

# Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene

Frederick S. B. Kibenge,<sup>1</sup> Molly J. T. Kibenge,<sup>2</sup> Patricia K. McKenna,<sup>1</sup> Paul Stothard,<sup>3</sup> Rebecca Marshall,<sup>1</sup> R. Roland Cusack<sup>4</sup> and Sandi McGeachy<sup>5</sup>

<sup>1,2</sup>Department of Pathology and Microbiology<sup>1</sup> and AVC Inc.<sup>2</sup>, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PEI, Canada C1A 4P3

<sup>3</sup>Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

<sup>4</sup>Veterinary Pathology, Nova Scotia Department of Fisheries and Aquaculture, Truro, Nova Scotia, Canada

<sup>5</sup>New Brunswick Department of Fisheries and Aquaculture, Fredericton, New Brunswick, Canada

Infectious salmon anaemia virus (ISAV), an orthomyxovirus-like virus, is an important fish pathogen in marine aquaculture. Virus neutralization of 24 ISAV isolates in the TO cell line using rabbit antisera to the whole virus and comparative sequence analysis of their haemagglutinin (HA) genes have allowed elaboration on the variation of ISAV isolates. The 24 viruses were neutralized to varying degrees, revealing two major antigenic groups, one American and one European. Sequence analysis of the HA gene also revealed two groups of viruses (genotypes) that correlated with the antigenic groupings. The two HA subtypes had nucleotide sequence identity of only  $\leq 79.4\%$  and amino acid sequence identity of  $\leq 84.5\%$  whereas, within each subtype, the sequence identities were 90.7% or higher. This grouping was also evident upon phylogenetic analysis, which revealed two distinct phylogenetic families. Between the two groups, the amino acid sequence was most variable in the C-terminal region and included deletions of 4–16 amino acids in all isolates relative to ISAV isolate RPC/NB-980 280-2. In order to view the relationships among these sequences and the HA sequences of the established orthomyxoviruses, a second phylogenetic tree was constructed which showed the ISAV sequences to be more closely related to sequences from *Influenzavirus A* and *Influenzavirus B* than to sequences from *Influenzavirus C* and *Thogotovirus*. The extensive deletions in the gene of European ISAV isolates lead us to speculate that the archetypal ISAV was probably of Canadian origin.

## Introduction

Infectious salmon anaemia (ISA) virus (ISAV) is a new member of the family *Orthomyxoviridae*. It causes ISA in marine-farmed Atlantic salmon (*Salmo salar*), which is probably the most important virus disease of salmonids in the Northern hemisphere. The taxonomic position of ISAV at the genus

level within the family *Orthomyxoviridae* has not yet been resolved. Comparison of the ISAV putative polymerase protein (PB1) and nucleoprotein (NP) with those of other orthomyxoviruses revealed amino acid identity values of  $< 25\%$  (Krossøy *et al.*, 1999) and  $< 13\%$  (Snow & Cunningham, 2001), respectively, supporting the suggestion that ISAV be assigned to a new, fifth genus, *Aquaorthomyxovirus*, within the *Orthomyxoviridae* (Krossøy *et al.*, 1999).

The structural viral protein profile of ISAV has not been determined conclusively, as the gene coding assignment of the ISAV genome is not clearly known. Metabolic radiolabelling of synthesized proteins coupled with immunoprecipitation, which allows detection of both structural and non-structural viral proteins, revealed up to 12 viral proteins (Kibenge *et al.*,

**Author for correspondence:** Frederick Kibenge.

Fax +1 902 566 0851. e-mail kibenge@upei.ca

The GenBank accession numbers of the ISAV HA sequences reported in this paper are AF283995–AF283998, AF294870–AF294882, AF378179–AF378181, AF388581, AF388582, AF391126 and AF395337.

2001), indicating that ISAV has a similar protein profile to that of influenza viruses (Cox *et al.*, 2000). The ssRNA genome of ISAV consists of eight segments of negative polarity, ranging in size from 1.0 to 2.3 kb, with a total molecular size of approximately 14.5 kb (Mjaaland *et al.*, 1997). Rimstad *et al.* (2001) described a genomic segment encoding the putative haemagglutinin (HA) of ISAV. The protein was shown to be an HA by demonstrating its haemadsorptive properties for salmon erythrocytes when expressed in a salmon cell line. Krossøy *et al.* (2001a) identified the ISAV HA gene as RNA segment 6 and showed it to encode a 42.4 kDa protein that corresponds to the 43–46 kDa protein detected previously in purified ISAV preparations (Falk *et al.*, 1997; Kibenge *et al.*, 2000). Peptide analysis of the ISAV 43 kDa protein also identified this protein as being the same as that encoded by RNA segment 6 (Griffiths *et al.*, 2001). Sequence comparisons among different ISAV isolates have indicated the occurrence of a highly polymorphic region in this gene (Rimstad *et al.*, 2001; Krossøy *et al.*, 2001a). Among influenza viruses, the HA gene is reported to be the most variable (Webster *et al.*, 1992). However, the ISAV HA gene was shown to differ from that of influenza viruses A and B in that it is not cleaved post-translationally, and the fusion activity of ISAV may be associated with a protein encoded by a separate gene (Krossøy *et al.*, 2001a).

Orthomyxoviruses have the unique capacity to undergo a high degree of antigenic variation within a short period of time. Considerable variation occurs among the HA and neuraminidase (NA) antigens of influenza A viruses, whereas those of influenza B viruses exhibit less antigenic variation and antigenic variation is rarely observed among influenza C viruses (Matsuzaki *et al.*, 2000). In the case of ISAV isolates, there is no information about antigenic variation.

ISAV isolates have been shown previously to fall into two phenotypic groups based on their ability to replicate in the CHSE-214 cell line (Kibenge *et al.*, 2000, 2001), i.e. two CHSE phenotypes. Previous attempts to explain the molecular basis for this variation have focused on the ISAV 43 kDa protein (Griffiths *et al.*, 2001), the putative HA protein. Indeed, it was reported that the molecular mass of this protein was 38 kDa in CHSE-214-compatible isolates (CHSE-positive phenotype) and 40 kDa in a non-compatible isolate (CHSE-negative phenotype) and that this difference was explained by a 10 amino acid insertion in the CHSE-214-non-compatible isolate (Griffiths *et al.*, 2001). In the present study, it was hypothesized that differences in the ability of different isolates of ISAV to replicate in CHSE-214 cells may be related to differences in the viral receptor-binding protein HA and, since HA is the major antigen responsible for serotype differences in influenza viruses, we wished to determine whether antigenic differences between ISAV isolates were correlated with the CHSE phenotypes. We also wished to add the HA sequences of many ISAV isolates from different geographical regions to the database of available HA sequences. The putative HA sequence

was therefore examined to determine the level of sequence variation among different ISAV isolates. For these purposes, we compared 10 ISAV isolates that produce cytopathic effects (CPE) in CHSE-214 cells (CHSE-positive phenotype) with 14 that do not (CHSE-negative phenotype), for a total of 24 ISAV isolates, and analysed an additional eight previous HA entries in GenBank.

