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Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus

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Abstract

Infectious salmon anaemia virus (ISAV) is an orthomyxovirus causing a multisystemic, emerging disease in Atlantic salmon. Here we present, for the first time, detailed sequence analyses of the full-genome sequence of a presumed avirulent isolate displaying a full-length hemagglutinin-esterase (HE) gene (HPR0), and compare this with full-genome sequences of 11 Norwegian ISAV isolates from clinically diseased fish. These analyses revealed the presence of a virulence marker right upstream of the putative cleavage site R_{267} in the fusion (F) protein, suggesting a $Q_{266} \rightarrow L_{266}$ substitution to be a prerequisite for virulence. To gain virulence in isolates lacking this substitution, a sequence insertion near the cleavage site seems to be required. This strongly suggests the involvement of a protease recognition pattern at the cleavage site of the fusion protein as a determinant of virulence, as seen in highly pathogenic influenza A virus H5 or H7 and the paramyxovirus Newcastle disease virus.
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Introduction

Infectious salmon anaemia virus (ISAV) is an orthomyxovirus causing a multisystemic emerging disease in Atlantic salmon (*Salmo salar*). Natural outbreaks of infectious salmon anaemia (ISA) were reported for the first time in Norway in 1984 (Thorud and Djupvik, 1988) and it was considered a unique Norwegian disease problem for over 10 years. However, in 1996 a disease condition (hemorrhagic kidney syndrome) in Canadian farmed salmon turned out to be caused by ISAV (Lovely et al., 1999; Mullins et al., 1998). In the following years the disease was diagnosed in Scotland (Rodger and Richards, 1998), the Shetland Islands, the Faroe Islands and USA (Bouchard et al., 1999). Isolation of ISAV from a disease outbreak in Coho salmon (*Oncorhynchus kisutch*) from Chile has been reported (Kibenge et al., 2001). The presence of ISAV in Chile was officially verified in 2006, and recently an ISA disease outbreak was officially confirmed in Atlantic salmon (http://www.oie.int/eng/en_index.htm).

The morphological, physiochemical and genetic properties of ISAV are consistent with those of the *Orthomyxoviridae* (Mjaaland et al., 1997; Falk et al., 1997), where ISAV is classified as the type species of a new genus *Isavirus* (Kawaoka et al., 2005) within this family. Present knowledge indicates that wild salmonids represent important natural reservoirs for ISAV. Natural disease outbreaks have only been described in farmed Atlantic salmon, although ISAV has been demonstrated both in

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wild salmonid and non-salmonid fish (Plarre et al., 2005; Raynard et al., 2001; Cunningham et al., 2002; MacLean et al., 2003) and the virus may, under experimental conditions, persist and replicate in other salmonid (*Salvelinus alpinus*, *Oncorhynchus mykiss*, *Oncorhynchus keta*, *Oncorhynchus kisutch*) (Snow et al., 2001; Rolland and Winton, 2003; Nylund et al., 1995, 1997) and non-salmonid fish (*Clupea harengus*, *Gadus morhua*) (Nylund et al., 2002; Grove et al., 2007).

As in influenza A and B viruses, the ISAV genome consists of eight negative sense, single-stranded RNA segments encoding at least 10 proteins (Mjaaland et al., 1997; Falk et al., 2004; Clouthier et al., 2002), although the organization of ISAV genes and gene products is unique. The functional organization of the two major ISAV surface glycoproteins differs from influenza A and B viruses; the ISAV hemagglutinin-esterase (HE) encoded by segment 6 is responsible for receptor-binding and release (Falk et al., 2004; Krossøy et al., 2001; Rimstad et al., 2001), while the segment 5 encoded fusion (F) protein is responsible for fusion of viral and cellular membranes (Aspehaug et al., 2005). For influenza A and B however, the receptor-binding and fusion functions reside in the hemagglutinin (HA) while receptor-destroying activity resides in the neuraminidase (NA) (Colman et al., 1983; Palese et al., 1974; Wiley and Skehel, 1977, 1987). The viral polymerases (PB2, PB1 and PA) are encoded by segments 1, 2, and 4 (Clouthier et al., 2002; Krossøy et al., 1999; Snow et al., 2003), while segment 3 encodes the nucleoprotein (NP) (Aspehaug et al., 2004; Snow and Cunningham, 2001). At least two proteins are encoded from each of the two smallest genomic segments: the un-spliced mRNA of segment 7 produces a non-structural protein with interferon antagonistic properties (Biering et al., 2002; McBeath et al., 2006; García-Rosado et al., in press), while ORF2 is suggested to encode for a nuclear export protein (Kibenge et al., 2007). The presence of a third ORF has also been suggested (Kibenge et al., 2007). From segment 8, the matrix protein is produced from the smaller ORF1 (Biering et al., 2002; Falk et al., 2004), while the larger ORF2 encodes an RNA binding structural protein with interferon antagonistic properties (García-Rosado et al., in press).

The HE surface glycoprotein is the molecule with the highest sequence variability and assumed to be of importance in virulence (Mjaaland et al., 2002, 2005; Devold et al., 2001; Cunningham et al., 2002; Nylund et al., 2007). Most of the variation in this molecule is, however, concentrated to a small highly polymorphic region (HPR) near the transmembrane domain (Krossøy et al., 2001; Cunningham et al., 2002; Rimstad et al., 2001; Devold et al., 2001; Mjaaland et al., 2002), suggested to result from differential deletions of a full-length avirulent precursor gene (HPR0) resulting in more or less pathogenic viruses, possibly as a consequence of transmission from a viral reservoir to densely populated farmed Atlantic salmon (Mjaaland et al., 2002). In fact, the presence of ISAV HPR0 isolate has been confirmed in healthy wild and farmed Atlantic salmon (Cunningham et al., 2002; Cook-Versloot et al., 2004; Anonymous, 2005; Nylund et al., 2007). HPR0 has not been detected in diseased fish with classical clinical and pathological changes consistent with ISA. Occasionally, however, detection of HPR0 has been associated with signs of proliferative gill inflammation (PGI) (Anonymous, 2005; Nylund et al., 2007). The possibility that these clinical signs could represent a new manifestation of ISA cannot be ruled out, and based on the lack of adequate experimental infection models with HPR0 virus, one should be careful to state that HPR0 is avirulent. However, circumstantial evidence indicates that infection with HPR0 virus in itself poses a lesser risk of disease outbreak than infection with ISAV from other HPR groups. All HE-HPRs described till today can be derived from a full-length HPR0 sequence, and virus isolated from ISA-diseased fish always contains a deletion in this region (Mjaaland et al., 2002; Nylund et al., 2007) (www.mattilsynet.no). The driving forces behind the differential deletion patterns in the ISAV HE could be analogous to a phenomenon described for influenza A NA, where varying lengths of the stalk region has been reported, a property that has been associated to host range adaptation (Air et al., 1990; Castrucci and Kawaoka, 1993).

