COMPARISON OF THE HAEMAGGLUTININ GENOTYPE WITH THE ANTIGENIC TYPE OF INFECTIOUS SALMON ANAEMIA VIRUS

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ABSTRACT

Infectious salmon anaemia (ISA) has been recognized as affecting salt-water farmed Atlantic salmon, *Salmo salar*, in Norway since 1984. The first disease outbreak outside of Norway occurred in the Bay of Fundy, New Brunswick, Canada, in 1996 and has since occured in Scotland, the USA and Denmark. The goal of this study was to further investigate the haemagglutinin (HA) genotype of infectious salmon anaemia virus (ISAV) in relation to its antigenic type. It was hypothesized that (a) ISAV, being an orthomyxovirus-like virus, may have similar characteristics to other members of *Orthomyxoviridae*; (b) genetic variation in the HA gene of ISAV has led to two antigenically distinct isolates and may be responsible for pathogenic differences; and (c) the CHSE-214 cell line may give a better assessment of virus neutralization than the TO cell line. The nucleotide and amino acid sequences of the HA gene of 62 ISAV isolates encompassing different geographical regions were compared and found to belong to two genotypes: a North American genotype and a European genotype. The two HA genotypes had nucleotide sequence identities of $\leq$80.4% and amino acid sequence identities of $\leq$88.2% whereas within each genotype, both sequence identities were 90.7% or higher. The amino acid sequence alignment of all isolates revealed one conserved *N*-glycosylation site, previously identified at position 333 to 335. There were two major mutation sites found between the two genotypes at amino acid positions 320 to 323 and positions 338 to 367, the latter being a highly polymorphic region. In order to correlate the ISAV genotype to the antigenic type, a virus interference system in the CHSE-214 cell line

was investigated for use in serotyping ISAV isolates. This system further indicated that the North American ISAV genotype is antigenically distinct from the European ISAV genotype. Antisera raised to the North American genotype had high neutralizing titres to isolates of that genotype, while antisera raised to the European genotype had low neutralizing titres to isolates of the North American genotype. In conclusion, this study supports the original observation that the two HA genotypes of ISAV do differ antigenically.

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COMMONLY USED ABBREVIATIONS

Term	Abbreviation
Standard units of measurement	
amplitude	Amp
base pair	bp
degree Celsius	°C
equal to or greater then	≥
equal to or less then	≤
kilo base	Kb
kilo Dalton	kDa
molarity	M
microCurie	mCi
microgram	µg
microliter	µL
milliamplitude	mAmps
milliliter	mL
millimole	mM
nanometer	nm
normal (concentration)	N
revolutions per minute	rpm
room temperature	RT
ultraviolet	UV
watt	W
Metal ions and compounds	
distilled deionized water	dd H_2O
ethylene diamine tetra acetate	EDTA

phosphate buffered saline with antibiotics	PBS 'A'
sodium hydroxide	NaOH
tris, boric acid, EDTA buffer	TBE
tris, EDTA buffer	TE
tris buffered hydrochloric acid	Tris-HCl
radioactive phosphate isotope	^{32}P
radioactive sulphur isotope	^{35}S

Other

adenine	A
alpha	α
beta	β
Chinook salmon embryo cell line	CHSE-214
complimentary DNA	cDNA
cytopathic effect	CPE
cytosine	C
deoxyribonucleic acid	DNA
deoxynucleotide triphosphate	dNTP
diadenosine triphosphate	dATP
dideoxynucleotide triphosphate	ddNTP
Fc receptor	FcR
fetal bovine serum	FBS
five prime	5'
guanine	G
haemagglutinin protein (*Influenzavirus A*, *Influenzavirus B*, *Isavirus*)	HA
haemagglutinin protein (*Influenzavirus C*)	HEF

head kidney leucocyte cell line	TO
highly polymorphic region	HPR
hydrogen ion activity (negative logarithm)	pH
infectious haematopoietic necrosis virus	IHNV
infectious pancreatic necrosis virus	IPNV
infectious salmon anaemia	ISA
infectious salmon anaemia virus	ISAV
infective dose	$TCID_{50}$
neuraminidase protein	NA
matrix proteins	M1, M2
monoclonal antibody	MAb
non-structural protein	NS1
nuclear export protein	NEP
nucleoprotein	NP
open reading frame	ORF
percent	%
polymerase chain reaction	PCR
polymerase proteins	PB1, PB2, PA
post infection	p.i.
receptor destroying enzyme	RDE
ribonucleic acid	RNA
ribonuclease	RNase
restriction fragment length polymorphism	RFLP
reverse transcriptase-polymerase chain reaction	RT-PCR
salmon head kidney cell line	SHK-1
Thermus aquaticus	*Taq*
three prime	3'

thymine	T
virus neutralization	VN

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 History of Infectious Salmon Anaemia

The infectious salmon anaemia virus (ISAV) has been infecting farmed Atlantic salmon, *Salmo salar*, in New Brunswick, Canada, since the late 1990s. In the summer of 1996, an apparently new disease to New Brunswick, Canada, caused high mortalities in salt-water farmed Atlantic salmon (Bouchard et al., 1999). The disease was diagnosed as haemorraghic kidney syndrome (HKS) although it had similar characteristics to those of infectious salmon anaemia (ISA), a disease first recorded in Norway in 1984 (Nylund et al., 1997). However, failure to isolate any bacterial, parasitic or viral agent from the tissues of dead fish hampered proper diagnosis (Jones and MacKinnon, 1999). Later in 1997, fish suffering from HKS were diagnosed as having ISA (Lovely et al., 1999).

Since the occurrence of ISA in Canada, the virus has been identified in Nova Scotia (Hatt, 1999; Ritchie et al., 2001b), Scotland (Rodger et al., 1998), the Faroe Islands, Denmark (Anon., 2000), and Maine, USA (Bouchard et al., 2001; Clouthier et al., 2002). The virus has also been detected in farmed rainbow trout, *Onchorynchus mykiss*, in Ireland (Anon., 2002) and Coho salmon, *Onchorynchus kisutch*, in Chile (Kibenge et al., 2001a). It has been reported that in 1999, ISA outbreaks have cost the Norwegian aquaculture industry $11 Million (U.S. dollars), the Canadian aquaculture industry $14 Million and the 1998-1999 outbreak season in Scotland a reported $32 Million (Hastings et al., 1999).

1.2 Pathogenesis of ISAV infection

ISAV is naturally transmitted horizontally and not vertically, although passive transfer from infected egg debris to hatching fry can occur (Melville and Griffiths, 1999). ISAV has also been shown to be transmitted experimentally by the following ways: (a) cohabitation of diseased fish with healthy fish and (b) injection of liver homogenate from infected fish into healthy fish (Evensen et al., 1991). ISAV has been shown to spread horizontally from fish to fish by the shedding of virions in bodily fluids such as blood, gut contents, epidermal mucus and urine from infected fish (Totland et al., 1996; Rolland and Nylund, 1998). Epidemiological studies have shown that factors such as cohabitation of healthy fish with infected live fish, exposure to animal wastes and slaughter processing plant effluent (Vägsholm et al., 1994) as well as the movement of well boats in amongst ISAV infected sites (Murray et al., 2002) have contributed to the spread of ISA. Although the spread of ISAV between net pens is usually slow (Rimstad and Mjaaland, 2002), it has been recommended that net pen sites be spaced no less than 5-6 km apart, and that waste waters from fish slaughter houses be throughly disinfected (Jarp and Karlsen, 1997). Salmon lice, *Lepeophtheirus salmonis*, have also been suggested to play a role in the dissemination of ISA (Rolland and Nylund, 1998). Fish suffering from clinical ISA display pale gills, ascites, petechiae in the visceral fat, congestion of the liver and spleen, severe anaemia, internal haemorraghing and lesions of the liver and kidney; mortality levels range from less than 5 % to 100% of the experimentally infected fish population (Evensen et al., 1991; Byrne et al., 1998; Rimstad et al.,

1999; Opitz et al., 2000).

ISAV causes the endothelial cells lining the blood vessels to lose integrity, which may explain the haemorraghic lesions and high mortality rate associated with the virus (Nylund et al., 1995b; Nylund et al., 1997). From haematological examinations, a great reduction in the number of circulating leucocytes and erythrocytes along with higher than normal numbers of immature erythrocytes have been noted (Evensen et al., 1991). The observation that intact virus particles are present in leucocytes suggests that these cells are potential targets for the virus (Nylund et al., 1995b). The cellular receptor for ISAV is not presently known (Rimstad and Mjaaland, 2002). Falk et al. (1997) were able to demonstrate that (from the staining of ISAV-infected SHK-1 cells with polyclonal rabbit anti-ISAV serum) there was a prominent nuclear presence of ISAV antigens after 24 hours, post infection. It is possible, therefore, that replication of the viral genome and transcription take place in the nucleus, as in the case of orthomyxoviruses (Rimstad and Mjaaland, 2002). A low-pH dependent fusion of ISAV with endosomal membranes has been demonstrated, which is analogous to the initial replication steps of the influenza viruses (Eliassen et al., 2000). ISAV is relatively short lived in the environment, persisting for approximately 20 hours at 6°C, but can survive in tissues for up to 4 days at the same temperature (Nylund et al., 1994a).