## Methods

■ **Cells and viruses.** Twenty-four ISAV isolates from different geographical regions and from both CHSE phenotypes (Kibenge *et al.*, 2000) were chosen for the study. ISAV isolates were propagated and titrated in CHSE-214, SHK-1 and TO cell lines as described previously (Kibenge *et al.*, 2000, 2001). The ISAV isolates that were used in this study and their characteristics are listed in Table 1.

■ **Anti-ISAV rabbit antibodies.** Rabbit polyclonal antisera to purified ISAV were prepared as described previously (Kibenge *et al.*, 2000) using ISAV isolates 'Back Bay 98', RPC/NB-980049, 7833-1 and RPC/NB-980 280-10. Briefly, for each virus isolate, one 250 g rabbit (Charles River Canada) was inoculated subcutaneously three times at 3-week intervals with 100 µg purified virus, once in Freund's complete adjuvant and twice in Freund's incomplete adjuvant. Pre-inoculation serum and sera collected every 3 weeks until after the last injection were tested for the presence of neutralizing antibodies in the virus-neutralization test.

■ **Virus-neutralization (VN) test.** VN tests were carried out on TO cell monolayers in 48-well culture plates. Cell monolayers were grown at room temperature (22 °C) in HMEM (Eagle's minimum essential medium containing Hanks' salts; BioWhittaker) supplemented with 292 µg/ml L-glutamine (Sigma), 1% non-essential amino acids (NEAA; Sigma), 100 µg/ml gentamicin (Sigma) and 10% foetal bovine serum (FBS) (Wergeland & Jakobsen, 2001). For maintenance medium, FBS was reduced to 5%. To set up a VN test, serial 2-fold dilutions of ISAV antiserum and an equal volume of virus suspension containing 100 TCID<sub>50</sub> were added to TO cell monolayers drained of medium and incubated at room temperature for 1 h before addition of 500 µl fresh maintenance medium to each well. After incubation for a further 10 days at 16 °C, cultures were examined microscopically for CPE to determine the VN test results.

■ **RT-PCR for ISAV.** Viral RNA was extracted from 250 µl cell culture lysate by using TRIZOL LS reagent (Canadian Life Technologies) following the manufacturer's protocol. The PCR primer pair ISAV HA1F/ISAV HA1R was designed originally from the nucleotide sequence of ISAV isolate Glesvaer/2/90 (Rimstad *et al.*, 2001) by using Primer Detective version 1.01 (Clontech). This sequence was subsequently shown to belong to the ISAV HA gene, RNA segment 6. The primer pairs used in this study consisted of either primers ISAV HA1F (nucleotides 70–91; sense, 5' AAATAACCCTGACACCACCTGG 3') and ISAV HA1R (nucleotides 1061–1082; antisense, 5' ACAGAGC-AATCCCAAAACCTGC 3') or primers ISAV SEG6FP (nucleotides 2–19; sense, 5' GCAAAGATGGCAGGATTC 3') and ISAV SEG6RP (nucleotides 1173–1192; antisense, 5' CGTTGTCTTCTTTCATAATC 3'). 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

**Table 1.** VN titres of four rabbit anti-ISAV antisera against different ISAV isolates

Virus titres in CHSE-214 cells were measured by end-point CPE and are expressed as  $\log_{10}$  TCID<sub>50</sub>/ml. –, Not titrated because virus is non-cytopathic in CHSE-214 cells. VN titres are expressed as the reciprocal of the highest dilution of antiserum that neutralized 100 TCID<sub>50</sub> of virus completely. Homologous VN titres are printed in bold. ND, Not done.

ISAV isolate designation and origin*	Titre in CHSE-214 cells	VN titre with rabbit polyclonal antiserum against:			
		'Back Bay 98'	RPC/NB-980 049-1	7833-1	RPC/NB-980 028-10
(9) Back Bay 98 (Canada)	5·80	<b>640</b>	3840	480	80
(7) RPC/NB-980 049-1 (Canada)	4·50	160	<b>2560</b>	120	960
(11) 7833-1 (Chile)	5·25	30	1280	<b>640</b>	120
(8) RPC/NB-970 877-2 (Canada)	–	480	3840	160	240
(10) HKS-36 (Canada)	–	240	1920	640	320
(6) RPC/NB-990 002-1 (Canada)	4·50	160	1280	120	640
(5) RPC/NB-990 508-3 (Canada)	3·16	60	960	960	> 1280
(4) RPC/NB-980 458-1 (Canada)	6·50	160	320	160	ND
(13) RPC/NB-990 681-3 (Canada)	3·83	160	120	480	40
(12) NBISA01 (Canada)	5·77	60	320	640	160
(3) DFO-1 (Canada)	–	4·50	10	480	160
(1) RPC/NB-980 280-2 (Canada)	–	30	320	80	960
(2) RPC/NB-980 028-10 (Canada)	–	< 10	40	< 10	> <b>1280</b>
(21) HI/92 (Norway)	–	–	20	30	80
(20) Glesvear/2/90 (Norway)	–	< 10	< 10	80	< 10
(14) 390/98 (Scotland)	–	< 10	< 10	40	15
(15) 832/98 (Scotland)	–	30	ND	ND	60
(16) 912/99 (Scotland)	–	20	20	< 10	20
(17) 301/98 (Scotland)	–	10	20	< 10	20
(24) U5575-1 (Canada)	–	< 10	20	< 10	< 10
(18) 485/9/97 (Norway)	1·83	< 10	< 10	15	15
(22) 1490/98 (Scotland)	–	< 10	< 10	< 10	< 10
(23) 835/9/98 (Norway)	–	ND	ND	ND	< 10
(19) 810/9/99 (Norway)	–	ND	ND	ND	15

\* Origin refers to geographical origin only. Numbers in parentheses are used in Table 3 to refer to these sequences.

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 a 1% 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 TA cloning kit (Invitrogen Life Technologies) in preparation for sequencing.

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

Reference sequences were obtained from GenBank for representatives of the genera *Influenzavirus A* [A/parakeet/Narita/92A/98 (H9N2), accession no. AB049160], *Influenzavirus B* (B/Lee/40, accession no. K00423), *Influenzavirus C* (C/California/78, accession no. K01689) and *Thogotovirus* (Dhori/Indian/1313/61, accession no. M34002).

■ **Phylogenetic analysis of ISAV.** Sequences were aligned by using CLUSTAL X with the default settings (Thompson *et al.*, 1997). Phylogenetic trees were generated from the aligned sequences by using CLUSTAL X and the neighbour-joining method (Saitou & Nei, 1987). Alignment regions containing gaps were excluded from the analysis. The results were analysed by using the bootstrap method (1000 replicates) to provide confidence levels for the tree topology.

