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# Characterisation of ISAV proteins from cell culture

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ABSTRACT: The characterisation of 2 infectious salmon anemia virus (ISAV) proteins is described. Proteins were harvested from ISAV-infected Chinook salmon embryo (CHSE)-214 cell culture by continuous elution denaturing gel electrophoresis, enabling the harvest of specific molecular weight fractions. Through the use of a polyclonal antiserum to ISAV, it was possible to identify a potentially autolytic major antigen of 72 kDa and a glycosylated protein of approximately 38 kDa which varied in size depending on cell line compatibility. N-terminal amino acid sequencing of the glycosylated proteins suggests that it is encoded by segment 6 of the ISAV genome. Further, sequence analysis of the glycosylated protein account for the variable molecular weight and may explain differences in host cell compatibility.

KEY WORDS: SAV · Proteins · Electrophoresis · Glycoprotein



## INTRODUCTION

Infectious salmon anemia virus (ISAV) has proven to be the most economically important acute disease of Atlantic salmon aquaculture in the northern hemisphere. Losses in New Brunswick, Canada, alone are estimated to be over 70 million Canadian dollars (New Brunswick Department of Agriculture, Fisheries and Aquaculture, pers. comm.). Originally identified in Norway (Thorud & Djupvik 1988), ISAV was identified in Canada in 1997 (Lovely et al. 1999), Scotland in 1998 (Rodger et al. 1998) and Chile in 1999 (Kibenge pers. comm.). It is now well established that nucleotide sequence differences exist between ISAV isolates from Europe and those from New Brunswick, Canada (Krossoy et al. 1999, Lovely et al. 1999). However, it was recently determined that an isolate from Atlantic salmon in Nova Scotia was more similar to European isolates than those of neighboring New Brunswick (Ritchie et al. 2001). In addition, it has been suggested that isolates made from Atlantic salmon in eastern Canada could be distinguished on the basis of a change in protein profile that was further reflective of an ability to grow on either the salmon head kidney (SHK-1) or Chinook salmon embryo (CHSE)-214 cell lines (Kibenge et al. 2000). Given the economic importance of ISAV, it is becoming increasingly important to identify ways of purifying key antigens from various isolates and for characterising the influence of these proteins on vaccine efficacy. In the current communication, continuous elution gel electrophoresis is used to fractionate proteins produced by ISAV cultured in the cell line CHSE-214. The series of fractions suggest autolytic behavior originating from a dominant 72 kDa antigen. In addition, a 38 kDa antigen is determined to be a glycosylated product of ISAV segment 6 (GenBank accession no. AF220607). Moreover, sequencing analysis of this gene revealed a direct relationship between a C-terminal sequence insertion and protein size, and a possible explanation for host cell compatibility.

## MATERIALS AND METHODS

**Cell culture.** ISAV strain AHL 1 was cultured in cell line CHSE-214 by the method of Jones et al. (1999). A residual pellet of 1 ml wet volume of ISAV-infected CHSE-214 cells was washed twice in tissue culture grade water (Gibco BRL, Gaithersburg, MD) at 8000 × *g*

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for 5 min to remove excess serum albumin from the cell pellet. The pellet was resuspended in 2 ml of Laemmli buffer (Laemmli 1970) and heated for 3 min at 94°C. Cell pellet residue was removed by centrifugation at 8000 × *g* for 10 min. Fractionation of antigens was achieved by denaturing gel electrophoresis using a Model 491 Prep Cell (BioRad Laboratories, Hercules, CA) with a 40 ml volume of 8% acrylamide separating gel and 2 ml volume of 4% separating gel. One ml of soluble cell culture material at a constant current of 40 mA was applied. Collection of samples began at 700 volt hours (VH) at a rate of 0.6 ml $min^{-1}$. One fraction was collected every 6 min. A total of 1200 µl from every third fraction was precipitated with 1/10 volume 100% trichloroacetic acid (TCA) and prepared for protein analysis as previously described (Wood et al. 1986). 877 is an ISAV isolate which can only grow on SHK-1 cells whereas isolate 049 can grow on CHSE and SHK-1 cells (Kibenge et al. 2000). Both isolates were prepared from ISAV-infected fish tissue obtained through routine surveillance in New Brunswick Department of Aquaculture, Fisheries and Agriculture.