For both avian influenza A virus and paramyxovirus Newcastle disease virus, mutations in proteolytic cleavage sites in HA_0 and F, respectively, are important for virulence (reviewed in Steinhauer, 1999; Peeters et al., 1999; Gotoh et al., 1992; Glickman et al., 1988). For instance, in avian influenza A virus HA of the subtypes H5 and H7, sequential insertion of several basic amino acid residues at the proteolytic cleavage site leads to systemic infection and multi-organ failure in poultry (Bosch et al., 1981; Senne et al., 1996). Several ISAV isolates have been reported to contain variations in their F gene caused by insertions near the putative cleavage site of the molecule (Devold et al., 2006). Whether these insertions lead to analogous alterations in the cleavage specificity of the virus, with potential changes in tissue or organ tropism, is not known.

The combination of surface molecules and the internal proteins might have unpredictable influence on the virulence and pathogenicity of a virus (Tumpey et al., 2005). The evolution of influenza viruses arises mainly through antigenic drift, where mutations results in the seasonal variation, and antigenic shift; the reassortment of gene segments with the possible emergence of new virulent strains (Desselberger et al., 1978; Li et al., 2004; Lindstrom et al., 1998; Matsuzaki et al., 2003; McCullers et al., 2004; Webster et al., 1982). Only a few examples of recombination have been reported (Khatchikian et al., 1989; Orlich et al., 1994; Hirst et al., 2004; Bergmann et al., 1992; Wagner et al., 2001; Fields and Winter, 1982; Jennings et al., 1983). In three of the cases recombination involves an insertion in the HA gene of influenza A subtype H7 near the cleavage site of the protein (Khatchikian et al., 1989; Orlich et al., 1994; Hirst et al., 2004). The detailed mechanisms driving the evolution of ISAV are not known, although there are reports suggesting that both reassortment and recombination might be of importance (Devold et al., 2006).

Here we present phylogenetic and sequence analyses of all coding regions of 12 Norwegian ISAV isolates, including the low- or avirulent HPR0 genotype, giving us an opportunity to map several molecular mechanisms behind the evolution of this virus. In particular, a new marker for virulence was identified at

position 266 in the F protein, right upstream of one of the putative cleavage sites, R_{267}, suggesting involvement of a protease recognition pattern as a determinant of virulence.

Results

The full-genome sequence of 12 different ISAV isolates known to vary in virulence was analysed, in order to understand some of the molecular mechanisms behind the evolution of ISAV, i.e. a) to determine the genetic relationships between different ISAV isolates and b) uncover the presence of potential virulence traits in the ISAV genome. In light of the large differences in virulence, the ISAV genome was highly conserved, with pair wise nucleotide sequence identities ranging from 89.0 to 99.7%, and amino acid identities between 92.3 and 99.5% (Fig. 1). For all gene segments, at least 50% of the amino acid substitutions led to different amino acid changes. The highest sequence variation was within the HPR region of the HE gene (Fig. 1). A relatively high nucleotide sequence variation was found for segments 5 and 4 as well. Interestingly, most of the variation between segment 4 sequences could be ascribed to 45 nucleotides shared only between three of the isolates; ISAV5, ISAV11 and SK779/06 (HPR0 genotype), none of these leading to changes in amino acids (Fig. 1). Least sequence variation was found in the two smallest genomic segments, 7 and 8. On the other hand, a significantly higher proportion of these leads to changes in amino acids. This was particularly true for segment 7 ORF1 and segment 8 ORF2, each containing 11 amino acid differences, while in their alternative ORFs, this number was 5 and 2, respectively. Only in one case in each of these two gene segments did a nucleotide produce an amino acid change in both their ORFs (Fig. 1).

The results from the phylogenetic analyses performed separately for each gene segment are presented in Fig. 2A. Several isolates diverging in virulence clustered together in several segments with high bootstrap support (ISAV5 and ISAV11 in segments 1, 2, 3, 4 and 7, and ISAV4 and ISAV10 in segments 1, 2, 4, 6, 7 and partially in segment 5) (Fig. 2A). Furthermore, a common ancestor is suggested for segment 6 in ISAV6, ISAV5 and ISAV3, but not ISAV11. An analogous situation was observed in segment 5 for the cluster containing ISAV8, ISAV10, ISAV11 and SK779/06, while here ISAV5 was excluded. Segment 3 from ISAV4 and ISAV10 was placed in separate clusters, supporting reassortment of this gene segment as well. Similarly, analyses of ISAV1 suggest reassortment events involving segments 2 and 4 (Fig. 2A). In segment 5, the monophyletic group formed by ISAV8/ISAV10/ISAV11/SK779/06 had strong support (Fig. 2A). Three nucleotides, shared only by these four isolates, lead to changes in amino acids in the F protein when compared to ISAV5 (Table 1), where two of these located in close proximity to one of the two putative cleavage sites of this protein, R_{267} (Aspehaug et al., 2005). We