## Results

### Two major antigenic groups of ISAV identified among various isolates from different geographic regions by virus neutralization

All viruses replicated to high titre and produced complete CPE in the TO cell line. The CPE, which consisted of cell rounding and lysis, appeared at 4 days p.i. and was complete

**Table 2.** GenBank accession numbers and the corresponding designations used in Figs 1–4

ISAV isolate designation and origin*	GenBank accession no.	Designation
1. RPC/NB-980 280-2 (Canada)	AF294870	Can-1
2. RPC/NB-980 028-10 (Canada)	AF294871	Can-2
3. DFO-1 (Canada)	AF294872	Can-3
4. RPC/NB-980 458-1 (Canada)	AF294873	Can-4
5. RPC/NB-990 508-3 (Canada)	AF294874	Can-5
6. RPC/NB-990 002-1 (Canada)	AF294875	Can-6
7. RPC/NB-980 049-1 (Canada)	AF294876	Can-7
8. RPC/NB-970 877-2 (Canada)	AF294877	Can-8
9. Back Bay 98 (Canada)	AF283995	Can-9
10. HKS-36 (Canada)	AF294878	Can-10
11. 7833-1 (Chile)	AF294879	Chil-1
12. NBISA01 (Canada)	AF283996	Can-11
13. RPC/NB-990 681-3 (Canada)	AF294880	Can-12
14. 390/98 (Scotland)	AF283997	Scot-1
15. 832/98 (Scotland)	AF388582	Scot-3
16. 912/99 (Scotland)	AF395337	Scot-6
17. 301/98 (Scotland)	AF388581	Scot-4
18. 485/9/97 (Norway)	AF378181	Nor-7
19. 810/9/99 (Norway)	AF378180	Nor-8
20. Glesvear/2/90 (Norway)	AF283998	Nor-1
21. HI/92 (Norway)	AF294882	Nor-2
22. 1490/98 (Scotland)	AF391126	Scot-5
23. 835/9/98 (Norway)	AF378179	Nor-9
24. U5575-1 (Canada)	AF294881	Can-13
25. Bay of Fundy 97 (Canada) <sup>a</sup>	AF302800	Can-14
26. Not specified (Canada) <sup>b</sup>	AX083268	Can-15
27. GA/TO Fish03 (Canada) <sup>c</sup>	AF297551	Can-16
28. Loch Nevis 98 (Scotland) <sup>a</sup>	AF302802	Scot-2
29. Bremnes 98 (Norway) <sup>a</sup>	AF302799	Nor-3
30. Sotra 93 (Norway) <sup>a</sup>	AF309075	Nor-4
31. Gullesfjord 93 (Norway) <sup>a</sup>	AF302801	Nor-5
32. Hitra 99 (Norway) <sup>a</sup>	AF302803	Nor-6

\* Origin refers to geographical origin only. *a*, Isolates reported by Krossøy *et al.* (2001*a*); *b*, sequence deposited by S. Griffiths and R. J. Ritchie; *c*, sequence amplified directly by RT-PCR from an apparently normal fish, deposited by F. Kibenge and M. Kibenge; these sequences were used in the phylogenetic tree in Fig. 3.

by 10 days p.i. Table 1 summarizes the characteristics of the ISAV isolates used and the VN titres obtained against the four different rabbit antisera. Ten ISAV isolates produced CPE in CHSE-214 cells (CHSE-positive phenotype) and 14 were non-cytopathic in CHSE-214 cells (CHSE-negative phenotype). The viruses were neutralized to varying degrees, revealing two major antigenic groups, one American and one European (with the exception of isolate U5575-1 from Canada). The American group, which was generally neutralized well by the four antisera, particularly by rabbit antiserum to ISAV isolate RPC/NB-980 049-1, consisted of 12 isolates from Canada and one isolate from Chile. The European group, which was neutralized poorly by all four rabbit antisera, consisted of five isolates from Norway, five from Scotland and one from Canada. It is considered that the two groups delineated by the

four rabbit antisera are sufficiently distinct to be recognized as separate serotypes (Table 1). However, VN results with specific antisera to viruses in the second group as well as the use of monospecific antisera will be necessary in order to demonstrate that there is no cross-neutralization between the two groups or even the existence of a third antigenic group.

#### Sequence variation of the ISAV HA gene among different ISAV isolates reveals two HA genotypes

RT-PCR amplification with primer pairs ISAV HA1F/ISAV HA1R and ISAV SEG6FP/ISAV SEG6RP yielding PCR products of approximately 1–1.2 kbp (predicted sizes 1031 bp and 1204 bp, respectively). The sequences determined from the PCR products obtained with primer pair ISAV HA1F/ISAV HA1R ranged from 1013 bp in Scottish isolate 390/98 to



Fig. 1. Sequence identity between HA from ISAV and members of *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C* and *Thogotovirus* (see Methods for details of reference sequences). (a) ISAV isolate U5575-1 (Can-13) and *Influenzavirus A* (Inf.A). (b) ISAV isolate RPC/NB 980-280-2 (Can-1) and *Influenzavirus C* (Inf.C). (c) Can-13 and *Thogotovirus* (Dhori). (d) Can-13 and *Influenzavirus B* (Inf.B). Gaps introduced to optimize the alignment are indicated by dashes. Residues between viruses with strong and weak similarity predicted by FASTA program are indicated by colons (:) and full stops (.), respectively.

1061 bp in Canadian isolate RPC/NB-980 280-2. All sequences were examined for authenticity by looking for open reading frames (ORFs) at least 30 residues long, starting with any amino acid codon, using the ORF Finder in the Sequence Manipulation suite (Stothard, 2000). All isolates contained a large ORF in reading frame 2 (ORF2) that spanned the whole sequence. In the Norwegian isolate Bremnes 98 (Nor-3), the HA ORF of which has been sequenced completely, this ORF (ORF2) spans nucleotide positions 8–1174 (Krossøy *et al.*, 2001a). ISAV isolates from Norway and Scotland also had a small ORF, 82 amino acids long, starting with a methionine codon in reading frame 1 (ORF1), which, in the Bremnes 98 isolate, spans nucleotide positions 358–606. This ORF1 was not found in any of the Canadian isolates or in the Chilean isolate. Reading frame 3 had no viable ORF in any of the ISAV isolates studied. The 24 sequences have been deposited in GenBank under the accession numbers listed in Table 2 (isolates 1–24).

The ISAV HA sequence was analysed for similarity to published HA sequences of selected viruses from *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C* and *Thogotovirus*, the four established genera in the virus family *Orthomyxoviridae* (see Methods for details of reference sequences). There was no nucleotide sequence identity between the HA sequences from ISAV and these orthomyxoviruses. However, there was some identity at the amino acid level that ranged from 23.0% in a 61 amino acid overlap with the sequence from *Thogotovirus* to 22.0% in a 91 amino acid overlap with the sequence from *Influenzavirus A*. This sequence identity was in the N-terminal region, spanning amino acid residues 24–140 of the ISAV HA for identities with the HA sequences from *Influenzavirus A*, *Influenzavirus C* and *Thogotovirus*, and amino acid residues 203–264 of the ISAV HA for identity with the sequence from *Influenzavirus B* (Fig. 1).