**Electrophoresis and Western blotting.** TCA precipitated pellets were dissolved in 20 µl of Laemmli buffer and 10 µl analysed on 8% Mini Protean II gels (BioRad) and Western blotted to polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) with a semi-dry transfer unit (BioRad) for 30 min at 20 V. Immediately after blotting, membranes were washed in 0.1% Ponceau S (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 min and destained with several washes of distilled water until blotted proteins were visible. Molecular weight standards and certain proteins were marked with pencil for reference points following immunostaining before removal of pink coloration by brief incubation (<30 s) in 0.1 N NaOH and a final wash in distilled water before air drying the membrane. Development of immunoreactive antigens was achieved by incubation of the dried blot with a 1 in 20 dilution of antibody 1 (polyclonal rabbit anti-ISAV: see below) in 1% casein tris-buffered saline pH 7.4 (cTBS) (BioRad) for 60 min, 2 washes of 10 s with TBS followed by incubation with a 1:2000 dilution of goat anti-rabbit immunoglobulin alkaline phosphatase (Pierce, Rockford, IL). Color development was achieved with 1-Step NBT/BCIP (Pierce) for approximately 3 min and terminated by washing in distilled water. All incubations were carried out in a final volume of 10 ml on a red rocker platform (Hoefer, San Francisco, CA). Documentation was achieved with an Imagemaster and associated software (Amersham Pharmacia Biotech). Estimation of molecular weights was achieved through comparison of migration of antigens with LMW protein standards (Amersham Pharmacia Biotech) or with Kaleidoscope or Precision broad range prestained standards (both BioRad). Estimation of molecular weight was achieved by calculation of relative mobility using first order language.

**Antiserum production.** Polyclonal antiserum to ISAV antigens was produced by intramuscular (i.m.) injection of a New Zealand rabbit with 0.5 ml of a mixture containing the pellet from 1.5 ml well of ISAV infected SHK-1 cells resuspended in 0.5 ml of phosphate-buffered saline (PBS) emulsified in 1.5 ml of Freund's incomplete adjuvant (FIA). After 4 and 8 wk the same volume of material was injected into the rabbit but consisting of 120 and 30 µg of viral particles purified by the method of Jones et al. (1999), resuspended in 0.5 ml PBS and emulsified with 1.5 ml of FIA. Five ml of blood were harvested from the peripheral ear vein at 10 wk and serum collected by allowing blood to clot followed by centrifugation. Antiserum was diluted 1:20 in cTBS and incubated for 2 h at room temperature with a drained monolayer of uninfected SHK-1 cells. The antibody solution was drained and similarly incubated with a monolayer of uninfected CHSE-214 cells. The adsorbed antiserum was clarified by centrifugation at 8000 × *g* for 10 min.

**Glycosylation.** Identification of glycosylated proteins was achieved using an enzymatic deglycosylation kit, incorporating PNGase F, O-glycosidase DS and NANase II, according to the instructions of the manufacturer (BioRad). Material for glycosylation consisted of washed pellets from 1.5 ml wells of SHK-1 cultures infected with either isolate 877 or 049, representing strains incapable or capable of growth on CHSE-214 cells respectively (Kibenge et al. 2000). For the purposes of analysis both strains were grown on the SHK-1 cell line as previously reported (Lovely et al. 1999). Washed cell pellets were lysed with 100 µl of lysis buffer (8 M urea, 4% w/v CHAPS, 40 mM Tris, pH 9.0) for 30 min at room temperature and centrifuged at 8000 × *g* for 10 min. Three parts of clarified lysate were mixed with 1 part 4 × Laemmli buffer and denatured by heating at 94°C for 3 min. A total of 5 µl was applied to 9% total acrylamide gels, Western blotted and developed as described above. Fractions containing the 38 kDa protein were concentrated from a volume of 2 ml to approximately 400 µl by spin filtration in an ultra-free MC (Microcentrifuge) tube with 10 kDa cut-off (Millipore, Bedford, MA) for 90 min at 2500 × *g* prior to inclusion in the deglycosylation assay. Control samples were prepared by subjecting identical samples to the same handling and buffer conditions with the exception of including the 3 deglycosylating enzymes.