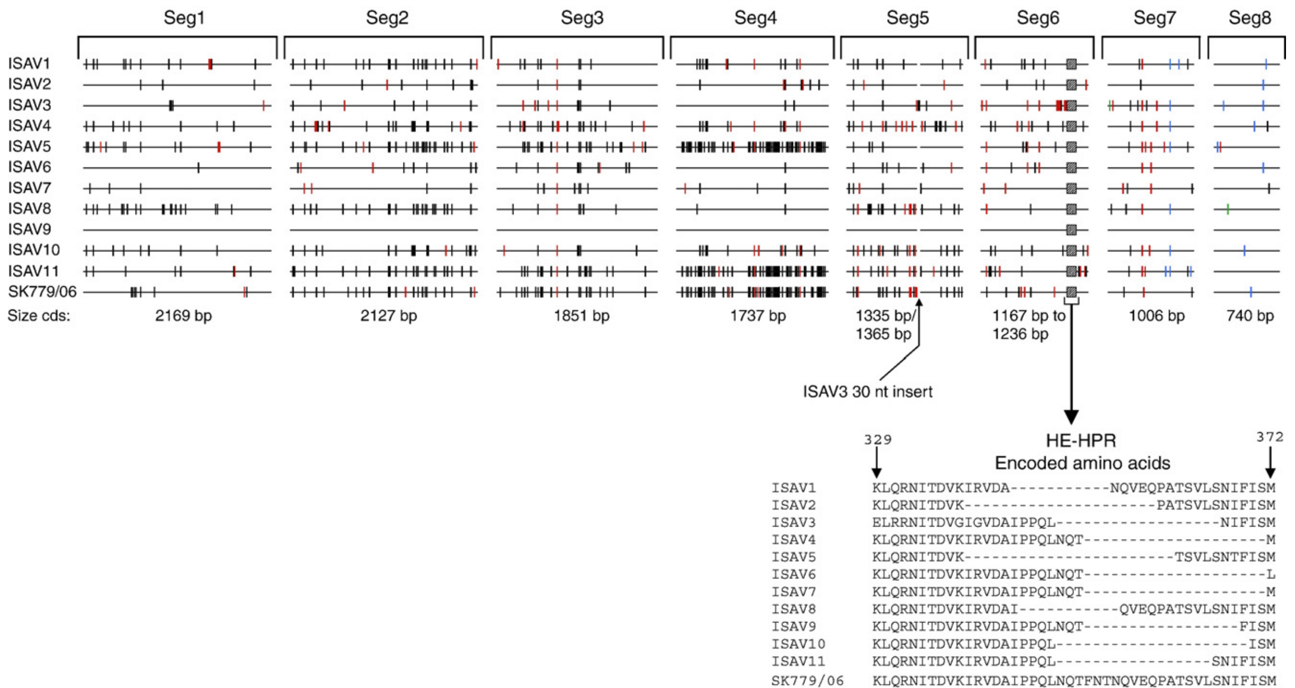


Fig. 1. ISAV genome sequence variation. Nucleotide and amino acid differences in each of the eight gene segments. The size of the coding region, shown at the bottom of each segment, varies only in segment 5 and 6. Each vertical line represents a positional nucleotide difference according to the reference isolate ISAV9. The position and sequences of the HE-HPR, aligned according to their deletion patterns, and the placement of the 10 amino acid insert in the ISAV3 F protein, are also shown. Red/blue/green lines represent nucleotide sequence differences leading to change in amino acid. For segments 7 and 8 the sequences covering both ORF's were used. Red lines represent changes only in their ORF1, blue lines changes only in their ORF2, and green lines amino acid change in both their ORFs, respectively.

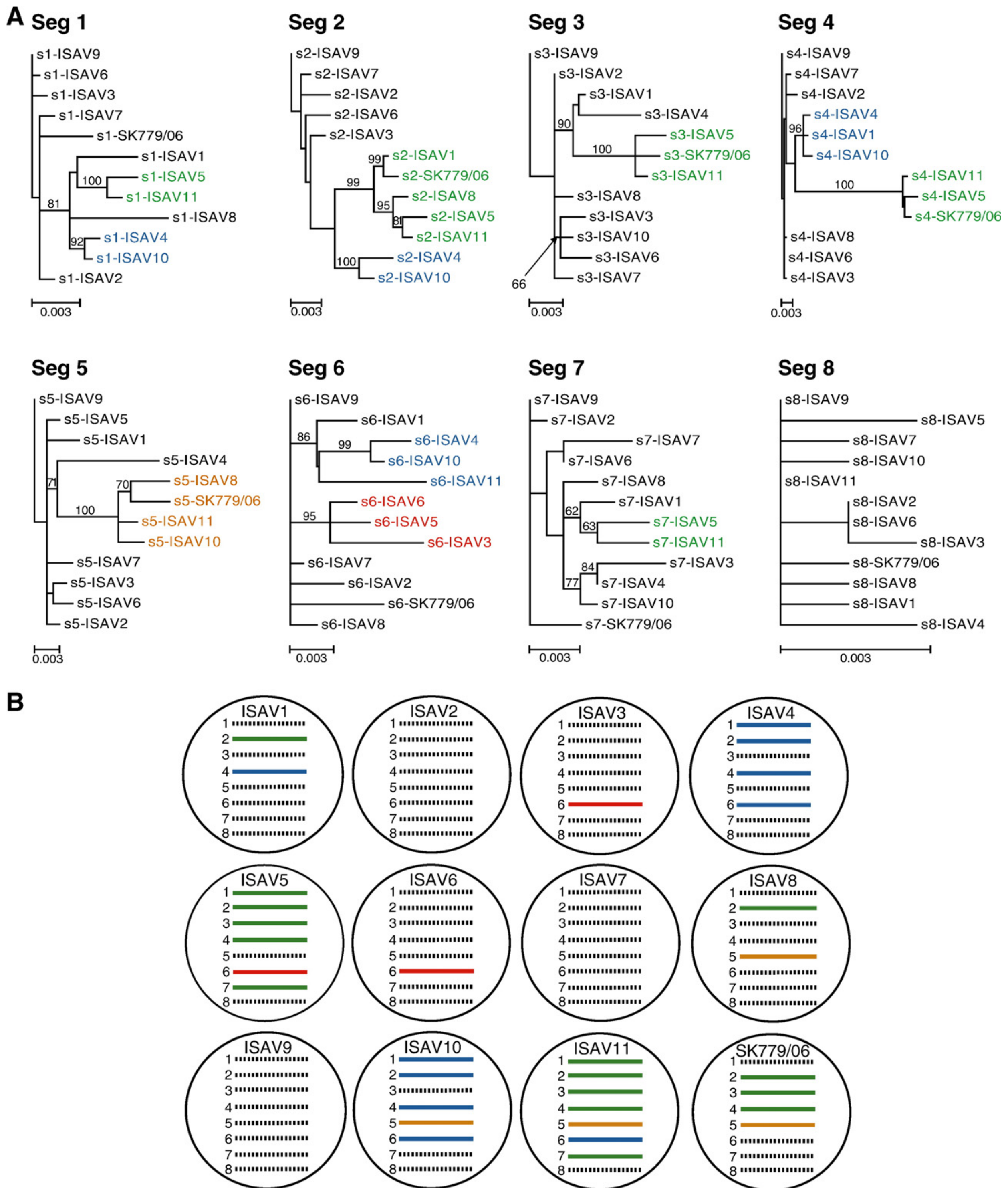


Fig. 2. ISAV gene reassortment. A) Phylogenetic analyses were performed using the coding sequences from all eight gene segments. The HPR region in gene segment 6 (HE) and the 30 nucleotide insert in segment 5 (F) of ISAV3 were not included in the analyses. The nucleotide sequences covering both ORF's of segment 7 and 8 were used. Phylogenetic trees were constructed by maximum likelihood (ML) using PHYML, the GTR model of nucleotide substitution and rooted to ISAV9. Bootstrap values were calculated from 100 replicas using the neighbour-joining method and the F84 model of nucleotide substitution. Isolates' gene segments shown in blue, green, red or orange differ significantly from that of ISAV9, and are depicted to have arisen from a common origin. The same colour coding, as well as the prerequisites for the conclusions, are detailed in panel B and its legends. B) Genetic relationship between ISAV gene segments. The data is based both on results from phylogeny, using bootstrap values above 80, and direct sequence analyses. Lower bootstrap values were accepted in cases were the common origins, or reassortment, of gene segments had the additional support from detailed sequence analyses. Gene segments likely to share common origins are drawn in the same colour. Dotted lines = relationship between gene segments is not known.