The FASTA program was used to perform pairwise comparisons between the nucleotide and deduced amino acid sequences from the 24 ISAV isolates sequenced in this study

and the eight previous GenBank entries of ISAV putative HA nucleotide and deduced amino acid sequences (Krossøy *et al.*, 2001a; Rimstad *et al.*, 2001). Pairwise nucleotide sequence identities among the 24 ISAV isolates sequenced in this study are listed above the diagonal in Table 3 in decreasing order in comparison to ISAV isolate RPC/NB-980 280-2. The corresponding pairwise deduced amino acid sequence identities of ORF2 are listed below the diagonal in Table 3. The sequence identity data revealed two groups of viruses (genotypes), an American genotype of 13 viruses (12 from Canada and one from Chile), and a European genotype of 11 viruses (five from Norway, five from Scotland and one from Canada). To correspond to nomenclature established for influenza virus NA and HA genes, the term subtype will be used to distinguish the two HA genotypes. The two HA subtypes had a nucleotide sequence identity of only  $\leq 79.4\%$  and amino acid sequence identity of  $\leq 84.5\%$  (Table 3) whereas, within each subtype, the sequence identities were 90.7% or higher (Table 3). Within the European subtype, ISAV isolates 390/98, 832/98 and 912/99 from Scotland were 100% identical (Table 3).

All the available amino acid sequences of ORF2 were aligned using CLUSTAL W (Thompson *et al.*, 1994). The full alignment is available as supplementary material at JGV Online (<http://vir.sgmjournals.org/>) and selected regions of the alignment are shown in Fig. 2. Between the American and European HA subtypes, there were twice as many amino acid changes in the second half of the protein as were found in the first half and most involved one or two residues distributed randomly. The change of N to S at position 155 resulted in loss of a potential glycosylation site,  $^{155}\text{NPT}^{157}$ , in the European HA subtype (Fig. 2a); the American HA subtype therefore has three potential *N*-glycosylation sites compared with two in the European HA subtype. However,  $^{155}\text{NPT}^{157}$  is probably not used (Krossøy *et al.*, 2001a; Feldmann *et al.*, 1988) and, since one of the *N*-glycosylation sites is located at the cytoplasmic side of the predicted transmembrane region, that leaves only the  $^{333}\text{NIT}^{335}$  potential *N*-glycosylation sites that is shared by

**Table 3.** Percentage nucleotide and deduced amino acid sequence identity of the putative viral HA gene of ISAV

Values above the diagonal are nucleotide sequence identities; values below the diagonal are deduced amino acid sequence identities. ISAV strains are numbered as listed in Tables 1 and 2. Only sequence identities among isolates sequenced in the present study are shown.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	—	99·6	99·4	99·4	99·3	99·3	99·3	99·2	99·1	99·0	99·0	98·7	98·6	79·2	79·2	79·2	79·2	78·9	78·8	78·8	78·7	78·5	78·2	78·1
2	95·5	—	99·7	99·7	99·6	99·6	99·4	99·5	99·4	99·2	99·3	99·1	98·9	79·4	79·4	79·4	79·4	78·6	79·0	78·5	78·5	78·3	78·4	78·4
3	95·2	99·7	—	99·7	99·7	99·6	99·6	99·4	99·5	99·4	99·6	99·2	99·2	79·4	79·4	79·4	79·4	78·6	79·0	78·5	78·5	78·3	78·4	78·5
4	95·2	99·7	100	—	99·9	99·9	99·7	99·8	99·8	99·7	99·6	99·2	99·2	79·4	79·4	79·4	79·4	78·6	79·0	78·5	78·5	78·3	78·4	78·5
5	94·9	99·4	99·7	99·7	—	99·8	99·6	99·7	99·6	99·4	99·5	99·1	99·3	79·3	79·3	79·3	79·3	78·5	78·9	78·4	78·4	78·2	78·3	78·4
6	94·9	99·4	99·7	99·7	99·4	—	99·6	99·7	99·6	99·4	99·5	99·1	99·1	79·3	79·3	79·3	79·3	78·5	78·9	78·4	78·4	78·2	78·3	78·4
7	94·3	98·8	99·1	99·1	98·8	98·8	—	99·5	99·4	99·2	99·3	98·9	98·9	79·3	79·3	79·3	79·3	78·8	78·9	78·2	78·2	78·1	78·3	78·2
8	94·9	99·4	99·7	99·7	99·4	99·4	98·8	—	99·7	99·5	99·6	99·0	99·0	79·4	79·4	79·4	79·4	78·6	79·0	78·5	78·5	78·3	78·4	78·5
9	94·3	98·8	99·1	99·1	98·8	98·8	98·3	99·4	—	99·4	99·5	99·3	98·9	79·3	79·3	79·3	79·3	78·5	78·9	78·4	78·4	78·2	78·3	78·4
10	94·6	99·1	99·4	99·4	99·1	99·1	98·5	99·7	99·1	—	99·3	98·7	98·7	79·2	79·2	79·2	79·2	78·2	78·8	78·1	78·1	78·0	78·2	78·2
11	94·1	98·5	98·8	98·8	98·5	98·5	98·0	99·1	98·5	98·8	—	98·8	98·8	79·1	79·1	79·1	79·1	78·3	78·7	78·2	78·2	78·0	78·1	78·2
12	93·5	98·0	98·3	98·3	98·0	98·0	97·4	98·0	98·5	97·7	97·1	—	98·4	79·2	79·2	79·2	79·2	78·6	78·8	78·3	78·3	78·1	78·2	78·4
13	93·5	98·0	98·3	98·3	98·5	98·0	97·4	98·0	97·4	97·7	97·1	96·5	—	78·7	78·7	79·2	78·7	77·9	78·2	77·8	78·0	77·6	77·7	77·8
14	84·4	84·3	84·5	84·5	84·3	84·3	83·7	84·3	84·3	84·3	83·7	84·3	82·8	—	100	100	99·8	98·5	99·6	98·0	98·8	96·2	99·0	95·2
15	84·4	84·3	84·5	84·5	84·3	84·3	83·7	84·3	84·3	84·3	83·7	84·3	82·8	100	—	100	99·8	98·5	99·6	98·0	98·8	96·4	99·0	92·9
16	84·4	84·3	84·5	84·5	84·3	84·3	83·7	84·3	84·3	84·3	83·7	84·3	82·8	100	100	—	99·8	98·5	99·6	98·0	98·8	96·4	99·0	92·9
17	84·1	84·0	84·3	84·0	84·0	84·0	83·4	84·0	84·0	84·0	83·4	84·0	82·5	99·4	99·5	99·5	—	98·5	99·4	98·0	98·8	96·4	98·8	92·9
18	84·4	83·7	84·0	84·0	83·7	83·7	83·1	83·7	83·7	83·4	83·1	83·7	82·2	96·3	96·3	96·3	96·3	—	98·1	95·7	98·7	95·2	97·5	95·7
19	84·1	84·0	84·3	84·3	84·0	84·0	83·4	84·0	84·0	84·0	83·4	84·0	82·5	99·1	99·1	99·1	98·5	95·4	—	97·7	98·4	95·9	97·4	94·8
20	77·9	80·8	81·0	81·0	80·8	80·8	80·5	80·8	80·8	80·8	80·2	80·8	79·3	94·4	94·4	94·4	94·4	92·0	93·5	—	98·4	95·7	97·2	93·1
21	81·6	83·7	84·0	84·0	83·7	83·7	83·1	83·7	83·7	84·0	83·1	83·7	82·2	98·5	98·5	98·5	98·5	96·6	97·6	93·8	—	95·3	97·8	93·4
22	83·9	83·7	84·0	84·0	83·7	83·7	83·4	83·7	83·7	83·4	83·1	83·7	82·2	93·8	94·1	94·1	94·1	92·7	93·2	91·8	93·5	—	95·3	94·8
23	82·2	82·2	82·5	82·5	82·2	82·2	81·6	82·2	82·2	82·2	81·6	82·2	80·8	97·3	97·3	97·3	96·7	93·7	98·2	92·1	95·9	91·5	—	94·2
24	81·0	84·5	84·5	84·5	84·3	84·3	83·7	84·3	84·3	84·0	83·7	84·5	82·8	93·6	93·6	93·6	93·6	93·4	92·7	90·7	93·0	94·2	91·0	—