**Amino acid sequencing.** N-terminal amino acid sequencing of bands excised from Coomassie Blue R-250 stained PVDF membranes (Millipore PSQ) was conducted by David McCourt, MidWest Analytical, St Louis, MO (www.mastl.com).

**RNA isolation.** SHK 1/7 cell cultures of New Brunswick ISAV isolates 877 and 049 were scraped from 24-well tissue culture plates (1 ml) and centrifuged for 30 min at 6500 × *g* at 4°C. All but 250 µl of supernatant was removed and extracted with the pellet for total RNA using TRIzol LS reagent (Gibco BRL) according to the manufacturer. RNA pellets were resuspended in 20 µl of DEPC-treated water.

**RT-PCR and sequencing analysis.** RT-PCR was performed using Ready-To-Go RT-PCR beads and protocol (Amersham Pharmacia Biotech). Briefly, RNA (4 µl) from isolates 877 and 049 was reverse transcribed using random hexamer primers (2.5 µg) in a total volume of 40 µl. The reverse transcription reaction was incubated at 42°C for 30 min, 94°C for 5 min, and then cooled to 4°C. The cDNA was then amplified using primers F1 (5′ ATGGCACGATTCATAATTTTATTCC 3′) and R2 (5′ CATGCTTTCCAACCTGCTAG 3′), which were designed from the published gene sequence (accession no. AF220607) for a Norwegian ISAV isolate with high homology to the first 24 amino acids of the 38 kDa protein studied in this paper. Final concentrations for these primers and $MgCl_2$ in the PCR reaction were 0.4 µM and 1.5 mM respectively. The cDNA was subjected to a 95°C 5 min incubation followed by 35 cycles of 94°C 1 min, 60°C 1 min, 72°C 2 min, and a final elongation incubation of 72°C 10 min before being cooled to 4°C. Reactions were performed in a Gene Amp 9600 thermocycler (PE Applied Biosystems). RT-PCR products were analysed on 1.2% low melting point agarose gels containing ethidium bromide (0.5 µg $ml^{-1}$) and purified by splicing out bands and subjecting them to the QIAquick gel extraction kit (Qiagen, Mississauga, ON). The bands were sequenced using 3.2 pmol of primers F1, R2, and R3 (5′ CATCAGAAGGATAAACACCC 3′), which was also designed from the published Norwegian sequence, and 4 µl of Big Dye Termnators (PE Applied Biosystems) mixed 1:1 with halfBD (BioCan Scientific, Mississauga). Sequencing reactions were electrophoresed on an ABI310 Genetic Analyser (PE Applied Biosytems), and sequences were analysed using the Sequencher program (Genecodes, Ann Arbor, MI). With the sequencing information obtained for the New Brunswick ISAV isolates more primers were designed for further sequencing and 3′ RACE. These primers included: F4 (5′ GATGATTGGTAAACTTGGCAGA 3′) and R4 (5′ CCATTCCCGTTCTTGGCTT 3′).

**3′ RACE.** Reverse transcription for 3′ RACE was performed in a volume of 20 µl using 1.9 µM of the oligo dT primer (5′ GGCCACGCGTCGACTAC$(T)_{17}$ 3′), 1 mM each dNTP, 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiotreitol, 200 U of Maloney murine leukemia virus (MMLV) reverse transcriptase, and 4 µl of RNA extract. The mixture was incubated for 60 min at 55°C followed by 10 min at 65°C. One µl of cDNA was then added to a 50 µl PCR reaction mixture containing 0.25 µM of primer AUAP (5′ GGCCACGCGTCGACTACT 3′), 0.25 µM of primer F4, 0.2 mM each dNTP, 2.5 U Taq DNA Polymerase, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl. Cycling conditions, gel analyses, band purification and sequencing conditions were as above. Primers F4 and AUAP were used for sequencing.

