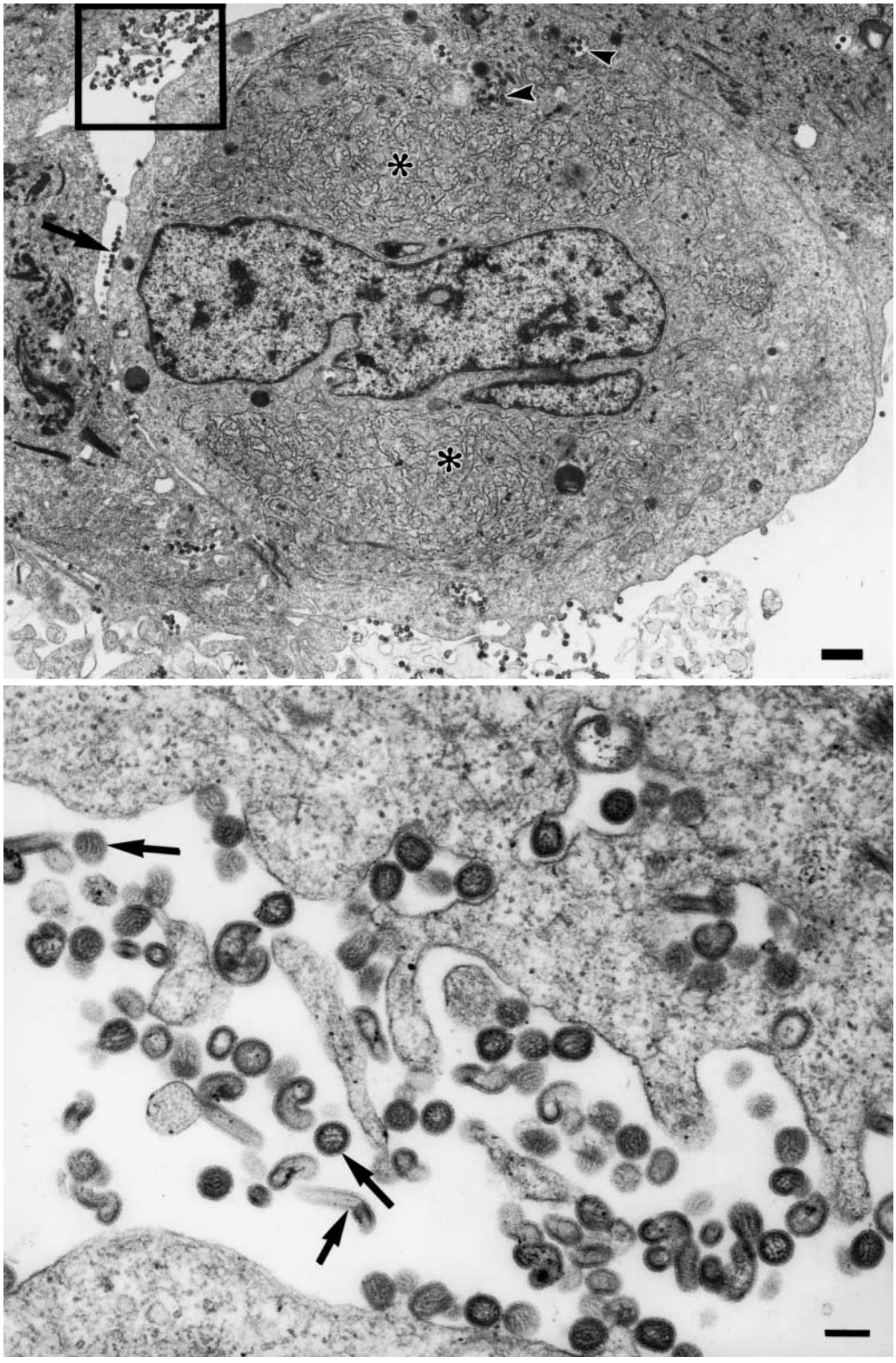

Fig. 2. Electron micrograph of TO cells inoculated with Chilean ISAV isolate 7833-1. (A) Ultrathin section of infected cells. Note the extended endoplasmic reticulum (asterisk), and numerous virus particles adherent to the outside of cells (arrow) and within the intercellular spaces (box), and a few virus particles in intracytoplasmic vacuoles (arrowheads). Scale bar = 0.86 µm. (B) Higher magnification of the box area in (A) showing individual pleomorphic enveloped virus particles, each with a uniform fringe surrounding several electron-dense nucleocapsids (arrows). Scale bar = 143 nm