Table 1
Comparisons of amino acid differences in selected pairs of ISAV isolates differing in virulence

Isolate pairs compared	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Segment 6[e]	Segment 7 ORF1	Segment 7 ORF2	Segment 8 ORF1	Segment 8 ORF2
ISAV1/SK779/06[a]	G/R_{488} R/K_{490} V/M_{611}	V/I_{140} V/M_{701} R/H_{707}	T/S_{9}	N/D_{192} N/S_{197} E/D_{303}	A/T_{58} N/G_{252} S/N_{263} L/Q_{266}	H/Y_{23} S/N_{154} N/T_{180} A/V_{290}	F/L_{133} L/V_{143}	R/K_{64} R/Q_{101}	“	D/N_{136} S/A_{200}
ISAV2/ISAV9[b]	“	K/R_{368}	S/N_{229}	V/M_{403} P/S_{481}	D/E_{76} R/G_{365}	C/F_{395}	“	“	“	L/P_{187}
ISAV1/ISAV11[c]	G/R_{488} R/K_{490} I/V_{577}	R/H_{707}	T/S_{9}	V/G_{164} N/D_{192}	A/T_{58} R/K_{164} N/D_{252} S/N_{263} G/E_{338}	I/M_{29} D/G_{73} A/V_{384} I/V_{392}	R/K_{149}	K/R_{48} R/Q_{101} I/V_{145}	“	S/A_{200}
ISAV4/ISAV7[d]	“	F/V_{61} D/N_{81} D/G_{94} D/N_{101} T/M_{147} N/K_{646}	S/A_{104} E/D_{235} L/F_{564}	T/S_{47} R/K_{209} L/I_{292} Y/H_{413} E/D_{471}	S/G_{65} R/Q_{73} A/T_{149} G/D_{196} Y/C_{222} A/V_{230} D/N_{255} E/G_{305} M/V_{427}	V/G_{14} T/N_{96} V/I_{143} K/T_{185}	V/I_{62} F/L_{133} D/E_{170} I/T_{184}	R/K_{64}	“	P/S_{154}

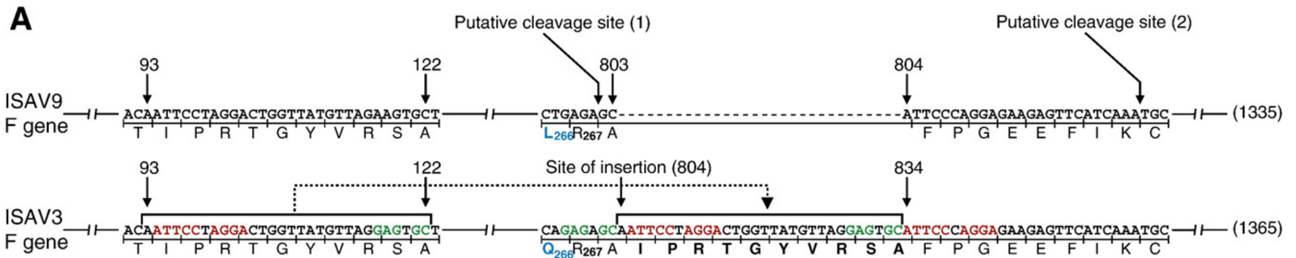
[a]The highly virulent ISAV1 isolate versus the low- or avirulent HPR0 genotype SK779/06.
[b]One high (ISAV2) and one low-virulent isolate (ISAV9).
[c]Two isolates (ISAV1 and ISAV11) inducing different types of immune responses.
[d]Two isolates (ISAV4 and ISAV7) sharing identical HE-HPR's.
[e]Sequence differences in the HE-HPR regions are not included in these comparisons.
Red = amino acid differences unique to the HPR0 genotype. Orange = amino acid differences found only in the low- or avirulent isolates ISAV8, ISAV10, ISAV11 and SK779/06 (shown in same colour as in Fig. 2A for comparisons). Blue = the Q_{266}/L_{266} virulence marker. “ = identical protein sequences.
Virulence- and immune response data for the selected ISAVs are from Mjaaland et al. (2005).

found no evidence of gene linkage in the reassortants. The results from phylogeny and sequence analyses are summarized in Fig. 2B.

In one isolate (ISAV3), a 30 nucleotide long insertion, resulting in the in-frame insertion of 10 amino acids, was found in segment 5 close to one of the putative cleavage sites (Fig. 3A). This inserted stretch was almost identical to a nucleotide sequence close to the 5′ end of the same gene. Sequence analyses at the insertion site revealed extensive sequence identity between the sequence surrounding the site of the insertion, and the 5′ and 3′-ends of the inserted nucleotide stretch itself (Fig. 3A), consistent with a sequence-based non-homologues replicase-driven recombination event. Phylogenetically, the ISAV3 F gene clustered with a contemporary isolate from 1989, isolated from a disease outbreak in the same geographical region (N5/89, Acc. no. AY853957) (Fig. 3B). These two isolates were identical in their HE gene sequences, including their HPR regions, and in their F gene (data not shown), except for a) the amino acid in position 266, which in the contemporary isolate was a leucine (L), as compared to a glutamine (Q) in ISAV3, and b) the presence of an insertion in ISAV3. In fact, in this study the Q_{266} amino acid right upstream of the putative R_{267} cleavage site was only shared between ISAV3 and the low- or avirulent HPR0 genotype, while in all the remaining 10 isolates (ISAV1, 2, 4–11) the amino acid in this position was L_{266} (Fig. 3A). Blast searches of all available whole or partial segment 5 sequences (57 in total) showed either a L_{266}, Q_{266}, or, in one case, H_{266}, at this position in the protein. None of the L_{266}, but all of the Q_{266}/H_{266} containing sequences had an insertion around the R_{267} cleavage site, at position 268 or at position 264 (H_{266} type only), except for one sequence from an isolate, where a Q_{266} was present in an F protein not containing insertion, (SF83/04, Acc. no. AY744392). However, this most likely represents a mixed infection of viruses with HPR0 and that of another HPR genotype, as two accession numbers are assigned for its HE gene, including a full-length HPR0 sequence.