**Cloning of the 38/40 kDa antigen.** For ISAV isolates 049 and 877 the upper primer used to amplify the 42 kd gene for cloning was 5′CAGGATCCGTACTATGGCACGATTCATAATTTTATTCC 3′; the lower primers were 5′ TTGGATCCGTCAAGCAACATACAGATTTGCAG 3′ (isolate 049) and 5′ TTGGATCCGTCAAGCAACAGACAGATTTGCAG 3′ (isolate 877). These primers amplified all of the sequenced open reading frames. PCR products were cloned into the Bam HI restriction site of plasmid pUK (AquaHealth Ltd, Charlottetown, PEI, Canada). To verify correct insert sequence and orientation within pUK, clones were sequenced using the above mentioned primers as well as 2 vector primers: A2-CMVF, 5′ TCAACGGGACTTTCCAAAAT 3′ and BGH reverse, 5′ TAGAAGGCACAGTCGAGG 3′.

**Expression of the recombinant 38/40 kDa antigen.** For eukaryotic expression clones were isolated from culture using SNAP midiprep kit (Invitrogen) and transfected into CHSE cells using Superfect reagent (Qiagen). Briefly, 1 d prior to transfection, CHSE cells were seeded into 24 well tissue culture plates at $6 \times 10^4$ cells $well^{-1}$ and incubated at 25°C. Cell growth medium consisted of MEM (Earle's salts) supplemented with 10% FBS, 0.01 M Hepes buffer, and 2 mM glutamine. Either 0.5, 1.0, or 2.0 µg of plasmid was diluted in Opti-MEM Reduced Serum Medium (Gibco) to a total volume of 60 µl. Superfect reagent was added to the mixtures to yield plasmid DNA:Superfect reagent ratios of 1:2. After vortexing, the mixtures were allowed to complex at room temperature for 15 min. Growth medium was then added (350 µl) and after mixing the total volume was transferred to CHSE cells which had been washed with growth media. Cells were incubated with the complexes for 6 h and then removed. Fresh growth media (1 ml) were added to the cells which were incubated at 25°C for 4 d before being harvested for Western blotting. Individual wells of transfected CHSE-214 were washed in PBS, and harvested by scraping in 1 ml PBS and pipetting into 1.5 ml microtubes. The cell suspension was pelleted by microcentrifugation at maximum speed for 3 min and resuspended in 50 l of Laemmli buffer and heated to 95°C for 5 min. 10 l of material were loaded onto 9% SDS-PAGE mini gels, electrophoresed, blotted and developed as described above.

## RESULTS

Fig. 1 illustrates the series of immunoreactive material harvested from CHSE-214 cells infected with ISAV isolate AHL 1 by continuous elution electrophoresis. The dominant 72 kDa antigen recognised by the anti-ISAV serum appears to generate the majority of lower molecular weight peptides (Fig. 1D, fractions 112 to 133), which themselves appear to retain an autolytic capacity (e.g. Fig. 1B, sample 76). In contrast the elution of a 38 kDa band coincides with the appearance of 2 larger molecular products following electrophoresis, which suggests that the lower molecular weight peptide is a subunit of the larger (Fig. 1B, fractions 49 to 55).

A Western blot of CHSE-214 compatible and non-compatible ISAV strains confirmed previous results that some growth compatibility isolates may be distinguished by relative electrophoretic migration of proteins around 40 kDa (Fig. 2, lanes 4 and 6) (Kibenge et al. 2000). AHL1 was comparable to the 049 isolate, consistent with their shared property of being able to grow on CHSE-214. Proteins from both growth compatibility isolates demonstrated a shift in mobility when deglycosylated (Fig. 2, lanes 5 and 7). No shift in mobility was detected for the 72 kDa protein or putative breakdown products.

Material from fraction 45 to 48 (38 to 40 kDa size range) was concentrated to prepare sufficient material for amino acid sequencing (Fig. 2, lane 1; deglycosylated material, lane 2). The first 24 amino acids were determined to be RLCLRNHPDTTWIGDSRSDQSRVN, corresponding to an unknown protein reportedly associated with a segment of a Norwegian isolate of ISAV (GenBank accession no. AF220607) with the exception of a single amino acid substitution at position 7, where histidine is substituted for tyrosine.