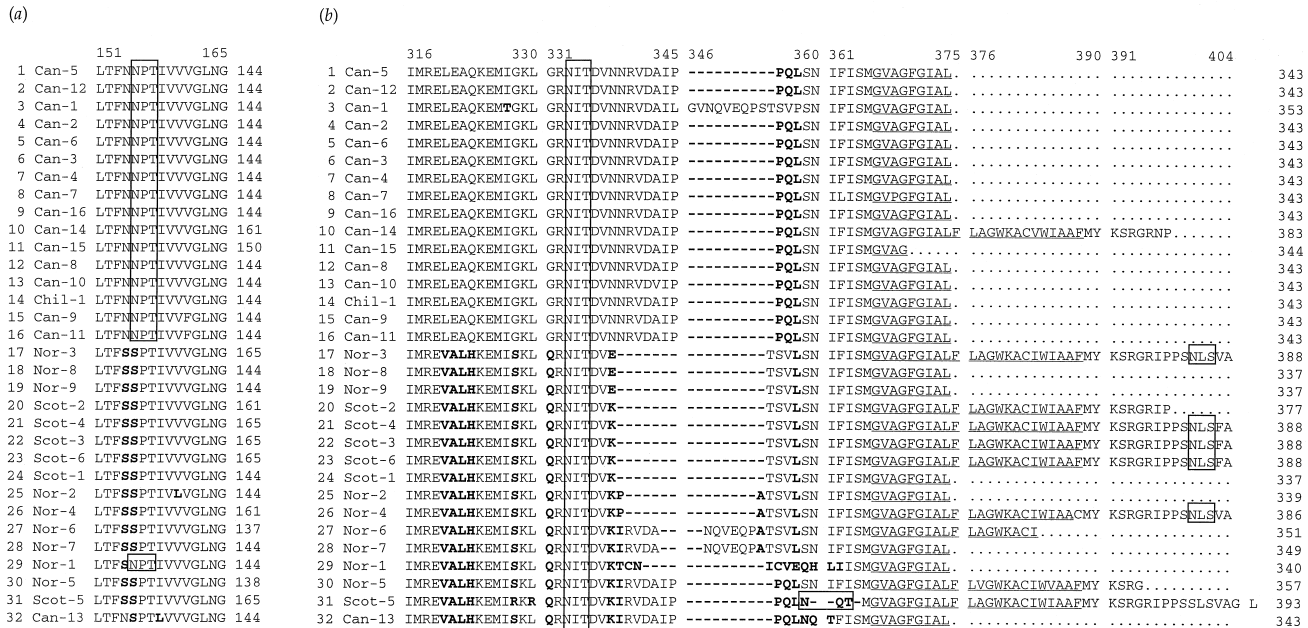


Fig. 2. Selected regions of an alignment of deduced amino acid sequences of the ISAV putative HA proteins of 32 ISAV isolates. (a) A region containing a potential glycosylation site, <sup>155</sup>NPT<sup>157</sup>, in the European subtype. (b) The hypervariable region (residues 339–354) in ORF2. Sequences that are not determined are indicated by dots, amino acid changes relative to ISAV isolate RPC/NB 980 280-2 (Can-1; line 3 of the alignment) are in bold and amino acid deletions are indicated by dashes. Potential N-glycosylation sites are boxed and a potential transmembrane region (Krossøy *et al.*, 2001a) is underlined. ISAV strains are referenced as listed in Table 2. The full alignment is available as supplementary material at JGV Online (<http://vir.sgmjournals.org/>).

all isolates (Fig. 2*b*). The longest stretch of amino acid changes between the two subtypes consisted of four residues, <sup>320</sup>LEAQ<sup>323</sup> in the American subtype and <sup>320</sup>VALH<sup>323</sup> in the European subtype. However, as shown in Fig. 2*b*), the alignment also revealed a hypervariable region (residues 339–354) in the C-terminal region that included a 4–16 amino acid deletion in all ISAV isolates relative to ISAV isolate RPC/NB-980 280-2 (Can-1). It is noteworthy that the only potential N-glycosylation site likely to be used, <sup>333</sup>NIT<sup>335</sup>, lies between these two major mutations. The most extensive deletion, of 16 residues, <sup>339</sup>NRVDAILGVNQVEQPS<sup>354</sup>, was seen between ISAV isolate RPC/NB-980 280-2 and Norwegian isolates Bremnes 98 (Nor-3), 810/9/99 (Nor-8) and 835/9/98 (Nor-9), and between the RPC/NB-980 280-2 isolate and the Scottish isolates 390/98 (Scot-1), Loch Nevis 98 (Scot-2), 832/98 (Scot-3), 301/98 (Scot-4) and 912/99 (Scot-6). The smallest deletion, of four residues, <sup>344</sup>ILGV<sup>347</sup>, was seen between RPC/NB-980 280-2 and Norwegian isolates Hitra 99 (Nor-6) and 485/9/97 (Nor-7). Thus, the HA ORF in ISAV isolate RPC/NB-980 280-2 is estimated to be 404 amino acids long, in contrast to the shortest, of 388 amino acid residues, in Norwegian isolates Bremnes 98, 810/9/99 and 835/9/98 and Scottish isolates 390/98, Loch Nevis 98, 832/98, 301/98 and 912/99.

Comparison of the ISAV Glesvaer/2/90 sequences between accession no. AF220607 of Rimstad *et al.* (2001) and

accession no. AF283998 determined in the present study showed a nucleotide sequence identity of 96.5% and an amino acid sequence identity in ORF2 of 93.2%, with most sequence divergence occurring in the hypervariable region, as seen with the Canadian isolates, but without any deletion. This result was not unexpected, since most likely we sequenced a different clone to that sequenced by Rimstad *et al.* (2001) within the Glesvaer/2/90 quasispecies, but it does indicate to us that this region is highly mutable.