For the isolates ISAV1–11 information on disease susceptibility and induced immune responses was available from a previously performed experimental trial (Mjaaland et al., 2005) while only field data were available for the HPR0 genotype SK779/06, as all efforts to culture this isolate have failed. In attempts to define candidate genes and areas of genes of importance in ISAV virulence, and in lack of a reverse genetics system for this virus, the clinical information was coupled with extended amino acid sequence analyses. These searches revealed that only eight amino acids were unique for the HPR0 isolate (Table 1, Fig. 1). Apart from the variation in the HPR region, there was only a very limited number of overall amino acid sequence variation between the highly virulent isolate ISAV1 and the low/avirulent HPR0 genotype (Table 1), of which only about one third leads to different amino acids.

Similarly, only very few amino acids differed between the high and low-virulent isolates ISAV1 and ISAV11, and between ISAV2 and ISAV9 (Table 1), and these were spread over several gene segments. In these cases, it is therefore tempting to suggest that the variations in the HPR are of vital importance for virulence. On the other hand, the two isolates



ISAV4 and ISAV7 share identical HPRs, but differ significantly in virulence. As they otherwise share relatively few amino acid differences in their genomes (Table 1), this shows that other genes may also be of importance in the virulence of ISAV.

Discussion

In this work we provide evidence shedding light on the mechanisms driving the evolution of ISAV. We present, for the first time, the full-genome sequence of a low/avirulent ISAV isolate not associated with classical ISA clinic or pathology, displaying a full-length hemagglutinin-esterase (HE) gene; the HPR0 genotype. Molecular and phylogenetic sequence analyses of the coding regions of 12 ISAV genomes differing in virulence provide evidence for the role of recombination and reassortment in virulence. In particular, we provide evidence for a new marker for virulence, next to one of the two potential cleavage sites, R_{267}, in the F protein: a Q_{266} (or in one case H_{266}) to L_{266} substitution seems to be a prerequisite for virulence. It is assumed that this single amino acid mutation may alter the recognition site and thereby the type of host proteases that will cleave the F protein, although no obvious switch in protease recognition pattern could be identified *in silico* through analysis of known amino acid patterns recognized by well-characterized proteases. As an alternative to the $Q_{266}/H_{266} \rightarrow L_{266}$ substitution in the acquisition of virulence, our data suggest that an insertion acquired by non-homologous recombination, right upstream or downstream of R_{267} is required, and that this site represents a recombinational hot-spot.

Recombination in the F gene has recently been described in seven ISAV isolates collected from field outbreaks during 2000–2002 (Devold et al., 2006). One isolate displayed an insertion positioned three amino acids upstream of R_{267}, while the six remaining isolates contained insertions at the same position as found in ISAV3, two amino acids downstream of this site. Five of the inserts were identical in sequence, including the presence of Q_{266}, while one isolate had a H_{266}. However, the significance of this in the virulence of ISAV has, for the first time, become evident due to the availability of sequence information of the low- or avirulent HPR0 genotype (SK779/06). We provide evidence suggesting that insertion of a short amino acid stretch in isolates containing Q_{266} or H_{266}, probably resulting in altered amino acid pattern around a putative cleavage site R_{267}, leads to acquisition of virulence. All isolates sequenced till today associated with disease outbreaks carrying Q_{266}/H_{266} contain an insertion, while the Q_{266}-carrying low- or avirulent SK779/06 does not.

The extensive sequence identity related to the recombination event leading to the 10 amino acid insertion at position 268, together with proposed mechanisms of recombination in RNA viruses (reviewed in Nagy and Simon, 1997; Lai, 1992), suggests that a switching of templates during viral replication has occurred. Recombination may have occurred during replication of RNA strands of either polarity, as there are sequence similarities between the 5′ and 3′ end of the insert and both regions flanking the site of insertion. *In silico* RNA structure prediction analyses, using both negative sense (−) and positive sense (+) viral RNA sequences, further suggests that the insertion does not lead to a significant change in the predicted RNA structure, which in turn could have impaired viral replication (data not shown). Finally, phylogenetic analyses of all available F (Fig. 3B) and HE genes (data not shown) revealed that the seven 2000–2002 isolates were neither closely related to nor contemporary with ISAV3, and that identical recombination events have occurred at least twice independently.

The relatively frequent occurrence of recombination in ISAV contrasts with other members of the orthomyxovirus family, where only a few cases of recombination have been documented so far (Khatchikian et al., 1989; Orlich et al., 1994; Hirst et al., 2004; Bergmann et al., 1992; Fields and Winter, 1982; Jennings et al., 1983). Three of these events involve insertions in the HA gene of subtype H7 of influenza A virus, one or two amino acids upstream of the HA1/HA2 cleavage site, and have been associated with increased cleavage rate and pathogenicity (Khatchikian et al., 1989; Orlich et al., 1994; Hirst et al., 2004). The effect of the $Q_{266}/H_{266} \rightarrow L_{266}$ substitution or alternatively, recombination, close to the cleavage site may therefore be analogous to the highly pathogenic influenza A viruses H5 and H7, where pathogenicity is acquired through the mutational change of cleavage specificity from a trypsin-like to furin-like recognition site following species crossover from waterfowl to domesticated poultry (Bosch et al., 1981; Senne et al., 1996). In addition, amino acids downstream of the cleavage site have also been shown to be of importance for cleavability of the HA in influenza A virus, (Horimoto and Kawaoka, 1995). Similar changes have been described in the cleavage site of the F protein of the avian paramyxovirus type 1 causing Newcastle disease in