The existence of reservoir hosts for ISAV includes other salmonids, such as brown trout, *Salmo trutta*, and rainbow trout. ISAV can propagate without causing clinical disease in brown trout and rainbow trout (Devold et al, 2000; Nylund et al., 1995a; Nylund et al., 1994b). Nylund et al. (1997) found that after ISAV had been

experimentally injected into rainbow trout, the fish showed only petechiae on the liver upon necropsy. Ascites was collected from a sampling of the infected rainbow trout and it was given to Atlantic salmon by an intraperitoneal injection (Nylund et al., 1997). All challenged Atlantic salmon died and showed typical signs of ISA (Nylund et al., 1997). Snow et al. (2001) have shown that ISAV is cleared from infected Arctic char, *Salvelinus alpinus*, at a much quicker rate as opposed to infected rainbow trout and brown trout, indicating that Arctic char are a less likely candidate as a reservoir host. Snow et al. (2001) also reported that all three salmonids were still RT-PCR positive for the virus at 40 days post infection. Nylund et al. (1995c) have reported that there was a greater overall resistance to ISA in two strains of wild Atlantic salmon as compared to a commercial aquaculture strain, indicating the possibility of ISA originating from wild stock. As the mortality rate of experimentally infected Atlantic salmon ranges from 5 to 100%, Atlantic salmon that survive the disease could be considered reservoir hosts, as there is a possibility that survivor fish could shed the virus for a period of time.

1.3 Characteristics of ISAV

ISAV particles are pleomorphic, but for the most part are spherical in shape with a mean diameter of 90-130nm (Falk et al., 1997; Sommer and Mennen, 1997). The virus particles have an envelope with surface glycoprotein spikes approximately 10nm long (Falk et al., 1997; Sommer and Mennen, 1997). The genome of ISAV is single stranded RNA with eight segments of negative polarity (Krossøy et al., 1999). Since ISAV and the influenza viruses share similarities, ISAV has been

included in the family *Orthomyxoviridae* and belongs to a new genus *Isavirus* (Anon., 2001) (Appendix A). It has been shown that 15°C is the optimal temperature for the virus to replicate and will not replicate at temperatures greater than 25°C (Falk et al., 1997).

1.4 Classification of ISAV

Since ISAV has a close resemblance to the Influenza A viruses, it had been thought that the genome segments might code for similar proteins. Several ISAV isolates from different geographical regions have been either fully or partially sequenced (in that all RNA segments are known) with the resulting information available in the GenBank database (Appendix B). Genome segment 1 encodes the putative polymerase protein, PB2 (Clouthier et al., 2002; Snow et al., 2003). Genome segment 2 of ISAV encodes the polymerase protein , PB1 (Blake et al., 1999; Krossøy et al., 1999) while genome segment 3 encodes the nucleoprotein, NP (Snow and Cunningham, 2001; Ritchie et al., 2001a; Clouthier et al., 2002). Segment 4 encodes the putative polymerase protein, PA (Ritchie et al., 2001a; Clouthier et al., 2002) and segment 5 encodes the putative receptor destroying enzyme, RDE (Clouthier et al., 2002) recently characterized as an acetylesterase (Kristiansen et al., 2002). Genome segment 6 encodes the haemagglutinin protein (Rimstad et al., 2001; Krossøy et al., 2001a; Griffiths et al., 2001). Genome segment 7 encodes the putative membrane proteins, M1 and M2 (Ritchie et al., 2002; Clouthier et al., 2002) and genome segment 8 encodes the putative non-structural proteins, NS1 and NEP (Suarez and Perdue, 1998; Blake et al., 1999;

Krossøy et al., 1999). However, work reported by Biering et al. (2002) suggests that genome segment 7 encodes a non-structural or minor structural protein and genome segment 8 encodes the 24 kDa major structural protein.

Both the Influenza A and B viruses have surface glycoprotein spikes called haemagglutinin (HA) and neuraminidase (NA) (Ito et al., 1991). ISAV has similar spikes, which are mushroom shaped and slightly shorter than the ones found on the surface of the Influenza A viruses (Falk et al., 1997). According to Falk et al. 66(1997), ISAV Glesvaer strain showed four major polypeptides with molecular weights of 71, 53, 43 and 24 kDa. Kibenge et al. (2000) found a similar protein profile for the 'Back Bay 98' and 'RPC/NB-049' ISAV isolates with molecular weights of 74, 53, 43 and 26.5 kDa whereas ISAV isolate Glesvaer had polypeptides of 71, 53, 46 and 26.5 kDa. Several investigators (Rimstad et al., 2001; Krossøy et al.; 2001a; Griffiths et al., 2001) have reported that the HA protein of ISAV is encoded by genome segment 6 and has a molecular mass of 42.4 kDa corresponding to the 43/46 kDa polypeptide reported by Falk et al. (1997) and Kibenge et al. (2000). Comparison of the ISAV HA sequence to selected viruses from the four established genera of the family *Orthomyxoviridae*: *Influenzavirus A, Influenzavirus B, Influenzavirus C* and *Thogotovirus* showed no nucleotide sequence identities between ISAV and the other orthomyxoviruses (Kibenge et al., 2001b). However, at the amino acid level, there was some identity ranging 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* (Kibenge et al., 2001b).

1.5 Genetic variation of orthomyxoviruses

In contrast to ISAV, the HA protein of Influenza viruses A and B is encoded by genome segment 4 (Ito, et al., 1991). The HA protein is a transmembrane glycoprotein (Tong, et al., 1998) and functions as (a) a ligand to bind to the receptor on the host cell surface (a glycoconjugate terminating in N-acetyl neuraminic acid) (Wiley and Shekel, 1987) and (b) to mediate the fusion of the viral envelope and intracellular membrane in low pH conditions (Tong, et al., 1998) allowing viral RNA entry into the cytoplasm. In order for fusion to occur, the HA must first undergo proteolytic cleavage (Kaverin, et al., 1996). The HA protein is a homotrimer consisting of the receptor binding HA1 monotrimer and the fusion HA2 monotrimer (Chen, et al., 1995). The HA protein of ISAV, however, does not undergo proteolytic cleavage (Krossøy et al., 2001). As it is involved with initial entry into the host cell, the influenza HA protein provokes the host to produce neutralizing antibodies against the virus (Smirnov et al., 1999) more specifically HA1 (Wiley and Skehel, 1987), and thus forms the basis for vaccines against influenza. It has also been suggested that perhaps the highly conserved antigenic sites on the different HA molecules may induce neutralizing antibodies to different subtypes of the influenza viruses (Smirnov et al., 1999).

The HA protein of the Influenza A viruses occurs in 15 different types (H1-H15) (Skehel and Wiley, 2000). If an animal cell is inoculated at the same time with two viruses with different HA subtypes, there may be an exchange or reassortment of the HA-coding RNA segment resulting in what is termed antigenic shift (Krystal, et al., 1983). Also, the HA genes within the subgroups themselves can undergo

mutations, resulting in what is known as antigenic drift (Krystal, et al., 1983). For example, during the antigenic drifts of the influenza viruses of the Hong Kong pandemic isolated between 1968 and 1999, the HA genes had an amino acid substitution rate of approximately 3.5 residues per year (Skehel and Wiley, 2000). More than half of these substitutions have been retained in viruses isolated in the subsequent years (Skehel and Wiley, 2000). A study performed by Karasin et al. (2000) found that, through phylogenetic analyses, the 1977 Colorado isolates and 1997 Ontario isolates of H3N2 from pigs, were wholly human influenza A viruses. However, influenza viruses isolated since 1998 from pigs in the mid-western USA have been found to be reassortments of human, swine and avian viruses, suggesting that the different HAs can be transmitted between different animal species (Karasin et al., 2000). The HA of Influenza B viruses also exhibit variation, although not as extensively as for the Influenza A viruses (Klenk et al., 1995). Influenza C viruses have a HEF protein which has the dual purpose of a fusion protein and a receptor-destroying enzyme (acetylesterase) (Klenk et al., 1995) and shares some characteristics of the acetylesterase of the ISA virus (Kristiansen et al., 2002). The NA protein of the Influenza A viruses occurs in 9 different types (N1-N9), with less variation occurring in Influenza B viruses (Klenk et al., 1995). Thogotoviruses have a single glycoprotein (encoded by genome segment 4) which shows no similarity to any influenza protein and has no apparent antigenic properties (Klenk et al., 1995; Kuno et al., 2001). Since the influenza virus is an RNA virus, subject to higher mutation rates than DNA viruses (RNA polymerases lack the proofreading ability) and because the HA protein is a surface glycoprotein

(which is exposed to antibodies), a high degree of antigenic variation can occur (Krystal, et al., 1983). This ability to alter the antigenic nature of the viral proteins results in the ability of the virus to reinfect hosts that are otherwise immune to the previous phenotype.