Primers were designed from AF220607 in order to compare nucleotide and amino acid sequences of the 049 and 877 strains (Fig. 3). The 2 strains shared approximately 99.5% homology at the nucleotide level across most of the gene. However towards the 3′ end, strain 049 diverged from 877 across a 39 nucleotide

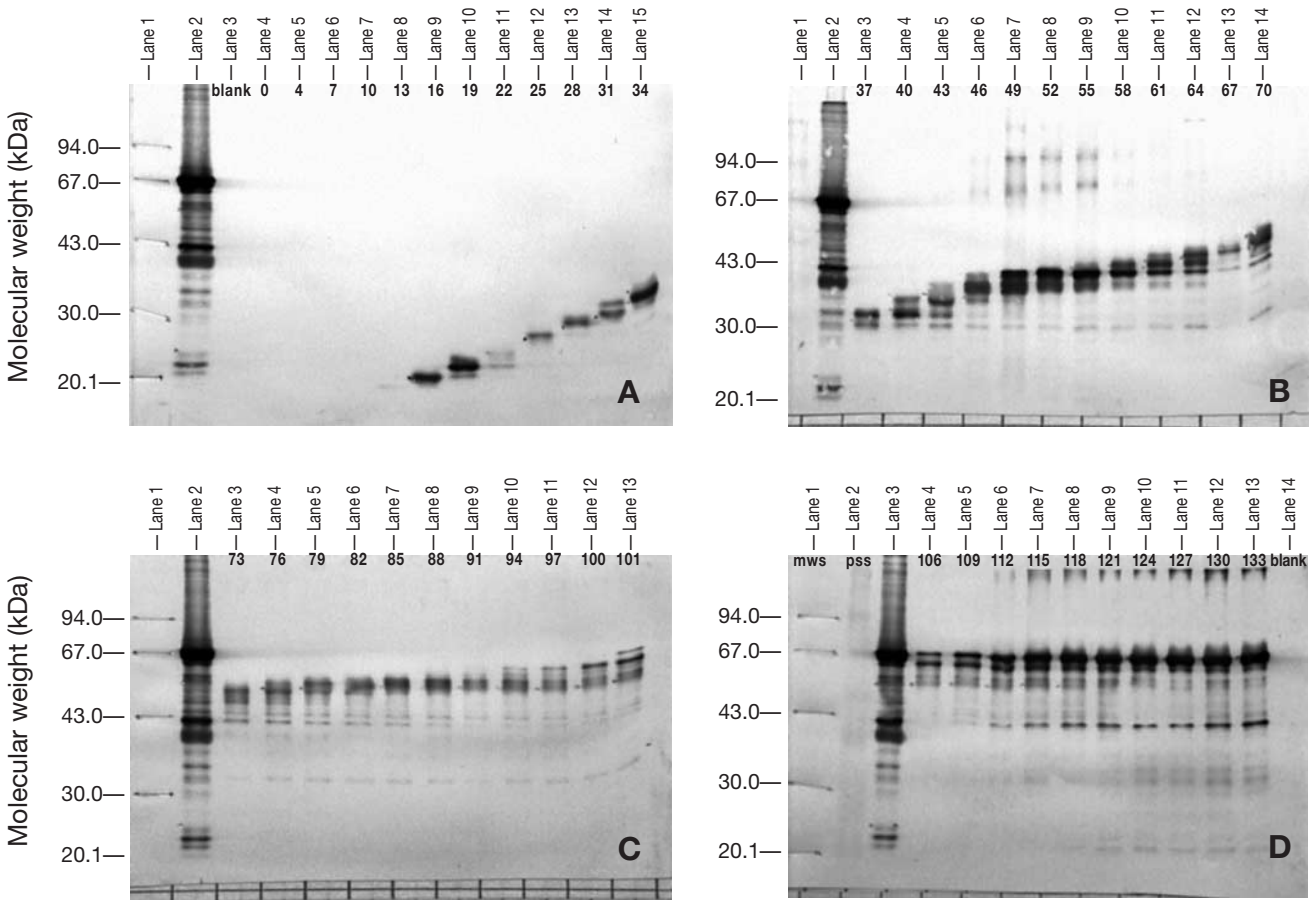


Fig. 1. Western blot of material harvested from continuous elution gel electrophoresis of ISAV-infected CHSE-214. Unfractionated material: lanes 2, 2, 2 and 3 in blots (A), (B), (C), amd (D) respectively. Values below lane notation indicate fraction number harvested after collection was initiated at 700 volt hours (VH). Molecular weight standards (mws): lane 1 in all blots. Prestained standards (pss), lane 2, blot (D)