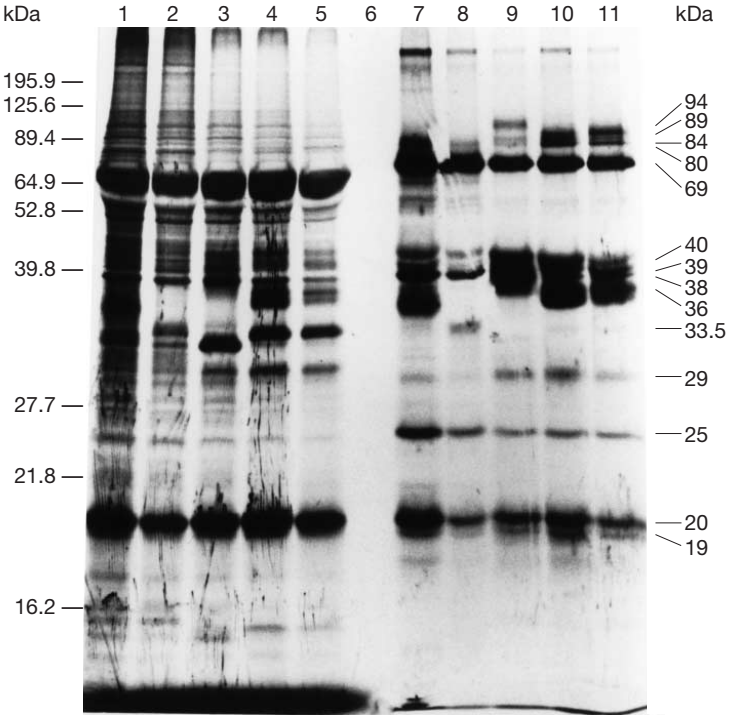

Fig. 3. SDS-PAGE of proteins labeled with [^{35}S]methionine in TO cells infected with different ISAV isolates. The prestained molecular weight marker proteins (BioRad) which ran in lane M are drawn in on the left. Lanes 1 to 5 contain Radiolabeled proteins synthesized in TO cells infected with ISAV isolates Glesvaer 2/90, 390/98, RPC-970 877-2, 'Back Bay 98', and 7833-1, respectively. Lane 6 contains immunoprecipate of TO-mock infected cells and lanes 7 to 11 contain labeled proteins as in lanes 1 to 5, immunoprecipitated with rabbit antiserum to ISAV isolate

obtained was used as a probe on colony blots of the 7833-1 cDNA library to identify related clones. These clones were sequenced and the cDNA inserts from the different clones were then used as probes to extend this sequence. The FASTA program (Pearson & Lipman 1988) was used to perform pairwise comparisons between sequences, allowing the compilation of 1845 nucleotides of the 7833-1 RNA segment 2 sequence from 4 cDNA clones. This sequence has been deposited in the GenBank database under accession number AF287950.

The FASTA program was also used to perform pairwise comparisons between the 7833-1 RNA segment 2 sequence and representative published RNA segment 2 sequences from ISAV isolates from Norway, Canada, and Scotland (Table 2). The Chilean sequence determined from the 4 cDNA clones spans nucleotide positions 309–2153 and amino acid positions 92–705 of the full length sequence of RNA segment 2 of Norwegian ISAV isolate Sotra 92/93 (Krossøy et al. 1999). It can be seen that the Chilean virus is most closely related to the Canadian virus, showing identities higher than 99% at both the nucleotide and amino acid levels. Alignment of the amino acid sequences using Multi Align Show in the Sequence Manipulation Suite (Stothard 2000) showed that the 614 amino acids of the Chilean virus in the area sequenced were 100% identical to those of the Canadian virus. Both viruses differed from the Scottish and Norwegian viruses in 15 residues each while at these positions, the Scottish virus differed from the Norwegian virus in 7 residues.

To obtain a large fragment of RNA segment 8 for sequence analysis, RT-PCR was performed using the primer pair Cun8F/Cun8R on RNA extracted from 7833-1. This primer pair is identical to that used by Cunningham & Snow (2000) and yields a product 878 bp long. This sequence has been deposited in the GenBank database under accession number AF312315. Ony partial RNA segment 8 sequences have been published for Canadian isolates; 435 bp for Bliss Harbour strain (Blake et al. 1999) and 592 bp for Bay of Fundy 97 strain (Lovely et al. 1999). Therefore, to obtain a comparative sequence length of a Canadian ISAV isolate, RNA extracted strain RPC/NB 877 and was subjected to RT-PCR with primer pair Cun8F/Cun8R under the same conditions, and the 878 bp product was then sequenced. This sequence has been deposited in the GenBank database under accession number AF312316.