The HA gene of the influenza viruses is under constant evolutionary pressure to escape neutralization by the host. As such, the antigenic variation of the influenza viruses is largely due to the variations in the conformation of the HA protein (Wiley and Shekel, 1987) driven by the neutralizing antibodies of the immune responses of the host (Lambkin and Dimmock, 1995). For influenza viruses, the HA receptor-binding site is a "pocket" located on the C-terminal end of the HA protein consisting of highly conserved amino acid residues with high homology among numerous strains of the influenza virus (Wiley and Shekel, 1987). It has been found that neutralizing antibody sites surround the receptor-binding pocket on the membrane-distal surface of the HA protein (Shekel and Wiley, 2000) and that there are as many as five antigenic sites on the HA gene of the influenza A virus, each composed of many non-overlapping epitopes (Lambkin and Dimmock, 1995). The proximity of the antibody-binding sites and the receptor-binding pocket are such that certain amino acid substitutions can influence the binding of the antibodies and the specificity of the receptor recognition (Wiley and Shekel, 1987). There are two features of the receptor binding pockets that make them antigenic, the loop-like structure of several of them and the attachment of carbohydrate side chains (Shekel and Wiley, 2000). The attachment of the carbohydrate side chains influences viral neutralization in two ways: (a) the carbohydrate side chains can be

derived from cellular enzymes, rendering the viral protein difficult to recognise as foreign and (b) during antigenic drift, amino acid substitutions lead to new carbohydrate attachment sites which lead to the abrogation of previous antibody binding sites (Shekel and Wiley, 2000). For example, two equine influenza virus strains isolated in Nigeria were found to be antigenically distinct from the European influenza counterparts as the result of amino acid substitutions in an antigenic site (Adeyefa et al., 1996). Other examples are: (a) a mutant mouse strain of influenza was found to have a increased pathogenicity than its wild-type counterpart due to amino acid substitutions (Smeek et al., 1996) and (b) amino acid substitutions were also responsible for an increase in pathogenicity in an avian influenza strain (Perdue et al., 1999).

1.6 Genetic variation of ISAV

The genetic variation of the different strains of ISAV is not fully known. Blake et al. (1999) found that through a comparison of partial cDNA nucleotide sequences of genome segments 2 and 8 Norwegian and Canadian isolates the variations were limited to one or two nucleotides substitutions. The authors suggested that the Canadian isolates of ISAV may be distinct mutants of the Norwegian isolates since the differences between the two groups were small (Blake et al., 1999). Kibenge et al. (2000) found further evidence that Canadian isolates of ISAV were different from the European isolates by using reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) on RNA segment 2. Snow and Cunningham (2000) compared genome segments 2 and 8 through phylogenetic analysis from Scottish isolates to the previously published

Norwegian and Canadian isolates and found that the Scottish isolates were more closely related to the isolates from Norway. Inglis et al. (2000) and Krossøy et al. (2001b) also confirmed these observations. Ritchie et al. (2001b) reported sequences from ISAV genome segment 8 from Atlantic salmon in Nova Scotia that were more homologous to Norwegian and Scottish isolates than to the New Brunswick isolate (>99% vs 89% at the nucleotide sequence level; >98% vs 80.5% at the amino acid sequence level).

A monoclonal antibody (MAb 3H6F8), produced by Falk et al. (1997) inhibited haemagglutination by virus, neutralized the virus in cell culture and could bind to the surface of ISAV particles in immune electron microscopy reactions. Krossøy et al. (2001a) were able to show that recombinant baculovirus-infected insect cells (containing the putative HA protein) had adsorptive properties for erythrocytes from Atlantic salmon, but not from brown trout. This is in accordance with the haemagglutinating properties of ISAV. Presently, the cellular receptor for ISAV is unknown. The HA of the influenza viruses attaches to sialoglycoproteins found on the surface of the permissive cells (Wiley and Shekel 1987) and as it has been found that there are certain types of sialoglycoproteins in salmon (Iwasaki et al., 1990). Kristiansen et al. (2002) demonstrated that ISAV was able to agglutinate mammalian (horse) erythrocytes along with trout and salmon erythrocytes. Following a preincubation of ISAV with 1 mM di-isopropyl fluorophosphate, a complete inhibition of elution occured for the trout and horse erythocytes indicating that the acetylesterase is the receptor destroying enzyme as the enzyme is required for the release of virions (Kristiansen et al., 2002). Since horse erythrocytes contain

only *N*-glycol neuraminic acid, this may be the cellular receptor for the virus (Kristiansen et al., 2002).

From an amino acid sequence alignment, Krossøy et al. (2001a) indicated a highly polymorphic region (HPR) between 5 isolates from Norway, 1 from Scotland and 1 from New Brunswick, Canada, that the authors suggested could be a useful epidemiological marker. This HPR is in close proximity to the predicted membrane-spanning region and may have a role in pathogenic variation among ISAV isolates. Devold et al. (2001) studied 30 isolates from Norway, collected from 1987 to 2001. In this study, the authors also included the previous 7 isolates from Krossøy et al. (2001a). A manual comparison of the HPR of the HA gene before 1993 showed that there were two HPRs. The 37 isolates were grouped phylogenically into two groups based on a full-length comparison of the HA gene. A comparison of the later isolates gave a clear indication of possible recombination events involving the two previous HPRs. Another phylogeny comparison based on possible recombination events resulted in the 11 HPR groups. The HPR groups (3, 4, 5, 6, 9, 10 and 11) always had amino acid sequences from the HPR2 group at the amino end and an amino acid sequence from the HPR1 group at the carboxyl end. Deletion of specific amino acid could be used to explain the existence of HPR7 and HPR8: a loss of 2 amino acids from HPR1 resulted in HPR7, while a 3 amino acid loss from HPR2 resulted in HPR8. In addition to possible recombination events within the HPRs, the authors also indicated possible recombination events in the 5' end of the flanking region close to the HPR. The authors concluded that when recombination occurred, for these 37 isolates, it always resulted in a HPR1-

associated 3' end and a HPR2-associated 5' end. The authors concluded that the different HPR groups were excellent examples of antigenic drift and could indeed help explain the epidemiology of the virus in Norway. However, evaluation of the HA and the acetylesterase protein from the other geographical regions are needed to fully explain any reassortment, deletion and insertion events of ISAV. Kibenge et al. (2000) has reported that ISAV can be divided phenotypically into two groups, a group which replicates in the CHSE-214 cell line with production of cytopathic effect (CPE) and one which does not: the majority of ISAV isolates from New Brunswick, Canada and one isolate from Chile have been found to replicate in the CHSE-214 cell line while the majority of ISAV isolates from Norway and Scotland and one isolate from Nova Scotia, Canada do not.

1.7 Subtyping of orthomyxoviruses

The influenza viruses are subtyped based on the antigenic properties of the HA and NA proteins (Wright et al, 1995). The similarities of the HA and the NA proteins at the amino acid level, ranges from 25% to 80% and 42% to 57%, respectively, between subtypes (Wright et al., 1995). The usual methods in determining the subtypes of the influenza viruses include virus growth in cell culture or embryonated chickens' eggs followed by haemagglutination inhibition tests, ELISA or immunofluorescence. However, a more rapid method for both the detection and the identification of the influenza virus subtypes is RT-PCR followed by sequencing methods. Wright et al. (1995) reported that they were able to detect 91% virus-positive samples of influenza virus types A and B, H1 and H3, from

performing RT-PCR as opposed to 87% virus-positive samples from cultured methods. Ellis et al. (1997) performed RT-PCR, RFLP and phylogenetic analysis on various influenza virus A, H3N2 strains from the influenza season of 1995-1996 in England. Through their work, they concluded that the strains had all evolved from one isolate; A/Thessaloniki/1/95. Rohm et al. (1996) found a novel HA subtype (H15) from a duck strain (DkUA83) when they compared the strain to other influenza virus A strains with the HAs H1-H14 through sequence analysis and double immunodiffusion assay. The subunits of HA were compared to H2, H4, H5, H7 and H14. Rohm et al. (1996) found that for HA1, DkUA83 was 67.5% to 71.3% homologous to the viruses with H7, 66.4% to the viruses with H4 and H14 and 69.6% with viruses with H2 and H5 at the amino acid level. When HA2 of DkUA83 was analyzed, it was found to be 82.8% to 90% homologous with the H7 influenza viruses. If a HA belongs to a certain subtype, the HA1 will have at least an 80% homology to the other viruses in that same subtype (Kawaoka, et al., 1990; Nobusawa et al., 1991), therefore Rohm et al. (1996) concluded that DkUA83 belonged to a new subtype, H15, which was probably derived from influenza viruses with the H7 subtype.