### Evolutionary relationships of ISAV based on the putative HA gene

The viral cDNA sequences of 24 ISAV isolates determined in this study and the eight other putative ISAV HA gene entries in GenBank were subjected to phylogenetic analysis (Fig. 3). All the sequences of ISAV isolates from Norway and Scotland and Canadian isolate U5575-1 (Can-13) form a distinct phylogenetic family with strong bootstrap support. Within this family, Can-13 appears to have diverged from the common ancestor of the European isolates. All these isolates also belonged to one subtype (Table 3) and those that were tested in VN in the present study belonged to the European antigenic group (Table 1). The remaining sequences (Can-1 to Can-12, Can-14 to Can-16 and Chil-1), which belonged to the American antigenic group (Table 1) and subtype (Table 3),

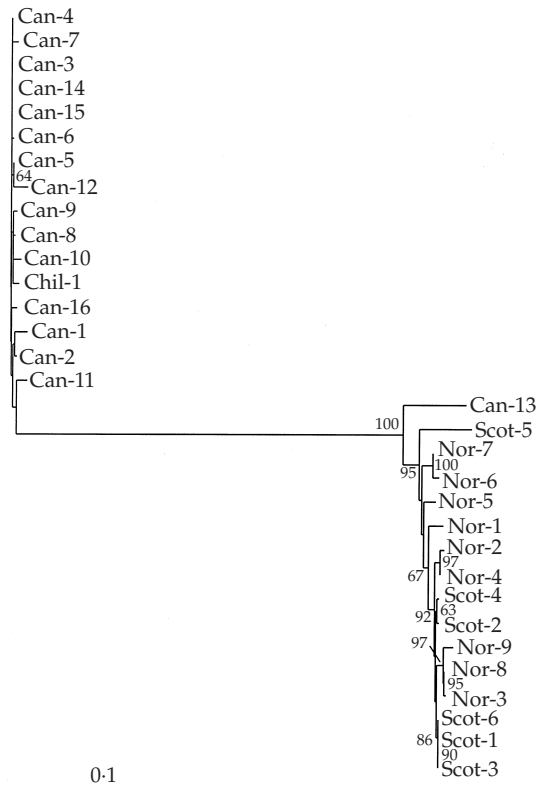


Fig. 3. Phylogenetic tree showing the relationships between 32 different ISAV isolates. The available nucleotide sequences of the putative HA gene of ISAV were aligned and analysed using CLUSTAL X (Thompson *et al.*, 1997). Percentage bootstrap values (1000 replicates) are shown for branch-points with greater than 60% bootstrap support. The high similarity of certain sequences makes some of the horizontal branches too short to display. Their absence causes crowded vertical branches in some cases (some lines look thicker than others). Bar, 0.1 expected substitutions per nucleotide site.

constitute a second family. The members of this second family are very similar to one another, as indicated by the short branch lengths separating them, and the majority of the sequences do not show significant subgrouping.

To view the relationships among the ISAV sequences and the HA sequences of the established orthomyxoviruses, a second phylogenetic tree was constructed. For simplicity, only two of the most diverged ISAV protein sequences, Can-1 and Can-13, were used in the analysis with sequences from *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C* and *Thogotovirus* (see Methods for details of reference sequences). As shown in Fig. 4, Can-1 and Can-13 showed significant grouping, as did the sequences from *Influenzavirus A* and *Influenzavirus B*. Although the ISAV sequences appear to be more closely related to members of *Influenzavirus A* and *Influenzavirus B* than to the other sequences, this grouping was often not observed using other alignment and tree-building techniques (data not shown). Overall, the HA sequences are very divergent, with Can-1 and Can-13 being the most closely related sequence pair.

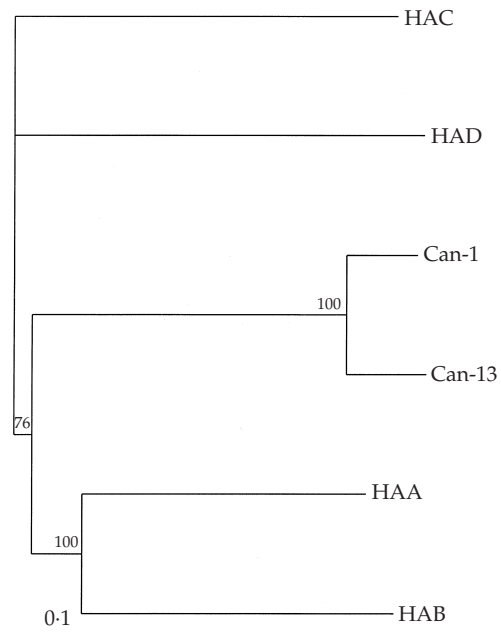


Fig. 4. Phylogenetic tree for the HAs of ISAV and members of the four established genera of the virus family *Orthomyxoviridae*. Protein sequences were aligned and analysed using CLUSTAL X (Thompson *et al.*, 1997). Percentage bootstrap values (1000 replicates) are shown for branch-points with greater than 60% bootstrap support. ISAV isolates RPC/NB 980-280-2 (Can-1) and U5575-1 (Can-13) are two of the most diverged of the ISAV isolates. The other members of *Orthomyxoviridae* are denoted as HAA (*Influenzavirus A*), HAB (*Influenzavirus B*), HAC (*Influenzavirus C*) and HAD (*Thogotovirus*).

## Discussion

In the present study, 24 ISAV isolates from different geographical regions, and belonging to two CHSE phenotypes, were subjected to *in-vitro* neutralization with rabbit polyclonal antisera to ISAV and sequence analysis of the putative HA gene in order to gain further understanding of the nature of phenotypic variation of ISAV. We show here that the degree of virus neutralization by four different rabbit antisera separates ISAV into at least two major antigenic types, one American and one European. Sequence analysis of the putative ISAV HA gene also revealed two groups of viruses corresponding to the antigenic groups (HA subtypes). This grouping was supported by phylogenetic analysis, which revealed two distinct phylogenetic families. The putative HA gene of ISAV shows dramatic nucleotide and deduced amino acid changes within the Canadian isolates and between Canadian and European isolates, with the presence of a hypervariable region in the C-terminal region. Although sequence analysis of this gene has been reported by others (Rimstad *et al.*, 2001; Krossøy *et al.*, 2001a), the present study included antigenic analysis, investigation of a possible association with CHSE phenotypes and the examination of many more ISAV isolates from different geographical regions than previously studied. We also add to the evidence supporting the conclusion that RNA segment 6 of ISAV encodes the HA protein by demonstrating limited

amino acid sequence similarity between the HA of ISAV and of members of the four established genera of the family *Orthomyxoviridae*. Thus, we demonstrate here for the first time that ISAV isolates vary significantly antigenically and that this antigenic variation is correlated with genetic variation of the putative HA gene.

Previous molecular analyses have shown that ISAV isolates from Canada and Europe vary significantly on RNA segments 2 and 8 (Blake *et al.*, 1999; Cunningham & Snow, 2000; Kibenge *et al.*, 2000, 2001; Inglis *et al.*, 2000). It has been generally accepted that, because of the close geographical proximity of Scotland and Norway, the Scottish and Norwegian isolates are more closely related to each other than to the Canadian isolates (Cunningham & Snow, 2000). The antigenic analysis and sequence analysis of the putative HA gene of 24 isolates in the present study shows that the groupings based on the HA gene do not separate the viruses clearly along geographical regions of origin, since ISAV isolate U5575-1 (Can-13) groups with the European subtype.