The FASTA program was used to perform pairwise comparisons between the 7833-1 RNA segment 8 sequence and representative published ISAV RNA segment 8 sequences. Comparison of the 7833-1 sequence

Table 1. ISAV proteins precipitated with ISAV-specific rabbit polyclonal antiserum

Protein no.	7833-1	Back Bay 98	RPC/NB 970-877-2	390/98	Glesvaer/ 2/90
1	89	85	94		
2	84	82	84		
3	80	80	80	80	80
4	69	69	69	69	69
5	40	40	40.5	41.5	41.5
6	39	39	39	40	40
7	38	38	38	39	39
8	36	36	36	33.5	35.5
9	29	30	30	29	29
10	25	25.5	25.5	25.5	25.5
11	20	20.5	20.5	20	20
12	19	19	19.5	19	19

Table 2. Sequence identity in RNA segment 2 of different ISAV isolates (upper right is percent nucleotide sequence identities and lower left is percent amino acid sequence identities)

ISAV strain	7833-1[a]	Bay of Fundy 97[b]	Scottish isolate[c]	Sotra 92/93[d]
7833-1[a]	–	99.8	84.4	84.3
Bay of Fundy 97[b]	99.7	–	84.5	84.5
Scottish isolate[c]	97.1	97.3	–	99.1
Sotra 92/93[d]	97.2	97.6	98.9	–

[a]Chilean ISAV isolate 7833-1, GenBank Accession number AF287950
[b]Canadian ISAV isolate Bay of Fundy 97, GenBank Accession number AF262399
[c]Scottish ISAV isolate, GenBank Accession number AJ242808
[d]Norwegian ISAV isolate Sotra 92/93, GenBank Accession number AJ002475

to those of Canadian isolates revealed 100% identity with Bliss Harbuor strain, 99.8% identity with RPC/NB 970-877-2, and 97% identity with the Bay of Fundy 97 strain. Lower nucleotide sequence identities of 88.9 and 87.8% were seen in comparisons between 7833-1 sequence with the Scottish isolate and Glesvaer/90 strain from Norway, respectively. The amino acid sequence identities in the NS1 and NS2 proteins are summarized in Table 3. Overall, it can be seen that among the different ISAV isolates, NS2 protein is less variable than NS1 protein (95 to 96% identity vs 75 to 78% identity between Canadian and Scottish/Norwegian ISAV). However, for both NS1 and NS2 proteins, the Chilean virus is most closely related to the Canadian viruses, showing identities of practically 100% (Table 3).

DISCUSSION

Infectious salmon anaemia virus is a new orthomyxovirus of fish. Although the disease it causes, ISA, was first recognized in 1984 in Norway (Thorud & Djupvik 1988), the virus was not isolated until 1995, when it was shown to cause CPE in a newly developed SHK-1 cell line (Dannevig et al. 1995). The development of this cell line facilitated molecular characterization of the virus (Mjaaland et al. 1997, Devold et al. 2000) and the development of sensitive diagnostic reagents and methods for detection and identification of ISAV such as monoclonal antibodies used in IFAT (Falk et al. 1998) and primer sequences used in RT-PCR (Mjaaland et al. 1997). Thus when ISA first occurred in Canada, Scotland, and the Faroe Islands, the virus was isolated and identified as ISAV relatively quickly (Rodger et al. 1998, Lovely et al. 1999). This paper reports the first isolation of ISAV in the southern hemisphere, outside the recognized ISAV geographic areas, and it is also the first report of isolation of ISAV from clinically sick fish other than Atlantic salmon. Presence of the virus in Coho salmon tissues was initially detected with ISAV-specific RT-PCR. Because the virus grew poorly in cell culture, its presence in cell culture was difficult to monitor otherwise. We also report a more detailed protein profile of ISAV determined using metabolic radiolabeling in cell culture and immunoprecipitation with ISAV-specific rabbit antiserum.

Positive ISAV RT-PCR results on fish samples that were negative in virus isolation was an interesting observation for the Chilean fish samples. Since all the samples examined were from Coho salmon, it was considered that the virus required Coho salmon cell cultures for adequate virus replication and development of CPE. However, recently, we have come across samples from both apparently normal and sick farmed Atlantic salmon from New Brunswick that were positive in RT-PCR but negative in virus isolation. There is

Table 3. Amino acid sequence identity in RNA segment 8 of different ISAV isolates (upper right are percent sequence identities of NS1 protein and lower left are percent sequence identities of NS2 protein)

ISAV strains	7833-1[a]	RPC/NB 970-877-2[b]	Bliss Harbour[c]	Scottish isolate[d]	Glesvaer/90[e]
7833-1[a]	–	99.6	100	77.9	76.1
RPC/NB 970-877-2[b]	99.5	–	100	77.5	75.6
Bliss Harbour[c]	100	100	–	73.8	71.4
Scottish isolate[d]	95.5	96.0	96.5	–	99.6
Glesvaer/90[d]	95.1	95.7	96.2	100	–