1.8 Virus neutralization

By definition antibody-based neutralization is: a "blockage of the activity of an organism or a toxin by antibody" (Tizard, 1996). Viruses need to enter the host cell in order to replicate (Janeway et al., 1997) therefore, the neutralization process can be thought of as a competition of the antibody and receptor located on the surface of the permissive cell for the virus (Tizard, 1996; Janeway et al., 1997). In

the case of the HA protein of the influenza viruses, the protein binds to the terminal sialic acid residues found in certain glycoproteins expressed on the target cell surface (Janeway et al., 1997). It has been shown that antibodies raised against the HA protein inhibit infection preventing the virus from binding to the cell receptors (Janeway et al., 1997). Neutralization tests therefore, are estimates of an antibody's ability to neutralize a virus (Tizard, 1996) by either preventing the virus from interacting with its receptors or by interfering with the fusion of the virus with the cell membrane (Janeway et al., 1997). The most common source of antibodies are found in serum samples, which are usually heat inactivated (56°C for 30 minutes) prior to use to destroy complement activity (Tizard, 1996). Antibodies can be produced against multiple epitopes found on any of the surface and internal viral proteins (Tizard, 1996). In antibody-based neutralization tests, the sera are added to the cells prior to the addition of virus, so that the antibodies are able to bind the virus before the virus is able to interact with the cellular receptors (Tizard, 1996).

The current cell lines available that support the growth of ISAV are the CHSE-214, the SHK-1, the ASK-2 and the TO cell lines. The SHK-1 cell line (Dannevig et al., 1995a, b), the TO cell line (Wergeland and Jakobsen, 2001) and the ASK-2 cell line (Rolland et al., 2002) are macrophage-like cells derived from salmon head kidney leucocytes. Salmonid leucocytes have been shown to contain receptors similar to the Fc receptors (FcR) found in their mammalian counterparts (O'Dowd et al., 1998). Joseph et al. (2003, in press) have observed poor neutralization capabilities of ISAV on SHK-1 and TO cells, suggested to be due to antibody-mediated entry of ISAV in these cells. These authors could restore virus

neutralization if the cells were exposed to staphylococcal Protein A before adding the virus-serum mixture, indicating that the antibody-mediated entry of ISAV in the cells may be Fc receptor-mediated. All ISAV isolates can replicate in the SHK-1 and TO cell lines but these cell lines can not be used for virus neutralization because of the possible Fc-receptor-mediated endocytosis of antibody coated virus. The CHSE-214 cell line does not show this phenomenon but not all ISAV isolates can grow with the production of CPE on the CHSE-214 cell line in this laboratory. Preliminary work with a mixed infection of a CHSE-positive ISAV isolate and a CHSE-negative ISAV isolate showed that the CHSE-negative isolate interfered with the growth of the CHSE-positive isolate inhibiting the appearance of CPE suggesting that this viral interference may be used as an alternative to antibody virus neutralization of CHSE-negative ISAV isolates (Dr. Fred Kibenge, personal communication).

1.9 Virus interference

It is possible for two viruses, such as the influenza viruses, to infect a cell at the same time, resulting in a reassortment of the two viruses (Flint et al., 2000). Alternatively, it is also possible that a co-infection of two viruses results in an interference between the two viruses, so that only one virus is able to successfully replicate in the host cells (Fields et al., 1996). Alonso et al. (1999) reported that infectious pancreatic necrosis virus (IPNV) interferes with the growth of infectious haematopoietic necrosis virus (IHNV) in the BF-2 cell line. IPNV had a five-fold increase in virus particles obtained from the dual infected cells over IHNV (Alonso et al., 1999). This was also verified by flow cytometry analysis as there was a four-

fold increase in the cells expressing the IPNV antigen over IHNV (Alonso et al., 1999). As an another example, Rott et al. (1981) reported an interference between a swine influenza virus A strain (SW) and an avian influenza A strain (FPV). Upon dual infection, FPV multiplied at normal yields, yet SW was very much suppressed (Rott et al., 1981). Rott et al. (1981) also noted that UV light-inactivated FPV did not interfere with the growth of SW and that in a dual infection with antisera raised against FPV, SW was able to grow. Therefore, FPV lost its interfering activity by becoming either inactivated or neutralized.

1.10 Virus inactivation

The methods chosen to inactivate viruses are all dependent on the presence of a lipid envelope (Burstyn and Hageman, 1996). A lipid envelope renders viruses more susceptible to environmental inactivation such as temperature, humidity, pH, UV and X-ray irradiation, repeated thawing and freezing cycles and lipid solvents and detergents. ISAV and IHNV, which are both enveloped viruses, are readily inactivated by various means (Murphy et al., 1999). IHNV is inactivated at temperatures above 50°C, exposure to lipid solvents or oxidizing agents and exposure to UV and X-ray irradiation (Fields et al., 1996). A study performed by Falk et al. (1997) found that ISAV is inactived by incubation at 56°C for only 5 minutes.

1.11 Hypothesis

The goal of this research project was to expand on the information known about the HA genotype of ISAV in relation to the antigenic type of ISAV. The hypotheses for this work are (a) that ISAV, being an orthomyxovirus-like virus, has

similar characteristics to those of members of the virus family *Orthomyxoviridae* and (b) that the CHSE-214 cell line may give a better assessment of viral neutralization than the TO and the SHK-1 cell lines.

If the HA protein is involved in the first crucial steps of viral infection as seen in influenza, the gene may be under pressure to evolve so that ISAV could re-infect its host species. As ISAV is an orthomyxovirus, it is possible that certain ISAV antigens are subject to similar antigenic shift. By extension, the HA of ISAV is a likely candidate for antigenic variation and determinants of pathogenicity. The genetic variation in the HA gene of ISAV has led to two antigenically distinct isolates which may be responsible for pathogenic differences observed among the ISAV isolates from different geographical regions.

It has been shown that the genotype can explain the antigenic type of the virus in that antisera raised against different ISAV isolates have different neutralizing capabilities. However, it is plausible that the CHSE interference system will provide a better system for studying virus neutralization of ISAV than the TO cell line. Further understanding of the genetic variation of the HA gene and its antigenic properties may lead to the development of more efficient vaccines against ISA to include strains from different geographical regions, if these strains also show differences in pathogenicity.

1.12 Objectives

The objectives of this research project based on these hypotheses are:

1) To compare the nucleotide and amino acid sequences of the HA gene

(genome segment 6) of several strains of ISAV encompassing different geographical regions.

2) To further correlate the ISAV HA genotype to the antigenic type using the CHSE-214 cell line.

2.0 MATERIALS AND METHODS

2.1.1 Virus and cell culture

Seven ISAV isolates from different geographical regions (Table 1) were chosen for this study. ISAV isolates were propagated in the TO cell line. One day old cell monolayers in 8 mL flasks were inoculated with the ISAV isolates. To the cell monolayers, 100 µL of virus in 900 µL maintenance media (5% FBS, 1% gentamycin, 0.1% L-glutamine in Hank's MEM) were added. The virus was absorbed at room temperature (RT) for one hour, followed by the addition of 7 mL of maintenance media. The flasks were incubated at 16°C and were monitored daily for CPE. When maximal CPE was detected, the flasks were frozen at -80°C. The virus was harvested by thawing the flasks once. The harvested virus was then aliquoted into cryostat tubes and frozen at -80°C until further used.

2.1.2 Virus titration

The purpose of virus titration is to establish the titre of the stock virus so that it can be appropriately diluted to a known amount for further use. The titration can be expressed as $TCID_{50}$ (median **T**issue **C**ulture **I**nfective **D**ose), which is the amount of virus needed to cause a cytopathic effect (CPE) in 50% of the cell monolayer infected with the virus. In virus neutralization tests, the quantity of 100 $TCID_{50}$ of the virus is added to the cell monolayers to establish a consistent amount of virus which is independent to the varying antibody titres which may occur between the virus anti-sera of the different ISAV isolates. To establish the 100 $TCID_{50}$ of ISAV, ten-fold dilutions of the virus in maintenance media are added to

Table 1: Geographic origin of ISAV isolates

Isolate	Country and/or province of origin	References
U5575-1	Nova Scotia, Canada	Kibenge et al., 2001b
390/98	Scotland	Kibenge et al., 2001b
Glesvaer	Norway	Falk et al., 1997
HI/92	Norway	Wergeland and Jakobsen, 2001
NBISA01	New Brunswick, Canada	Jones et al., 1999
7833-1	Chile	Kibenge et al., 2001a; 2001b
Back Bay '98	New Brunswick, Canada	Bouchard et al., 1999

the cell monolayers and allowed to incubate at RT for 1 hour. More maintenance media is added and the cells and virus mixture are incubated at 16°C until day 14 in case of CHSE-214 cells or 10 days for TO cells, checking for CPE. At the final day of reading, an endpoint is reached and 100 $TCID_{50}$ can then be calculated using the Karber Formula.

Karber Formula:

$$\text{(negative log of highest virus concentration)} - \frac{\text{(sum of \% mortality at each dilution)}}{100} - 0.5 \text{ X (log of dilution)}$$

Example:

ISAV isolate U5575(1) on TO cells at day 10:

-1 - (((100+100+100+100+100+100+100+66)/ 100)) - 0.5 X log10)

-1 - (7.66-0.5)

-8.16

The antilog of .16 is 1.44; therefore 100 µL of virus is further diluted by the addition of 44 µL for a total of 144µL which is then 10^8. Ten-fold dilutions are then made to 10^2, which is 100 $TCID_{50}$.