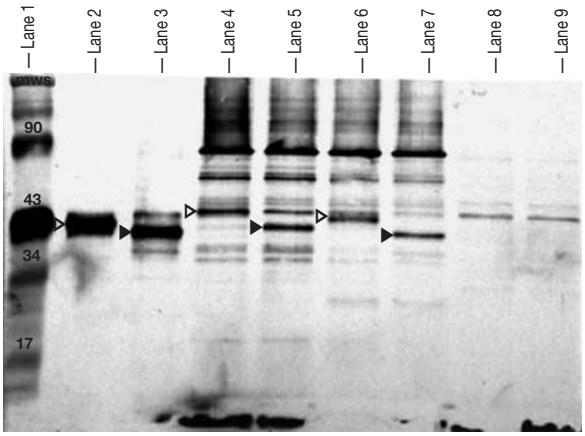


Fig. 2. Western blot of immunoreactive material harvested from; gel elution series fraction 45 (lanes 2 and 3); isolate 877 (lanes 4 and 5); isolate 049 (lanes 6 and 7); uninfected SHK-1 cells (lane 8); equivalent samples incubated with PNGase F, O-glycosidase DS and NANase II (lanes 3, 5, 7 and 9); Kaleidoscope (BioRad) molecular weight standards (mws) (lane 1). Solids arrowheads indicate deglycosylated proteins with altered mobility from original location in gel (open arrowheads)

region, including a 28 nulceotide insertion, and a 2 nucleotide insertion. This translates to a 10 amino acid insertion, and several non-synonymous substitutions, in strain 877 compared to 049. Counting the insertion as 'one change', 877 shared 97.4% homology (375/385) with 049 at the amino acid level. Compared to the European strain (AF220607), however, 877 and 049 shared 84.0% (322/383) and 85.3% (327/383) amino acid identities respectively.

The open reading frames of both variant proteins were cloned into the pUK vector and used to transfect CHSE-214 cells. Both transfections resulted in the expression of proteins in the appropriate molecular weight range as determined by reactivity of Western blots with polyclonal antiserum to ISAV particles (Fig. 4).

## DISCUSSION

One observation arising from the continuous elution series of ISAV tissue culture proteins is that the major 72 kDa antigen may be associated with autolytic behavior. In short, most of the immunoreactive lower molecular weight peptides appear to have been derived from this progenitor with some peptides retaining autolytic activity following elution. However, further studies would need to be conducted to discount the possibility of a comigrating protease. If such autolytic activity can be confirmed, the 72 kDa antigen may be similar to that the 57 kDa major antigen of *Renibacterium salmoninarum*, the aetiologic agent of bacterial kidney disease (Griffiths & Lynch 1991). Further characterisation of the autolytic behavior and substrate

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220607  LLAPVYSRLCLRNYPDTTWIGDSRSDQSRVNPQSLDLVTEFKGVLQAKNGNGLLKQMSGR
049   -------------H-----------------Q-------N---I---------M------
877   -------------H-----------------Q-------N---I---------M------

220607  FPSDWYTPTTKYRILYLGTNDCTDGPTDMIIPTSMTLDNAARELYLGACRGDVRVTPTFV
049   ------Q---------I------E--N-V----------V--D-----------------
877   ------Q----------------E--N-V---------KV--D-----------------

220607  GAAIVGLVGRTDAVTGFSVKVLTFSSPTIVVVGLNGMSGIYKVCIAATSGNVGGVKLING
049   ---EL--I-----L----------NN---------------------S-------N-V--
877     ---EL--I-----L----------NN---------------------S-------N-V--

220607  CGYFNTPLRFDNFQGQIYVSDTFEVRGTKNKCVLLRSSSDTPLCSHIMRNVELDEYVDTP
049   ----SA-------K-------------------------NA---T--K--I---------
877   ----SA-------K-------------------------NA---T--K--I---------