[a]Chilean ISAV isolate 7833-1, GenBank Accession number AF312315
[b]Canadian ISAV isolate RPC/NB 970-877-2, GenBank Accession number AF312316
[c]Canadian ISAV isolate Bliss Harbour, GenBank Accession number AF095255
[d]Scottish ISAV isolate, GenBank Accession number AJ242016
[e]Norwegian ISAV isolate Glesvaer/90, GenBank Accession number AJ012285

anecdotal evidence of similar observations in Norway as well. This suggests that such discordant results are not unique to Coho salmon samples. It is possible that ISAV strains of low virulence and non-pathogenic strains grow poorly or not at all in currently available fish cell lines. A similar situation is known to occur in avian influenza viruses in that non-pathogenic strains do not produce plaques (CPE) in certain mammalian cell lines, a property that is directly related to the uncleavability of the haemagglutinin of these viruses, and is used to delineate virulent and avirulent avian influenza viruses (Klenk & Rott 1988).

The virus that was isolated from Coho salmon tissues was shown to be similar to Canadian ISAV isolates in its ability to grow in CHSE-214 cell line, in viral protein profile, and RNA segments 2 and 8 sequence. The very high degree of nucleotide sequence identity between the Chilean and Canadian viruses on the one hand and the Norwegian and Scottish viruses on the other suggests that ISAV strains occur in 2 geographic lineages: American and European. However, ISAV isolated recently from broodstock Atlantic salmon with clinical disease in Nova Scotia, Canada, closely resembled Norwegian ISAV (authors' unpubl. data).

The structural protein profile of ISAV has not been conclusively determined. Previous attempts to identify viral proteins of ISAV have utilized analysis of purified virus preparations in SDS-PAGE followed by silver staining (Falk et al. 1997) and/or Coomassie blue staining and Western blotting (Kibenge et al. 2000). These methods have been able to detect up to 4 major structural viral polypeptides. In the present study we used more sensitive methods, metabolic radiolabeling of synthesized proteins coupled with immunoprecipitation with ISAV-specific rabbit polyclonal antiserum followed by SDS-PAGE and autoradiography. This allowed us to detect up to 12 viral proteins. While some of these proteins may have precursor-product relationships, most of them must be mature proteins including non-structural proteins that could not be detected by traditional methods and show that ISAV has a similar protein profile to that of influenza viruses (Klenk et al. 1995). The 80–94 kDa cluster of proteins (Fig. 3) probably consists of the polymerase proteins. The calculated molecular weight of the PB1 RNA polymerase from the deduced amino acid sequence of the RNA segment 2 is 80.5 kDa (Krossøy et al. 1999). The 69 kDa band in the present study corresponds to the 71 kDa (Falk et al. 1997) and 74 kDa of previous reports (Kibenge et al. 2000). The 38–41.5 kDa cluster corresponds to the 43/46 kDa bands (Falk et al. 1997, Kibenge et al. 2000), and the band that is approximately 25 kDa in the present study corresponds to the 24 kDa (Falk et al. 1997) and 26.5 kDa of previous reports (Kibenge et al. 2000). Thus 3 new viral proteins of ISAV of 33.5–36, 29–30, and 19–20.5 kDa in size are demonstrated in the present study. The 33.5–36 kDa band is interesting in that it varies from a 33.5 kDa polypeptide in the Scottish ISAV isolate 390/89 to a 36 kDa protein in the Chilean isolate 7833-1 and Canadian ISAV isolates 'Back Bay 98' and RPC-970 877-2. The 29–30 and 19–20.5 kDa bands probably correspond to the non-structural proteins NS1 and NS2, respectively. The calculated molecular weights of these proteins from the deduced amino acid sequence of the RNA segment 8 are 23 kDa for NS1 and 18 kDa for NS2 (Mjaaland et al. 1997).

It is not known when and how ISAV was introduced into Coho salmon in Chile. ISAV can infect a wide range of fishes (Nylund et al. 1994, 1995, 1997), but in wild fish species, it usually produces an asymptomatic infection (Nylund et al. 1999). Within Norway, Scotland and the Canadian east coast (considered the normal geographic distribution), ISAV has been documented to cause disease outbreaks only in marine-farmed Atlantic salmon. Wild fish with virus but no disease are common (Nylund et al. 1999, Devold et al. 2000), suggesting that asymptomatic or mild infection usually occurs among wild fish in those regions. Similarly, substantial virulence in any other farmed fish species has not been reported. Therefore, an outbreak producing high mortality in Coho salmon is unusual for ISAV and may represent introduction from a native wild fish population or a new strain of ISAV. In both of these cases, migratory wild fish and importation of commercial fish eggs may play important roles in the natural transmission cycles.

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