2.1.3 RT-PCR for ISAV

Viral RNA of selected ISAV isolates was extracted from 300 µL cell culture lysate by using TRIZOL LS reagent (Canadian Life Technologies) following the manufacturer's protocols. RT-PCR was performed on the extracted RNA samples of the ISAV isolates. One primer pair targeting segment 6 of the ISAV genome was used. The following primer pair were used: ISAV HA1F (nucleotides 70-91; sense, 5'AAACTACCCTGACACCACCTGG3') and ISAV HA1R (nucleotides 1061-1082;

anti-sense, 5'ACAGAGCAATCCCAAAACATGC3') (Kibenge et al., 2001b). One-step RT-PCR was carried out using the Titan One-Tube RT-PCR system kit (Roche Molecular Biochemicals). Briefly, using conditions as previously described (Kibenge et al., 2001b), cycling was performed using 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 minutes and 94°C for 2 minutes, respectively, which was followed by 40 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 61°C for 45 seconds, extension at 72°C for 90 seconds with a final extension at 72°C for 10 minutes.

The RT-PCR products were resolved on a 1% agarose gel and visualized under 340 nm UV light after staining with ethidium bromide (Sambrook et al., 1989). The RT-PCR products were then excised from the agarose gel and purified using the High Pure PCR Product Purification Kit® (Roche Molecular Biochemicals) following the manufacturer's protocols. The PCR products were ethanol precipitated in preparation for cloning into the pCRII vector (Invitrogen Life Technologies).

2.1.4 TA Cloning

Cloning of the RT-PCR products of the ISAV isolates was performed using the TOPO TA Cloning kit (Invitrogen Life Technologies). The RT-PCR products precipitated in ethanol were pelleted for 30 minutes (14,000 rpm), washed with 70% ethanol (centrifuged for 10 minutes at 14,000 rpm) and spin vacuum dried for 8 minutes. The pellets were then resuspended in 4 µL of sterile distilled, deionized water (dd H_2O). To the resuspended pellet, 1 µL of the salt solution and 1 µL of the vector supplied by the kit were added. This was mixed gently and allowed to

incubate at RT for 5 minutes. Two microliters of this cloning reaction was added to a vial of *E. coli* DH5∝ competent cells, supplied from the kit, and were mixed gently. This was incubated on ice for 30 minutes. The cells with the cloning reaction were heat shocked at 42°C for 30 seconds and then placed on ice. Two hundred and fifty microliters of SOC medium (Invitrogen Life Technologies) at RT was added to the vial which was then placed in a shaking water bath at 37°C for 1 hour. One hundred microliters were then used to plate out onto an agar plate containing ampicillin and X-gal; 2 plates per transformation reaction were used. The plates were incubated overnight at 37°C. Master plates were made by transferring white colonies to fresh agar plates containing ampicillin and X-gal and incubated overnight at 37°C.

2.1.5 Isolation of plasmid DNA

Five milliliters of 2X YT broth containing 100 µL (100 µg/mL) of ampicillin were inoculated with the bacteria containing the recombinant plasmid. The cultures were incubated at 37°C overnight with constant shaking (250 rpm). The bacteria were then harvested by centrifugation at 14000 rpm for 30 seconds. To the bacterial pellet, 110µL of solution #1 (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl, pH 8.0) was added and vortexed vigorously and then, 220 µL of solution #2 (0.2 N NaOH, 1% SDS) was added. This was mixed and incubated on ice for at least one minute. After the ice incubation, 165 µL of solution #3 (3 M potassium, 5 M acetate) were added, vortexed and incubated on ice for another 5 minutes. The samples were then centrifuged for 5 minutes at 14000 rpm and the supernatant was transferred to new microfuge tubes. One milliliter of cold 100% ethanol was added

and an incubation of 5 minutes on ice was performed to precipitate the plasmid DNA. The plasmid DNA was then pelleted by centrifugation at 14,000 rpm for 5 minutes and the pellets were resuspended in 50 µL of TE buffer containing 20 µg/mL RNase and incubated at 37°C for 5 minutes. The plasmid DNAs were screened for inserts by restriction digestion using the enzyme *EcoRI*. Eight microliters of the isolated plasmid DNA was cut with 1 µL of *EcoRI* and 1 µL of the reaction buffer (GIBCO/BRL). The plasmid DNA, enzyme and buffer mixture were subjected to a one hour incubation at 37°C. The samples were resolved on a 1% agarose gel as previously described.

Plasmid DNA for sequencing was prepared by the alkaline-lysis method of Birnboim and Doly (1979). Fifty milliliters of 2X YT broth containing 1 mL of ampicillin (100 µg/mL) (Boehringer Mannheim) was inoculated with bacteria containing recombinant plasmid to ensure that at least 1 µg/µL of DNA for sequencing was obtained.

2.1.6 Plasmid DNA sequencing

The plasmid DNA was washed in 70% ethanol, centrifuged at 14,000 rpm for 10 minutes, vacuum dried for 8 minutes and resuspended in 100 µL of dd H_2O. Ten microliters of 2 M NaOH, 2 mM EDTA was added to the plasmid DNA. The tubes were then incubated in a 37°C water bath for 30 minutes, to denature the plasmid DNA. Eleven microliters of 3M Na acetate and 302.5 µL of 100% ethanol were then added to the tubes, which were then mixed and placed at -80°C overnight. The plasmid DNA was sequenced by a modification to the Sanger dideoxy/enzymatic

method, according to the protocols of the Sequenase Version 2.0 ^{T7}Sequencing™ kit (Amhersam Pharmacia Biotech). The kit used unmodified T7 DNA polymerase to extend oligonucleotide primers. Either a forward or a reverse primer (Universal primer M 13/pUC; reverse sequencing primer M 13/pUC, Boehringer Mannheim) was annealed to the denatured template for 2 minutes at 65°C, then cooled to >35°C. The annealed primer and template were incubated for 5 minutes at RT in a mixture containing T7 DNA polymerase and all four deoxynucleotriphosphates (dNTPs), of which dATP was radiolabelled (^{35}S-dATP∝S, 9.25 Mbq 1 mCi/mL, Amhersam Pharmacia Biotech). Extension of the primer was terminated in four separate reactions, each containing a single dideoxynucleotriphosphate (ddNTP) in addition to the four dNTPs. The termination reactions were incubated at 37°C for 5 minutes prior to addition of the stop solution.

2.1.7 Sequence analysis

All sequence reaction samples were resolved using an acrylamide gel in TBE (Tris, Boric acid, EDTA) sequencing buffer (pH 8.3). Electrophoresis was performed at a constant power of 55 W for at least 4 hours. Afterwards, gels were soaked in 10% methanol/10% acetic acid solution for 1 hour to remove urea and were then dried on Whatman 3 Cr chromatography paper (Fisher Scientific) with vacuum for 3.5 hours at 80°C (DrygelSr. Slab Dryer Model SE 1160, Hoefer Scientific). Dried gels were subjected to direct autoradiography using Kodak X-Omat film at RT.

The nucleotide sequences were manually read from the radiographs and were entered in the computer using Word Perfect 5.0. Nucleotide sequence analysis was carried out using the Sequence Manipulation suite (Stothard, 2000),

BLAST (National Center for Biotechnology Information) and FASTA (Pearson and Lipman, 1988).

Once the forward and reverse strands of the partial DNA sequences had been read, they were entered into the Sequence Manipulation Suite and the reverse strands were converted (using the reverse option in the DNA manipulation section) so that all partial sequences were read in 5' to 3' direction. The partial sequences, forward and reverse, were then entered into the BLAST program. After it was established that the partial sequences matched with genome segment 6 of ISAV, the whole segment 6 sequence was downloaded from the GenBank database. The sequences of segment 6 of the selected ISAV isolates were then analyzed using the ORF Finder program (Sequence Manipulation Suite) to obtain the amino acid sequence of the HA protein. Sequence similarities between isolates were then calculated using the FASTA program (Pearson and Lipman, 1988).

2.2.1 Rabbit and fish anti-sera to ISAV

The pooled fish-antiserum from experimentally infected rainbow trout to ISAV isolate U5575-1 was provided by Dr. Molly Kibenge. The rabbit-antiserum to ISAV isolate 7833-1 used was previously described (Kibenge et al. 2000, 2001b).