The two HA subtypes had a nucleotide sequence identity of only  $\leq 79.4\%$  and amino acid sequence identity of  $\leq 84.5\%$  whereas, within each subtype, the sequence identities were 90.7% or higher. Between the two subtypes, there were twice as many amino acid changes in the second half of the protein as were found in the first half and most involved one or two residues distributed randomly. Mutations with the potential to influence the structure/function of the ISAV HA protein include the change at the potential glycosylation site, <sup>155</sup>NPT<sup>157</sup>, in the European HA subtype, the four-residue mutation between the two subtypes, <sup>320</sup>LEAQ<sup>323</sup> in the American subtype and <sup>320</sup>VALH<sup>323</sup> in the European subtype, and the hypervariable region (residues 339–354) in the C-terminal region, which included a 4–16 amino acid deletion in all ISAV isolates relative to ISAV isolate RPC/NB-980 280-2 (Can-1). In previous reports on the ISAV HA gene, only small numbers of isolates were analysed and no definite pattern emerged. Consequently, none of those reports identified the 10-residue deletion in the Canadian isolates relative to ISAV isolate RPC/NB-980 280-2 (Can-1) or the four-residue deletion, <sup>344</sup>ILGV<sup>347</sup>, in Norwegian isolates Hitra 99 (Nor-6) and 485/9/97 (Nor-7) relative to isolate RPC/NB-980 280-2 (Can-1) reported in the present study. The putative ISAV HA has been suggested to have the same orientation as the influenza virus HA, with the first 334 amino acid residues making up the ectodomain and, depending on the virus strain, residues 351–366 to 374–388 making up the hydrophobic domain that could cross the virus envelope (Rimstad *et al.*, 2001; Krossøy *et al.*, 2001a). Thus, given their location in the ISAV HA protein, the <sup>320</sup>LEAQ<sup>323</sup> ↔ <sup>320</sup>VALH<sup>323</sup> and the 4–16 residue deletion mutations between the two HA genotypes (Fig. 2b) might account for the two HA antigenic types identified in the present study.

It is interesting to note that isolate U5575-1 (Can-13) appears to have diverged recently from the common ancestor

of the European isolates. This isolate is most similar to Norwegian isolate Gullesfjord 93 (Krossøy *et al.*, 2001a) (Nor-5) and the Scottish isolate 1490/98 (Scot-5) from the island of Skye (Figs 2 and 3). ISAV U5575-1 was isolated from a clinical ISA outbreak in Nova Scotia, Canada, whereas all other Canadian isolates are from New Brunswick. A previous report of ISAV similar to European isolates found in farmed Atlantic salmon in Nova Scotia was based on RT-PCR-amplified sequences of segment 8 from apparently normal fish, and virus was never isolated from those fish (Ritchie *et al.*, 2001). This is the first documentation of an ISAV isolate that confirms the presence of a European ISAV ancestor in North America. The extensive deletions in the HA gene of European ISAV isolates may suggest that the archetypal ISAV was probably of Canadian origin, for example the RPC/NB-980 280-2 (Can-1) isolate. Although the deletions described in this study may not necessarily infer a direction of evolution in this virus, previous phylogenetic analysis based on RNA segment 2 (PB1 gene) suggested that American and European ISAV isolates diverged around 1900, coincident with the introduction of rainbow trout, *Oncorhynchus mykiss*, to Europe from North America (Krossøy *et al.*, 2001b).

The putative HA gene of ISAV shows dramatic nucleotide changes within the Canadian isolates and between Canadian and European isolates. The putative archetypal ISAV, isolate RPC/NB-980 280-2, is found in Canada with the most complete amino acid sequence of the putative HA protein. Other isolates from Canada, Chile, Norway and Scotland have deletions relative to this isolate. This suggests that ISAV is still evolving and is yet to reach a state of adaptation where further nucleotide changes will not result in amino acid changes (i.e. when further changes will provide no selective advantage). While the sequence of the European isolates contained two ORFs, a small ORF1 and a large ORF2, all the Canadian isolates contained only the large ORF2. This indicated to us that ORF1 might not encode an essential protein, since it was absent in a significant group of ISAV isolates.

The interaction of a virus with its cellular receptor initiates a chain of dynamic events that will enable entry of the virus into the cell (Schneider-Schaulies, 2000). This interaction is critical, as it determines the host range of the virus, which may change with mutations in the virus. Whereas all the ISAV isolates studied to date are cytopathic in the SHK-1 and TO cell lines, only some of them cause CPE in the CHSE-214 cell line, allowing the grouping of ISAV into two phenotypes (Kibenge *et al.*, 2000). In the present study, we used a total of 24 different ISAV isolates to demonstrate that this phenotype is associated neither with the antigenicity of ISAV nor with the sequence variation of the putative HA gene. The lack of correlation between the CHSE phenotypes and the HA subtypes may be due to the finding reported by Krossøy *et al.* (2001a), that the ISAV HA does not appear to be cleaved post-translationally and therefore does not carry fusion activity. Among the established genera of *Orthomyxoviridae*, only

members of the genus *Thogotovirus* have no requirement for glycoprotein cleavage; however, virions in this genus contain only six or seven genome segments (Cox *et al.*, 2000). In influenza viruses, where the HA protein mediates virus entry into cells by a low pH-induced membrane-fusion event in endosomal vesicles, the fusion activity of the HA is dependent on its being cleaved and the susceptibility of cleavage of the HA has been correlated with virulence of virus in tissue culture and in animals (Palese & García-Sastre, 1999). Influenza virions with uncleaved HA are non-infectious (Klenk & Rott, 1988). It can therefore be speculated that the molecular basis for the ISAV CHSE phenotypes may be associated with the fusion protein encoded by a gene separate from the HA gene. However, there is anecdotal evidence as well of ISAV in both apparently normal and sick farmed Atlantic salmon that can be detected by RT-PCR but not by virus isolation using presently available fish cell lines (Kibenge *et al.*, 2001). The inference that the ISAV HA gene may not be correlated with virulence of the virus in tissue culture is further supported by observations that HA gene sequences directly amplified by RT-PCR either from an apparently normal fish (Can-16, accession no. AF297551) or from diseased fish (Selje A12 sample; Rimstad *et al.*, 2001) from which virus could not be isolated using SHK-1 cells were similar to the American and European HA subtypes, respectively. In fact, the Can-16 HA amino acid sequence differed from the American HA subtype only at residue 143, with the substitution of a serine for leucine (see full alignment available at <http://vir.sgmjournals.org/>). Thus, more in-depth analysis is necessary to clarify the molecular basis of the CHSE phenotypes of ISAV, particularly since, in many viruses, such tropism is associated with a single residue.

The limited amino acid sequence identity between the HA of ISAV and members of the four established genera of the family *Orthomyxoviridae* (Fig. 1) is interesting. For the sequences from *Influenzavirus A*, *Influenzavirus C* and *Thogotovirus*, the sequence similarity was due to the N-terminal region of ISAV HA, spanning amino acid residues 24–140 while, in the sequence from *Influenzavirus B*, it spanned amino acid residues 203–264 of the ISAV HA. The functional significance of these regions is not known. To view the relationships among the ISAV sequences and the HA sequences of the established orthomyxoviruses, we constructed a phylogenetic tree (Fig. 4). It is interesting that Can-1 and Can-13, which represent the two ISAV HA subtypes, showed significant grouping, as did *Influenzavirus A* and *Influenzavirus B*. Although the ISAV sequences appear to be related more closely to *Influenzavirus A* and *Influenzavirus B* than to the other sequences, this grouping was often not observed using other alignment and tree-building techniques and further supports the suggestion that ISAV be assigned to a new genus within *Orthomyxoviridae*.