Fig. 3. Sequence analyses of the recombination in gene segment 5 of ISAV3. A) A 30 nucleotide stretch, encoding 10 amino acids and located in the 5′-part of segment 5 (here illustrated by (+)RNA, although the event may occur on RNA strands of either polarity), was duplicated and inserted in close proximity to the putative cleavage sites of the encoded fusion protein in ISAV3. Sequence homologies between the insert sequence and sequences surrounding the insertion site are shown in the same colour (red or green). Relevant nucleotide positions, encoded amino acids in the one-letter code, and the location of the two putative cleavage sites in the F protein, are all indicated. The amino acid sequence encoded by the insertion sequence is shown in bold and the virulence marker L_{266}/Q_{266} in blue colour. The F gene sequence from the reference isolate ISAV9 is included for comparisons. B) A phylogenetic tree was constructed with maximum likelihood (ML) and the GTR model of nucleotide substitution using multiple sequence alignment of the coding sequences of segment 5 from ISAV1-11, SK779/06 (HPR0 genotype) and 57 other whole or partial segment 5 sequences acquired from GenBank. ISAV9 was used as root, and bootstrap values shown at selected nodes were calculated from 100 replicas using the neighbour-joining method and the F84 model of nucleotide substitution. To avoid gaps at the alignment ends, only the sequence parts stretching from nt 46-1356 in the ORFs were used. All F genes encoding the marker Q_{266}, alternatively H_{266}, are in bold. Two genes without insertions but containing the Q_{266} marker are both in bold and underlined. Sequences having identical insertions to ISAV3 are marked with an asterisk.

poultry (Glickman et al., 1988; Peeters et al., 1999; Gotoh et al., 1992). In ISAV, it is not known whether cleavage of the F protein occurs after R_{267} or K_{276}, the two sites suggested by Aspehaug et al. (2005).

The alterations at the cleavage site of the ISAV F protein, together with deletions in the HE-HPR region probably resulting in altered receptor-binding and -destroying activity, most likely represent viral adaptation to Atlantic salmon leading to disease in densely populated fish farms. This may raise the question whether the reservoir for ISAV, previously suggested to be wild salmonid fish (Plarre et al., 2005; Nylund et al., 2003; Raynard et al., 2001), remains to be identified.

In orthomyxoviruses, reassortment of gene segments occurs frequently and is a major contributor to the evolution of these viruses and the emergence of new virulent strains (Guan et al., 2003; Wallensten et al., 2005; McCullers et al., 2004; Matsuzaki et al., 2003; Jones et al., 1987). The relative contribution of reassortment in the evolution and virulence of ISAV remains elusive. Our data indicate, however, that the ISAV5 genotype is more virulent than those isolates whom it forms monophyletic groups with in several gene segments, although not segment 5 (ISAV8, ISAV10, ISAV11 and SK779/06) (Mjaaland et al., 2005). This may suggest that reassortment of segment 5 has contributed to the virulence of ISAV5. Although amino acid differences present in the other gene segments may contribute to virulence as well, the contribution from gene segment 5 is strengthened by the three encoded amino acid differences between ISAV5 and ISAV8/ISAV10/ISAV11/SK779/06. More importantly, two of these differences are located in close proximity of R_{267}, and N_{263}/S_{263} in particular can be expected to be part of a protease recognition motif with cleavage at position 267. The potential additional involvement in protease recognition and cleavage efficiency of these mutations should therefore not be excluded as a factor influencing the virulence of ISAV5, as compared to the low-virulent isolates.

The limited sequence variability distributed throughout the genomes of the ISAV isolates together with the preferred substitutions to different amino acids, gives extra significance to the changes actually present. Analyses of all protein sequences encoded by SK779/06 and comparing with all ISAVs of the present study, including results from protein blast searches, revealed that only a few amino acid differences encoded in the SK779/06 genome were unique for this isolate. The majority of these were located in the polymerase genes, and their potential involvement in viral replicative efficiency should not be ruled out (Tumpey et al., 2005). Except for the HE-HPR region, relatively few amino acid differences exist between the high virulent isolate ISAV1 and SK779/06, and between several other high and low-virulent isolates. This probably reflects the importance of the HE-HPR region in virulence of these isolates, in addition to the $Q_{266}/H_{266} \rightarrow L_{266}$ substitution in the F protein, which seems to be an absolute prerequisite for virulence. On the other hand, isolates with identical HE-HPR's, like ISAV4 and ISAV7, also vary significantly in virulence, reflected by differences in their ability to induce protective immune responses (Mjaaland et al., 2005). This case clearly illustrates that gene regions other than the HE-HPR determine virulence. The highest percentages of amino acid substitutions, as compared to the number of variable nucleotide sites, were found in gene segments 7 and 8, and most of these where placed in the proteins suggested to be involved in interferon type I antagonism, likely to be involved in virulence as well (McBeath et al., 2006; reviewed in Krug et al., 2003; García-Rosado et al., in press). In conclusion, our results indicate that highly parallel mechanisms for viral adaption and virulence are operating between ISAV and avian influenza A viruses.

Materials and methods

Virus strains

Eleven of the ISAV isolates (ISAV1-11) originate from different regions along the Norwegian coast from the time period 1989–1997 (Mjaaland et al., 2002, 2005), and represent a collection of isolates differing highly in virulence, according to a previously performed experimental trial using standardised experimental fish (half-siblings with identical MHC class I and II genotype) (Mjaaland et al., 2005). With the exception of ISAV7, inducing the lowest overall mortality (2.5%), the numbering in the remaining 10 isolates is directly related to their virulence as determined by their induced mortality; ISAV1 inducing highest mortality (46.8%) and ISAV11 the lowest (3.8%) (Mjaaland et al., 2005). The low virulence of ISAV7 and ISAV11 was most likely due to their efficiency in inducing protective cellular immune responses (Mjaaland et al., 2005). The twelfth isolate (SK779/06), with the low- or avirulent HPR0 genotype, was detected in 2006 by RT-PCR of tissues from farmed Atlantic salmon located in a farm with no classical signs of ISA, although suffering from signs of proliferative gill inflammation. Of the twenty-nine fish tested from this population, only one tested ISAV (HPR0) positive.

Viral RNA isolation

For isolation of RNA from the ISAV1-11 isolates, Atlantic salmon kidney tissue from ISAV-diseased fish was homogenized and used directly for inoculation onto salmon head kidney cells (SHK-1) as described earlier (Dannevig et al., 1995; Mjaaland et al., 2002). Culture supernatants were passed two or three times. Maintaining (passaging) ISAV in a salmon cell line for longer periods of time (up to a year), in between introducing repeated freeze/thaw cycles has shown that the virus is very resilient to mutational change in cell culture over time, as monitored by its HE sequence (unpublished results from our laboratory using ISAV4, the Norwegian reference isolate). In addition, all isolates in this study, except for the HPR0 genotype (SK779/06), had all been maintained in SHK-1 cells and passaged 2–3 times prior to usage in an experimental trial (see previous subsection) (Mjaaland et al., 2005). Viral RNA was extracted from the final supernatants by using the QIAmp Viral RNA Mini Kit (Qiagen). Due to the very low amount of viral

RNA present in the HPR0-positive fish, and the lack of success in attempts to culture this strain due to its avirulent/low-virulent nature, total RNA was isolated directly from fish tissue using RNeasy Mini Kit (Qiagen).