220607  NTGGVYPSDGFDSLHGSASVRTFLTDALTCPDIDWSRIDAASCEYDSCPKMVKDFDQTSL
049   -------------------I-----E-----GV--D--------------L--E----G-
877   -------------------I-----E-----GV--D--------------L--E----G-

220607  GNTDTLIMREVALHKEMISKLQRNITDVKIRVDAI•••••••••PPQLNQT••••MGVAG
049   -----Q----LEAQ----G--G------NN-----•••••••••----•SNIFIS-----
877   -----Q----LEAQ---TG--G------NN-----LGVNQVEQPSTSVPSNIFIS-----

220607  FGIALFLAGWKACIWIAAFMYKSRGRIPPSSLSVA*
049   -------------V------------N--AN-Y--*
877   -------------V----C-------N--AN----*
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Fig. 3. Protein comparison of European (AF220607), 049 and 877 strains. •: sequence deletion; –: conservation of an amino acid; *:end of sequence considered

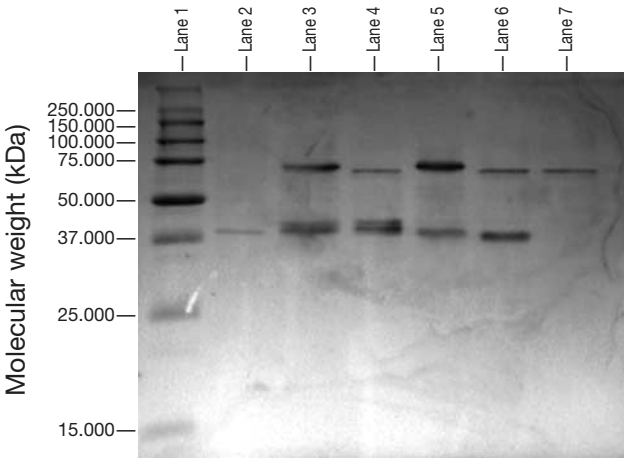


Fig. 4. Western blot of CHSE-214 transfected with pUK877/40 (lane 4), pUK049/38 (lane 6) or unloaded pUK (lane 7); SHK-1 cell cultures infected with isolate 877 (lane 3) or 049 (lane 5); uninfected SHK-1 (lane 2). Precision prestained standards (BioRad) (lane 1)

specificity associated with these major antigens may be of importance for disease studies since both will encounter salmonid host cell determinants that lead to the internalisation of the pathogen.

Of the remaining proteins a 38 kDa protein was determined to be glycosylated as indicated by a shift in mobility following deglycosylation. That a 38 kDa protein in CHSE-214 compatible isolates AHL1 and 049 in addition to a 40 kDa protein associated with the CHSE-214 non-compatible isolate 877 are affected by this behavior suggests that the 2 are variants of the same gene. This protein is likely to be the 43 kDa protein previously described (Falk et al. 1997), and the target of a widely used monoclonal antibody (Falk et al. 1998, Falk pers. comm.). However, it was not possible to determine immunoreactivity of this protein to the monoclonal antibody by Western blot, probably due to irreversible denaturation of the target epitope following SDS-PAGE. Nevertheless, glycosylation of the 38 kDa protein and its detection with antiserum to whole viral particles suggests that the protein is associated with the viral envelope.

Sequence analysis of the p38 protein and associated gene revealed that, like other ISAV sequences, the Norwegian sequence (AF220607) was significantly different from the North American strains (877/049), showing 86% similarity at the protein level to the most closely related North American strain (049). However, the protein homology is lower than the 99% similarity between ISAV proteins expressed from segments 2 and 8 from different geographic regions (Ritchie et al. 2001). This alone may be indicative of a cell surface protein. However, a more surprising finding was a 10 or 13 amino acid insertion in strain 877 (compared to 049 or AF220607 respectively) towards the C-terminal of the protein. The insertion in 877 explains the increase in size of the expressed protein (40 kDa compared to 38 kDa). In addition, the absence of 10 amino acids from CHSE-214 compatible strain 049 may have resulted in a change in receptor avidity that enables infection of the atypical host cell line. Using the results presented here, further investigation into host-virus receptor interaction is now possible which will ultimately provide a better understanding of ISAV infection.

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