2.2.2 Virus interference in the CHSE-214 cell line

It has been previously shown that there are ISAV isolates that can replicate and produce CPE in the CHSE-214 cell line (such as 7833-1 and NBISA01) and those which do not (such as U5575-1) (Kibenge et al., 2000a). Preliminary studies using mixtures of the two phenotypes of ISAV in the CHSE-214 cell line suggested

that the CHSE negative phenotype inhibited the production of CPE by the CHSE positive phenotype (Dr. Fred Kibenge, personal communication). As TO, SHK-1 and ASK-2 cell lines can not be used in antibody-based neutralization studies (Chapter 1.8), the following experiment was designed to confirm this observation with the intention of exploiting the potential interference phenomenon in virus neutralization tests on ISAV using the CHSE-214 cell line. Three hundred microliters of virus, either U5575-1 or 7833-1, was added to 2.7 mL of maintenance media. The virus was added to a one day-old CHSE-214 cell monolayer in 25 mL flask and incubated at RT for 1 hour. Seventeen milliliters of maintenance media was then added. The cells inoculated with the virus were incubated at 16°C for 14 days or until maximum CPE was observed. Isolates U5575-1 and 7833-1 had previously been titrated on TO cells and so the same procedure was performed using a 1:1 mixture of 100 $TCID_{50}$ of both U5575-1 and 7833-1, as well as a 1:1 mixture of 100 $TCID_{50}$ heat-inactivated (56°C for 30 minutes, Falk et al. (1997)) U5575-1 and 100 $TCID_{50}$ of 7833-1 as previously described and monitored for 14 days. To establish that the heat-inactivated virus had been inactivated, 100 µL of heat-inactivated U5575-1 was added to 900 µL of maintenance media and then added to a one day-old TO cell monolayer and allowed to incubate at RT for 1 hour. Seven milliliters of maintenance media was then added. The TO cells were incubated at 16°C for 10 days.

To determine the point at which U5575-1 interferes with 7833-1 and NBISA01, U5575-1 was added to the 48 well plates just prior to the addition of 7833-1 and NBISA01. A "checkerboard" titration test was set up with U5575-1

titrated left to right from 10^{-1} to 10^{-8} whereas 7833-1 was titrated top to bottom from 10^{-2} to 10^{-7} (Table 2) with 100 µL U5575-1 being added to the cells just prior to adding 100 µL of 7833-1. The same "checkerboard" titrations were repeated with U5575-1 (1000 $TCID_{50}$ to 0.001 $TCID_{50}$) along with 7833-1 (1000 $TCID_{50}$ to 0.01 $TCID_{50}$) and also NBISA01 (1000 $TCID_{50}$ to 0.01 $TCID_{50}$).

2.2.3.1 Virus titration of ISAV and IPNV in CHSE-214 cells

Five hundred microliters of cells in growth medium were added to each well of a 48 well plate and the plate was sealed. The cells were allowed to monolayer at RT overnight and were then inoculated with 7833-1 and NBISA01. Ten-fold dilutions of each isolate were prepared starting from 10^{-1} to 10^{-8}. Four hundred and fifty microliters of maintenance medium was added to 8 dilution tubes. Fifty microliters of virus sample was added to the first tube and mixed well. Then, 50 µL from the first tube (10^{-1}) was transferred to the second tube (10^{-2}) and then 50 µL of this was transferred to the third tube (10^{-3}) and so on up to 10^{-8}. One hundred microliters of each dilution of the virus was added to the cells in triplicate. The plate was then incubated at RT for 1 hour. Five hundred microliters of maintenance medium was added to the monolayers following the incubation. The plate incubated at 16 °C for 14 days. The Karber Formula was used to calculate the 100 $TCID_{50}$/unit volume of the virus. U5575-1 had been previously titrated on TO cells in the same manner, with a final reading for CPE at day 10. IPNV was titrated on CHSE-214 cells similar to the ISAV isolates.

Table 2: Example of a checkerboard titration setup for ISAV isolates U5575(1) and **7833-1**.

U5575-1	1000 $TCID_{50}$	100 $TCID_{50}$	10 $TCID_{50}$	1 $TCID_{50}$	0.1 $TCID_{50}$	0.01 $TCID_{50}$	0.001 $TCID_{50}$	0.0001 $TCID_{50}$
7833-1 100 $TCID_{50}$	1000 $TCID_{50}$ **100 $TCID_{50}$**	100 $TCID_{50}$ **100 $TCID_{50}$**	10 $TCID_{50}$ **100 $TCID_{50}$**	1 $TCID_{50}$ **100 $TCID_{50}$**	0.1 $TCID_{50}$ **100 $TCID_{50}$**	0.01 $TCID_{50}$ **100 $TCID_{50}$**	0.001 $TCID_{50}$ **100 $TCID_{50}$**	0.0001 $TCID_{50}$ **100 $TCID_{50}$**
10 $TCID_{50}$	1000 $TCID_{50}$ **10 $TCID_{50}$**	100 $TCID_{50}$ **10 $TCID_{50}$**	10 $TCID_{50}$ **10 $TCID_{50}$**	1 $TCID_{50}$ **10 $TCID_{50}$**	0.1 $TCID_{50}$ **10 $TCID_{50}$**	0.01 $TCID_{50}$ **10 $TCID_{50}$**	0.001 $TCID_{50}$ **10 $TCID_{50}$**	0.0001 $TCID_{50}$ **10 $TCID_{50}$**
1 $TCID_{50}$	1000 $TCID_{50}$ **1 $TCID_{50}$**	100 $TCID_{50}$ **1 $TCID_{50}$**	10 $TCID_{50}$ **1 $TCID_{50}$**	1 $TCID_{50}$ **1 $TCID_{50}$**	0.1 $TCID_{50}$ **1 $TCID_{50}$**	0.01 $TCID_{50}$ **1 $TCID_{50}$**	0.001 $TCID_{50}$ **1 $TCID_{50}$**	0.0001 $TCID_{50}$ **1 $TCID_{50}$**
0.1 $TCID_{50}$	1000 $TCID_{50}$ **0.1 $TCID_{50}$**	100 $TCID_{50}$ **0.1 $TCID_{50}$**	10 $TCID_{50}$ **0.1 $TCID_{50}$**	1 $TCID_{50}$ **0.1 $TCID_{50}$**	0.1 $TCID_{50}$ **0.1 $TCID_{50}$**	0.01 $TCID_{50}$ **0.1 $TCID_{50}$**	0.001 $TCID_{50}$ **0.1 $TCID_{50}$**	0.0001 $TCID_{50}$ **0.1 $TCID_{50}$**
0.01 $TCID_{50}$	1000 $TCID_{50}$ **0.01 $TCID_{50}$**	100 $TCID_{50}$ **0.01 $TCID_{50}$**	10 $TCID_{50}$ **0.01 $TCID_{50}$**	1 $TCID_{50}$ **0.01 $TCID_{50}$**	0.1 $TCID_{50}$ **0.01 $TCID_{50}$**	0.01 $TCID_{50}$ **0.01 $TCID_{50}$**	0.001 $TCID_{50}$ **0.01 $TCID_{50}$**	0.0001 $TCID_{50}$ **0.01 $TCID_{50}$**
0.001 $TCID_{50}$	1000 $TCID_{50}$ **0.001 $TCID_{50}$**	100 $TCID_{50}$ **0.001 $TCID_{50}$**	10 $TCID_{50}$ **0.001 $TCID_{50}$**	1 $TCID_{50}$ **0.001 $TCID_{50}$**	0.1 $TCID_{50}$ **0.001 $TCID_{50}$**	0.01 $TCID_{50}$ **0.001 $TCID_{50}$**	0.001 $TCID_{50}$ **0.001 $TCID_{50}$**	0.0001 $TCID_{50}$ **0.001 $TCID_{50}$**

2.2.3.2 Inactivation of IPNV

Mixed infection of ISAV isolate 7833-1 and pH-inactivated IPNV was performed to determine if the virus inference was ISAV specific or not. IPNV was diluted 1 in 10 in 0.85% saline, pH 12.22 and was left to incubate overnight at RT. The pH-inactivated IPNV was then brought to pH 7.23 before addition to the cell monolayers. Ten-fold dilutions of the inactivated IPNV were added to separate 48-well plates as in the titration procedures, just prior to the addition of 100 $TCID_{50}$ of 7833-1 to the monolayers. The cells were then monitored daily for development of CPE until day 14.

To ensure that IPNV had been completely inactivated, an 8 mL flask of CHSE-214 cells was inoculated with 100 µL of the inactivated IPNV in 900 µL of maintenance media and left to incubate for 1 hour at RT. Seven milliliters of maintenance media were then added. The flask was monitored daily for CPE until day 14.

2.2.4 Virus neutralization of ISAV

As previously reviewed (Chapter 1.9), it is known that viruses can interfere with other viruses in cell culture, in that the infectivity of virus A is reduced in the presence of virus B. If two different viruses are found to interfere with each other, it should be possible to remove the interference by antibody-based neutralization against one of the viruses (in that the antibody will bind to the virus, so that it can no longer be infective) then the other virus will be able to infect the cell culture, provided the antibody blocks binding or uptake two viruses are antigenically

different. To prove that neutralizing the CHSE-negative isolate (U5575-1) enables the CHSE-positive isolates (7833-1 and NBISA01) to cause CPE, the following virus neutralization (VN) tests were performed. The VN tests used fish antiserum to U5575-1 and rabbit antiserum to 7833-1 in one day-old CHSE-214 cell monolayers in 48-well plates. Two-fold dilutions of sera from 1:10 to 1:1280 in maintenance medium were made in dilution tubes. The 100 $TCID_{50}$/100 µL virus sample and back titrations of the virus sample (100, 10, 1 and 0.1 $TCID_{50}$/100 µL) also were prepared. The growth media were removed from the monolayers, the sera were added, followed by the addition of the 100 $TCID_{50}$/100µL virus preparation (either 100 $TCID_{50}$ of NBISA01, 100 $TCID_{50}$ of 7833-1 or the mixtures of U5575-1 and 7833-1 and U5575-1 and NBISA01 (as determined from the "checkerboard" titration procedures) as well as 100 $TCID_{50}$ IPNV). One hundred microliters of the back titrations of the 100 $TCID_{50}$ virus samples were added in duplicate per dilution of the virus. As for the cells which were inoculated with the ISAV isolate mixtures, cells monolayers in duplicate were inoculated with the CHSE-positive and CHSE-negative mixture with the CHSE-positive isolate only and the CHSE negative isolate only as the control wells. The cells were incubated at RT for one hour in the laminar flow hood, then 0.5 mL maintenance medium was added to the cell monolayers and the plate was sealed, placed at 16°C and monitored daily for CPE until day 14.