In conclusion, we show that the ISAV isolates vary significantly in the putative HA gene and that this genetic variation is correlated with antigenic variation revealing two

HA subtypes, one American and one European. The two HA subtypes have a nucleotide sequence identity of only  $\leq 79.4\%$  and amino acid sequence identity of  $\leq 84.5\%$  whereas, within each subtype, the sequence identities are 90.7% or higher.

We thank the following for supplying us with the ISAV isolates: Dr Steve Griffiths [Research and Productivity Council (RPC), Fredericton, New Brunswick, Canada], Dr Carmencita Yason (Regional Diagnostic Virology Laboratory, Atlantic Veterinary College, Charlottetown, PEI, Canada), Dr Heidrun Wergeland (Bergen High Technology Center, Bergen, Norway), Dr Knut Falk (National Veterinary Institute, Oslo, Norway), Dr Birgit Dannevig (National Veterinary Institute, Oslo, Norway) and Dr Alastair McVicar and Mrs Katrina Burnside (Fish Health Inspectorate, FRS Marine Laboratory, Aberdeen, UK). The authors wish to thank Dr Oystein Evensen (Alpharma AS) for giving access to the TO cell line. Financial support for this work was provided by a strategic grant from the Natural Sciences and Engineering Research Council of Canada, AVC Inc., Charlottetown, PEI, and Alpharma AS, Norway.

## References

- Blake, S., Bouchard, D., Keleher, W., Optiz, M. & Nicholson, B. L. (1999). Genomic relationships of the North American isolate of infectious salmon anaemia virus (ISAV) to the Norwegian strain of ISAV. *Diseases of Aquatic Organisms* **35**, 139–144.
- Cox, N. J., Fuller, F., Kaverin, N., Klenk, H.-D., Lamb, R. A., Mahy, B. W. J., McCauley, J., Nakamura, K., Palese, P. & Webster, R. (2000). Family *Orthomyxoviridae*. In *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 585–597. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.
- Cunningham, C. O. & Snow, M. (2000). Genetic analysis of infectious salmon anaemia virus (ISAV) from Scotland. *Diseases of Aquatic Organisms* **41**, 1–8.
- Falk, K., Namork, E., Rimstad, E., Mjaaland, S. & Dannevig, B. H. (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *Journal of Virology* **71**, 9016–9023.
- Feldmann, H., Kretzschmar, E., Klingeborn, B., Rott, R., Klenk, H. D. & Garten, W. (1988). The structure of serotype H10 hemagglutinin of influenza A virus: comparison of an apathogenic avian and a mammalian strain pathogenic for mink. *Virology* **165**, 428–437.
- Griffiths, S., Cook, M., Mallory, B. & Ritchie, R. (2001). Characterisation of ISAV proteins from cell culture. *Diseases of Aquatic Organisms* **45**, 19–24.
- Inglis, J. A., Bruce, J. & Cunningham, C. O. (2000). Nucleotide sequence variation in isolates of infectious salmon anaemia virus (ISAV) from Atlantic salmon *Salmo salar* in Scotland and Norway. *Diseases of Aquatic Organisms* **43**, 71–76.
- Kibenge, F. S. B., Dybing, J. K. & McKenna, P. K. (1991). Rapid procedure for large-scale isolation of plasmid DNA. *Biotechniques* **11**, 65–67.
- Kibenge, F. S. B., Lyaku, J. R., Rainnie, D. & Hammell, K. L. (2000). Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. *Journal of General Virology* **81**, 143–150.
- Kibenge, F. S. B., Garate, O. N., Johnson, G., Arriagada, R., Kibenge, M. J. T. & Wadowska, D. (2001). Isolation and identification of infectious

- salmon anaemia virus (ISAV) from Coho salmon in Chile. *Diseases of Aquatic Organisms* **45**, 9–18.
- Klenk, H.-D. & Rott, R. (1988)**. The molecular biology of influenza virus pathogenicity. *Advances in Virus Research* **34**, 247–281.
- Krossøy, B., Hordvik, I., Nilsen, F., Nylund, A. & Endresen, C. (1999)**. The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the *Orthomyxoviridae*. *Journal of Virology* **73**, 2136–2142.
- Krossøy, B., Devold, M., Sanders, L., Knappskog, P. M., Aspehaug, V., Falk, K., Nylund, A., Koumans, S., Endresen, C. & Biering, E. (2001 a)**. Cloning and identification of the infectious salmon anaemia virus haemagglutinin. *Journal of General Virology* **82**, 1757–1765.
- Krossøy, B., Nilsen, F., Falk, K., Endresen, C. & Nylund, A. (2001 b)**. Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. *Diseases of Aquatic Organisms* **44**, 1–6.
- Matsuzaki, Y., Mizuta, K., Kimura, H., Sugawara, K., Tsuchiya, E., Suzuki, H., Hongo, S. & Nakamura, K. (2000)**. Characterization of antigenically unique influenza C virus strains isolated in Yamagata and Sendai Cities, Japan, during 1992–1993. *Journal of General Virology* **81**, 1447–1452.
- Mjaaland, S., Rimstad, E., Falk, K. & Dannevig, B. H. (1997)**. Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. *Journal of Virology* **71**, 7681–7686.
- Palese, P. & García-Sastre, A. (1999)**. Influenza viruses (*Orthomyxoviridae*): molecular biology. In *Encyclopedia of Virology*, 2nd edn, pp. 830–836. Edited by A. Granoff & R. G. Webster. San Diego: Academic Press.
- Pearson, W. R. & Lipman, D. J. (1988)**. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences, USA* **85**, 2444–2448.
- Rimstad, E., Mjaaland, S., Snow, M., Mikalsen, A. B. & Cunningham, C. O. (2001)**. Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin. *Journal of Virology* **75**, 5352–5356.
- Ritchie, R. J., Cook, M., Melville, K., Simard, N., Cusack, R. & Griffiths, S. (2001)**. Identification of infectious salmon anaemia virus in Atlantic salmon from Nova Scotia (Canada): evidence for functional strain differences. *Diseases of Aquatic Organisms* **44**, 171–178.
- Saitou, N. & Nei, M. (1987)**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989)**. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider-Schaulies, J. (2000)**. Cellular receptors for viruses: links to tropism and pathogenesis. *Journal of General Virology* **81**, 1413–1429.
- Snow, M. & Cunningham, C. O. (2001)**. Characterisation of the putative nucleoprotein gene of infectious salmon anaemia virus (ISAV). *Virus Research* **74**, 111–118.
- Stothard, P. (2000)**. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* **28**, 1102–1104.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994)**. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997)**. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876–4882.
- Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. (1992)**. Evolution and ecology of influenza A viruses. *Microbiological Reviews* **56**, 152–179.
- Wergeland, H. I. & Jakobsen, R. A. (2001)**. A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). *Diseases of Aquatic Organisms* **44**, 183–190.

---

Received 10 May 2001; Accepted 9 August 2001