RT-PCR, cloning and sequencing

All viral gene segments were amplified by a two-step RT-PCR. First-strand cDNA synthesis was carried out using segment specific forward primers (Table S1) or random hexamers together with Superscript III reverse transcriptase (Invitrogen). For the PCR reaction, 1–11 µl cDNA, segment specific primers and Easy-A High Fidelity PCR Cloning Enzyme (Stratagene) were used producing complete ORF's including initiation and stopcodons (Table S1). For most segments also parts of the 5′ and 3′ non-translated regions were amplified. PCR cycling conditions were 95 °C/2 min, followed by 35–38 cycles of 95 °C/40 s, 50 or 55 °C/30 s and 72 °C/1–2.5 min followed by a final extension step at 72 °C/7 min. PCR products were resolved by agarose gel electrophoresis, visualized by ethidium bromide staining, purified using QIAquick Gel Extraction Kit (Qiagen) and cloned into pCR2.1-TOPO/pCR4-TOPO according to the manufacturer's protocol (Invitrogen). Plasmids from 3–6 positive clones per isolate were isolated from bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen). Sequencing was performed either by using an ABI PRISM Big Dye Terminator Cycle Sequencing kit in an automatic sequencer (ABI Prism 377), by capillary sequencing using the MegaBACE DYEnamic ET dye terminator kit (MegaBACE 500) (Amersham), or plasmid DNA was sent to GATC-Biotech AG (Konstanz, DE). If the sequencing data from the selected clones proved inconclusive, additional clones were selected for sequencing.

Due to the very low amounts of viral RNA present in the HPR0-positive fish tissues, the only way to obtain the complete coding sequences of the SK779/06 HPR0 isolate was to produce small overlapping PCR fragments, ranging from 287 bp to 804 bp in size (Table S1). Moreover, to obtain sufficient amounts of PCR product needed for sequencing, nested PCR was performed on each of the reactions. The two-step RT-PCR and agarose gel purification were performed as described above. Nested PCR was performed on 1–2 µl of purified PCR product from the first PCR reaction. PCR cycling conditions were as described above, except for an annealing temperature of 52 °C, and 40 amplification cycles. In a number of cases PCRs were repeated with cDNAs from separate RT-reactions for further sequence verification. Purified nested PCR products were sent to GATC-Biotech AG for sequencing.

Phylogenetic analyses

Multiple sequence alignments of the coding regions of all the eight ISAV gene segments were performed in AlignX (Vector NTI Advance™ 9 package, InforMax, Inc.) and BioEdit (Tom Hall, Ibis Therapeutics). Phylogenetic analyses were performed by maximum likelihood (ML) using the program PHYML (Guindon and Gascuel, 2003) (available at http://phylemon.bioinfo.cipf.es/cgi-bin/phyml.cgi). FindModel (http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html) (developed from a Modeltest script, by Posada and Crandall, 1998), together with pair wise comparisons of nucleotide substitution models using likelihood ratio tests (LRTs), was used to determine the most appropriate model. As a consequence, the GTR model was chosen in all phylogenetic analyses, as it produced the highest likelihood scores (lnL values) of all the substitution models available in PHYML, being significantly better than the other models in LRT for some of the segments. Bootstrap values in the trees were calculated from neighbour-joining analyses and 100 replicas using the programs seqboot, dnadist and neighbour (F84) implemented in the PHYLIP 3.65 package (Felsenstein, 2005). All trees were drawn using MEGA4 software (Tamura et al., 2007) (available at http://www.megasoftware.net/) and rooted to the reference isolate ISAV9 whose genomic sequence is highly similar to the consensus sequence from multiple sequence alignment of all coding regions in the genomes of the 12 ISAV isolates. The HPR region in segment 6 which lies between positions 985 and 1114 when aligned according to the HPR0 genotype (SK779/06), and a 30-nucleotide insert only present in segment 5 of ISAV3, was excluded from the sequence alignments as adequate evolutionary models for deletions or insertions are not available and therefore not included in software for phylogenetic analyses. Mostly, bootstrap values set above 80 were used as cut-off value to indicate close relationship between virus isolates. In some cases though, due to the highly conserved nature of the ISAV genome, lower values combined with detailed sequence analysis was used to depict common origins between gene segments.

GenBank accession numbers

The nucleotide sequences obtained in this study have the following accession numbers in GenBank: DQ785175–DQ785286 and EU118815–EU118822.

Computer analyses

Protein blast searches (http://www.ncbi.nlm.nih.gov/BLAST/) and nucleotide blast (blastn) searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted using ISAV9 coding sequences as query. RNA secondary structure predictions of viral (−)RNA and (+)RNA were performed using the Mfold program (version 3.2) (Zuker, 2003; Walter et al., 1994). Default parameters were used in the predictions with the exception of temperature, which was set to 15 °C. PeptideCutter (http://au.expasy.org/tools/peptidecutter/) was used in the search for potential protease cleavage sites or motives in the fusion protein (Gasteiger et al., 2003).

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Appendix A

Table S1
Primers used to amplify the open reading frames (ORF's) from the genomes of ISAV1 to ISAV11 and SK779/06 (HPR0 genotype)