3.0 RESULTS

3.1 TA cloning of ISAV isolates

RT-PCR with the primer pair ISAV HA1F/ISAV HA1R was used to amplify a 1031 base pair (bp) fragment from genome segment 6 of the seven ISAV isolates listed in Table 1. An agarose gel of RT-PCR amplification products is shown in Figure 1. The cloned ISAV RT-PCR products were sequenced. Figure 2 shows an agarose gel with three clones of U5575-1 RT-PCR products. All other positive clones from the other ISAV isolates gave similar results (data not shown).

3.2 Nucleotide and amino acid sequence similarities of ISAV isolates

Only the terminal (end) sequences of the RT-PCR amplified products of ISAV isolates U5575-1(Nova Scotia) and 390/98 (Scotland) were determined in this study as clones of the other isolates yielded sequences of poor quality, as the sequences did not produce homologies to known ISAV HA sequences. The nucleotide sequence of isolates U5575-1 and 390/98 matched to the ISAV genome segment 6 in the GenBank database when interrogated using the BLAST program. The amino acid sequences of U5575-1 and 390/98 predicted from the nucleotide sequences revealed an open reading frame (ORF) which spanned almost the entire sequence between nucleotide positions 2 and 1030. The ORF encoded a protein of 340 amino acids long for U5575-1 and 390 amino acids long for 390/98. The genome segment 6 nucleotide and amino acid sequences of all the other isolates analyzed were obtained from the GenBank database (Table 3).

Figure 3 shows an example of the pairwise nucleotide similarity between

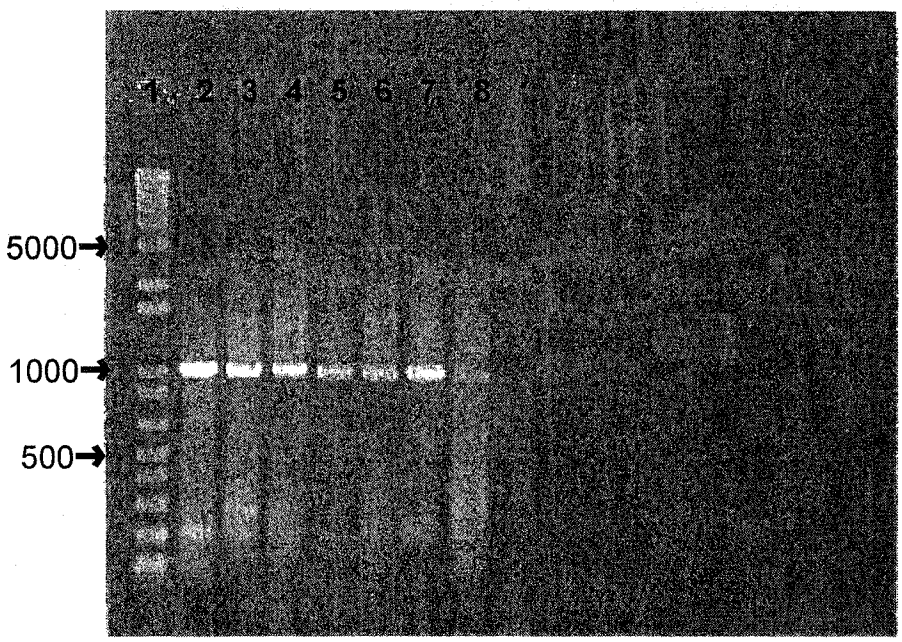


Figure 1: Agarose gel of RT-PCR amplified cDNAs of ISAV isolates. Lane 1: 1 Kb DNA ladder. Lanes 2-8 contain: (2)7833-1 (Chile), (3) ILAV (Norway), (4) U5575-1 (Nova Scotia, Canada), (5) MT-ISAV (New Brunswick, Canada), (6) HI/92 (Norway), (7) NBISA01 (New Brunswick, Canada) and (8) 390/98 (Scotland), respectively.

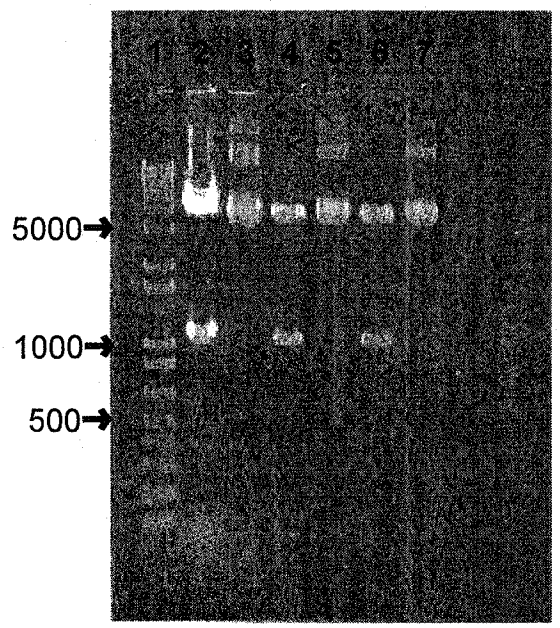
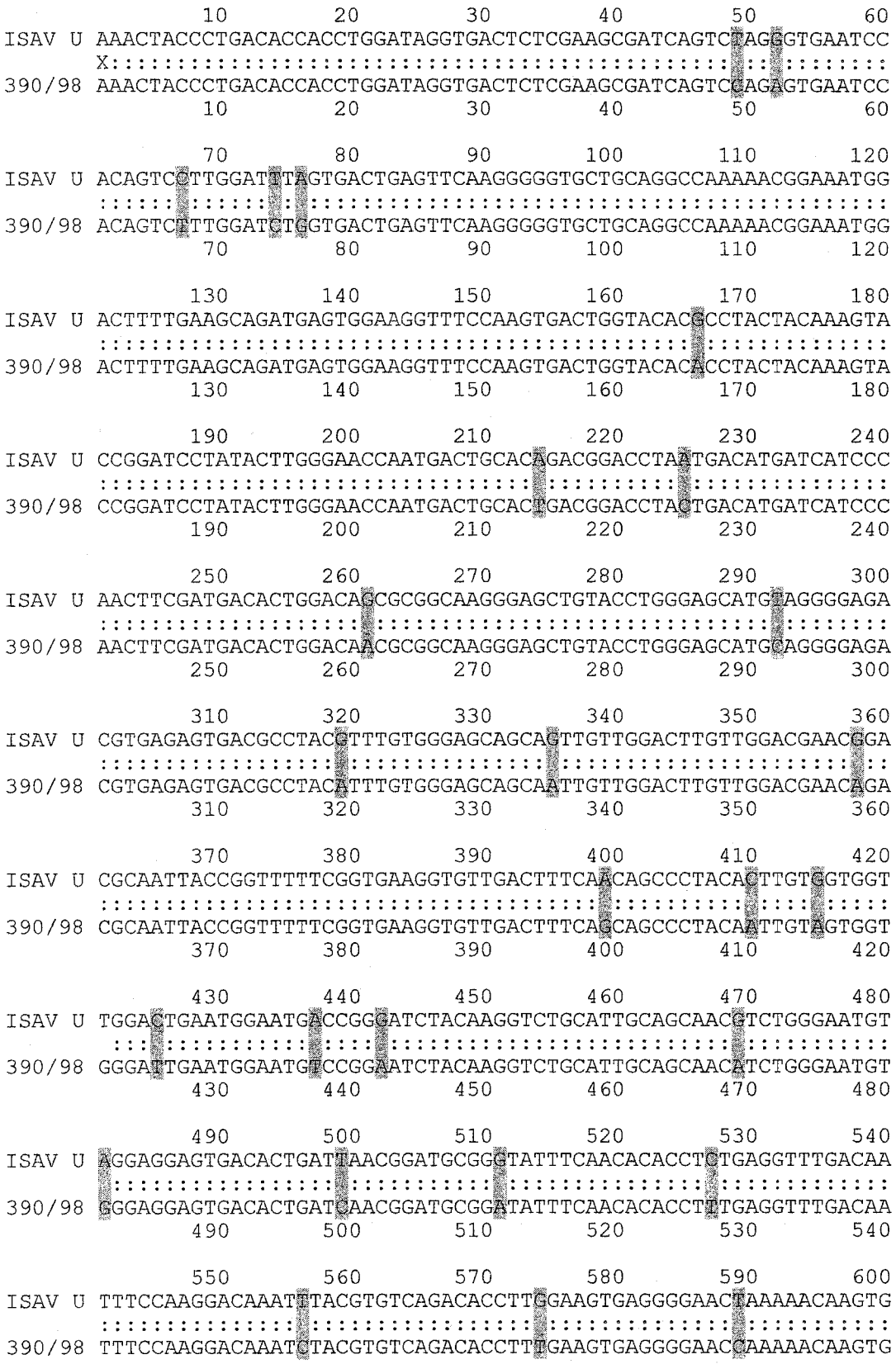


Figure 2: Agarose gel of positive TA clones of ISAV isolate U5575(1). Lane 1: 1 Kb DNA ladder. Lanes 2-7 contain: (2) clone #1 cut with *EcoRI*; (3) clone #1 uncut; (4) clone #2 cut with *EcoRI*; (5) clone #2 uncut; (6) clone #3 cut with *EcoRI* and (7) clone #3 uncut, respectively.