Genomic segments and gene product(s)	Primer name	Primer sequence (5′–3′) and binding sites relative to ORF's
Segment 1 (PB2)	ORFseg1-F[a]	**C$_{(-9)}$AGCTAAGAATGGACTTTATATCAG**
	ORFseg1-R[a]	**G$_{(+24)}$CAATATTGACCAACACTCA**
	s1F[b]	CACC**ATGGACTTTATATCAGAAAACAC**
	s1IntR[b]	**CACCACCTTCTGAGACATGTTGAT**
	s1IntF[b]	**CAACTACCGAGGAGTACTGGAATGT**
	s1IntR2[b]	**GTGTGTGTTCTCAGGCTTTCCA**
	s1IntF2[b]	**GTCAAGAACACGTCAATAGACAGGAT**
	s1IntR3[b]	**TTATGTCCACCCCTGTGTTTTCT**
	s1IntF3[b]	**AGAGTCTGTACGAAAAGGCAACCT**
	s1IntR4[b]	**CTACCTGTTGCCATCTTCTCCAT**
	s1IntF4[b]	**GGAGAGGTGTATGAAGAGAGGTCAAA**
	s1R[b]	**TTAAACACCATATTCATCCATCAGGT**
Segment 2 (PB1)	ORFseg2-F[a]	**C$_{(-15)}$GCTCTTTAATAACCATGGAAAC**
	ORFseg2-R[a]	**G$_{(+24)}$GGTATATACACCATCACAC**
	s2F[b]	CACC**ATGGAAACTCTAGTAGGTGGG**
	s2IntR[b]	**GTTTCCATATGTATCCTTTCACTTCTTGT**
	s2IntF[b]	**CAGAGGAAGAACCATGTCAGACC**
	s2IntR2[b]	**GCTACTGGTTTGACACTTTTTTGAGA**
	s2IntF2[b]	**TTTCCTAAGAGAACTTGGGATAAAGG**
	s2IntR3[b]	**TAAGAGCCATTTCGAGTCTTTGTCTT**
	s2IntF3[b]	**CAGTTTCACACCTGAGGCAGTGTA**
	s2IntR4[b]	**CTTTGAGTGCAATTCCATCGAAA**
	s2IntF4[b]	**AGAGACAATGTCTATACCTGAGAGCAT**
	s2R[b]	**TCAAACATGCTTTTTCTTCTTAATCA**
Segment 3 (NP)	ORFseg3-F[a]	**C$_{(-10)}$GTATAAGAGATGGCCGATA**
	ORFseg3-R[a]	**C$_{(+77)}$CCAAACCCAATTATATCATAG**
	s3F[b]	CACC**ATGGCCGATAAAGGTATG**
	s3IntR[b]	**TTGCCTCTTTCCGTCTTCATC**
	s3IntF[b]	**AAGGTGAAATGAGCATCTGG**
	s3IntR2[b]	**CTGGTCTGCTGACAACACTGACT**
	s3IntF2[b]	**AGGATCAAAGGCAACTGAATCAAG**
	s3IntR3[b]	**ACACATGTTCTAGCCACAGACATT**
	s3IntF3[b]	**TCAGTGTTGGCAATGGACTTCAT**
	s3IntR4[b]	**CCTGATAGCGCCTTGGTTGA**
	s3IntF4[b]	**GCTGAGGCTGTCCAATGTGAA**
	s3R[b]	**TTTCAAATGTCAGTGTCTTCCTCCT**
Segment 4 (PA)	ORFseg4-F[a]	**C$_{(-4)}$AAGATGGATAACCTCCGTG**
	ORFseg4-R[a]	**C$_{(+1)}$TCATTGGGTACTGACTGCA**
	s4F[b]	CACC**ATGGATAACCTCCGTGAA**
	s4IntR[b]	**TGATTGATCTTCATTGGTATTCCAT**
	s4IntF[b]	**GGGTCTAGTCGAAACAGGAGTT**
	s4IntR2[b]	**AGCAGCAATTCTTGCAGGAAGT**
	s4IntF2[b]	**CGCAGTTCTGTGAAATTGATGAA**
	s4IntR3[b]	**AGTTGCTGTGCCATCCAGTC**
	s4IntF3[b]	**GGAGACACAAGAAAGGAAGGATACT**
	s4R[b]	**TTATTGGGTACTGACTGCAATTTTC**
Segment 5 (F)	ORFseg5-F[a]	**A$_{(+1)}$TGGCTTTTCTAACAATTTTA**
	ORFseg5-R[a]	**T$_{(0)}$TATCTCCTAATGCATCCC**
	s5F[b]	CACC**ATGGCTTTTCTAACAATTT**
	s5IntR[b]	**CCCTACCAAGGACAACATCATGTCG**
	s5IntF[b]	**GAAGCAGACTTGAGGATTCA**
	s5IntR2[b]	**TTCACCTTGTTGATCTGGTCTA**
	s5IntF2[b]	**AGCATCCGCAGAAGATGTGAA**
	s5R[b]	**TTATCTTCTAATGCATCCCCACAG**
Segment 6 (HE)	ORFseg6-F[a]	GGGCTAGCA**$_{(+1)}$TGGCACGATTCATAATT**
	ORFseg6-R[a]	GGGGTACCGTA**$_{(-3)}$GCAACAGACAGGCTCGAT**
	s6F[b]	CACC**ATGGCACGATTCATAATTT**
	s6IntR[b]	**CTTCAAAGGTGTCTGACACGTA**
	s6IntF[b]	**GGAATCTACAAGGTCTGCATTG**
	s6R[b]	**TTAAGCAACAGACAGGCTCGATG**

Table S1 (*continued*)

Genomic segments and gene product(s)	Primer name	Primer sequence (5′–3′) and binding sites relative to ORF's
Segment 7 ORF1 (non-structural)	Seg7-F[a]	TATTGGTCTCAGGGA$_{(-21)}$**GCTAAGATTCTCCT**
	Seg7-R[a]	ATATGGTCTCGTATTA$_{(-100)}$**GTAAAAATTCTCCTTTT**
Segment 7 ORF2 (putative nuclear export protein)	s7F[b]	CACC**ATGGATTTCACCAAAGTGTA**
	s7IntR[b]	**GAAAATCCATGTTCTCAGATGCAA**
	s7IntF[b]	**TGGGATCATGTGTTTCCTGCTA**
	s7R[b]	**TTAATTCTCATTACAAATGTATTTTTC**
Segment 8 ORF1 (M protein)	Seg8-F[a]	TATTCGTCTCAGGGA$_{(-21)}$**GCAAAGATTGGCTA**
	Seg8-R[a]	ATATCGTCTCGTATTA$_{(+145)}$**GTAAAAAAAGGCTTTTT**
Segment 8 ORF2 (structural protein)	s8F[b]	CACC**ATGAACGAATCACAATGGA**
	s8IntR[b]	**GTAGCATTGTCTTCAGGTCCTTCAA**
	s8IntF[b]	**AAAGTGGGCAATGGTGTATGGTA**
	s8R[b]	**TTATTGTACAGAGTCTTCCAATTGGTC**

[a]Primers amplifying the complete ORFs of ISAV1–ISAV11 using two-step RT-PCR. The majority of these primers are wholly or partially located to the non-translated regions of each gene segment, and the position numbering (subscript numbers) is for forward primers relative to coding sequence$_{(start)}$, and for reverse primers to coding sequence$_{(end)}$.

[b]Primers used to amplify the ORFs from SK779/06 (HPR0 genotype). All bind to translated regions and were designed to produce small overlapping fragments of coding sequence ranging from 287 bp to 804 bp in size. Amplification was performed using two-step RT-PCR, followed by nested PCR. For all primers the segment specific sequences are shown in bold.

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