Table 3. List of ISAV isolates analyzed using the FASTA program.

Strain number	Isolate	Country	Strain number	Isolate	Country
1	RPC/NB-980-280-2	Canada	32	Hitra	Norway
2	RPC/NB-980-028-10	Canada	33	21/96	Norway
3	DFO-1	Canada	34	25/97	Norway
4	RPC/NB-980-458-1	Canada	35	26/97	Norway
5	RPC/NB-990-508-3	Canada	36	27/97	Norway
6	RPC/NB-990-002-1	Canada	37	28/97	Norway
7	RPC/NB-980-049-1	Canada	38	7/92	Norway
8	RPC/NB-970-877-2	Canada	39	52/00	Norway
9	Back Bay 98	Canada	40	29/97	Norway
10	HKS-36	Canada	41	32/98	Norway
11	7833-1	Chile	42	40/98	Norway
12	NBISA01	Canada	43	41/98	Norway
13	RPC/NB-990-681-3	Canada	44	45/99	Norway
14	Bay of Fundy 97	Canada	45	49/99	Norway
15	Not specified	Canada	46	51/00	Norway
16	GA/TO	Canada	47	56/00	Norway
17	485/9/97	Norway	48	54/00	Norway
18	Gullesfjord	Norway	49	57/00	Norway
19	Glesvaer/2/90	Norway	50	1490/98	Scotland
20	390/98	Scotland	51	18/96	Norway
21	832/98	Scotland	52	22/96	Norway
22	912/99	Scotland	53	33/98	Norway
23	301/98	Scotland	54	37/98	Norway
24	810/9/99	Norway	55	38/98	Norway
25	HI/92	Norway	56	6/91	Norway
26	835/9/98	Norway	57	U5575-1	Canada
27	Sotra	Norway	58	47/99	Norway
28	Bremnes	Norway	59	9/93	Norway
29	Loch Nevis	Scotland	60	14/95	Norway
30	1/87	Norway	61	46/99	Norway
31	17/96	Norway	62	48/99	Norway



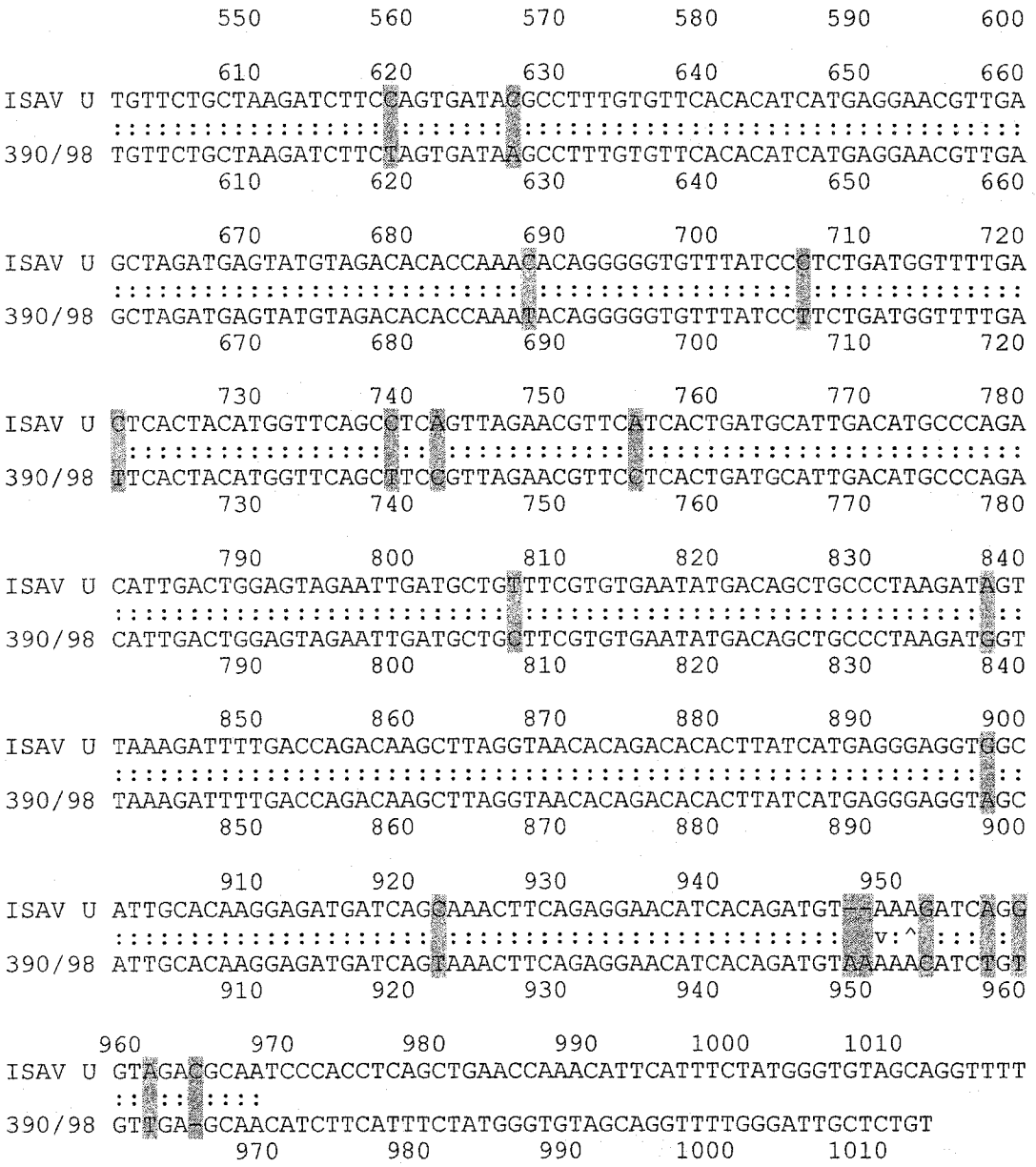


Figure 3: Nucleotide homology between ISAV isolates U5575-1 (ISAV U) and 390/98 (390/98). A indicates a mismatch or a deletion. Nucleotide matches indicated by colon (:). A change in nucleotide is depicted by a mismatch (blank) or a deletion by a dash (-); a deletion of two nucleotides at position 949 of U5575(1) can be seen and one nucleotide deletion at position 966 of 390/98.

isolates U5575-1 and 390/98 using the FASTA program. From this analysis, the two ISAV isolates are 95.2% identical at the nucleotide level. Between the two isolates there are only single nucleotide mismatches that occur throughout the sequence. Figure 4 illustrates the pairwise amino acid sequence similarity of ISAV isolates U5575-1 and 390/98 using the FASTA program. The ISAV isolates are 93.6% identical at the amino acid level. The mismatches are of only one or two amino acids in length and occur throughout the sequence. An area of amino acid deletions is seen at amino acid positions 345 to 350 of isolate 390/98. All the available sequences of genome segment 6 from GenBank database (Table 3) were analyzed using the FASTA program and the summary of the sequence identities are shown in Table 4 (Appendix E). The sequence homologies further support the two genotypes reported by Kibenge et al. (2001b). ISAV isolates from New Brunswick, Canada, and the single isolate from Chile (numbers 1-16) were found to belong to the North American genotype while ISAV isolates from Norway, Scotland, and the single isolate from Nova Scotia (numbers 17-62) belonged to the European genotype. The two HA genotypes have nucleotide sequence identities of $\leq$80.4% and amino acid sequence identities of $\leq$88.2% whereas within each genotype, both the nucleotide and amino acid sequence identities are 90.7% or higher.

The following two isolates from Norway were found to be 100% identical at the nucleotide level: Misund (number 61, from Sogn og Fjordane county) and 810/9/99 (number 24, county unknown). The following isolates from Norway were found to be 100% identical at the amino acid level: 485/9/97 (number 17, county

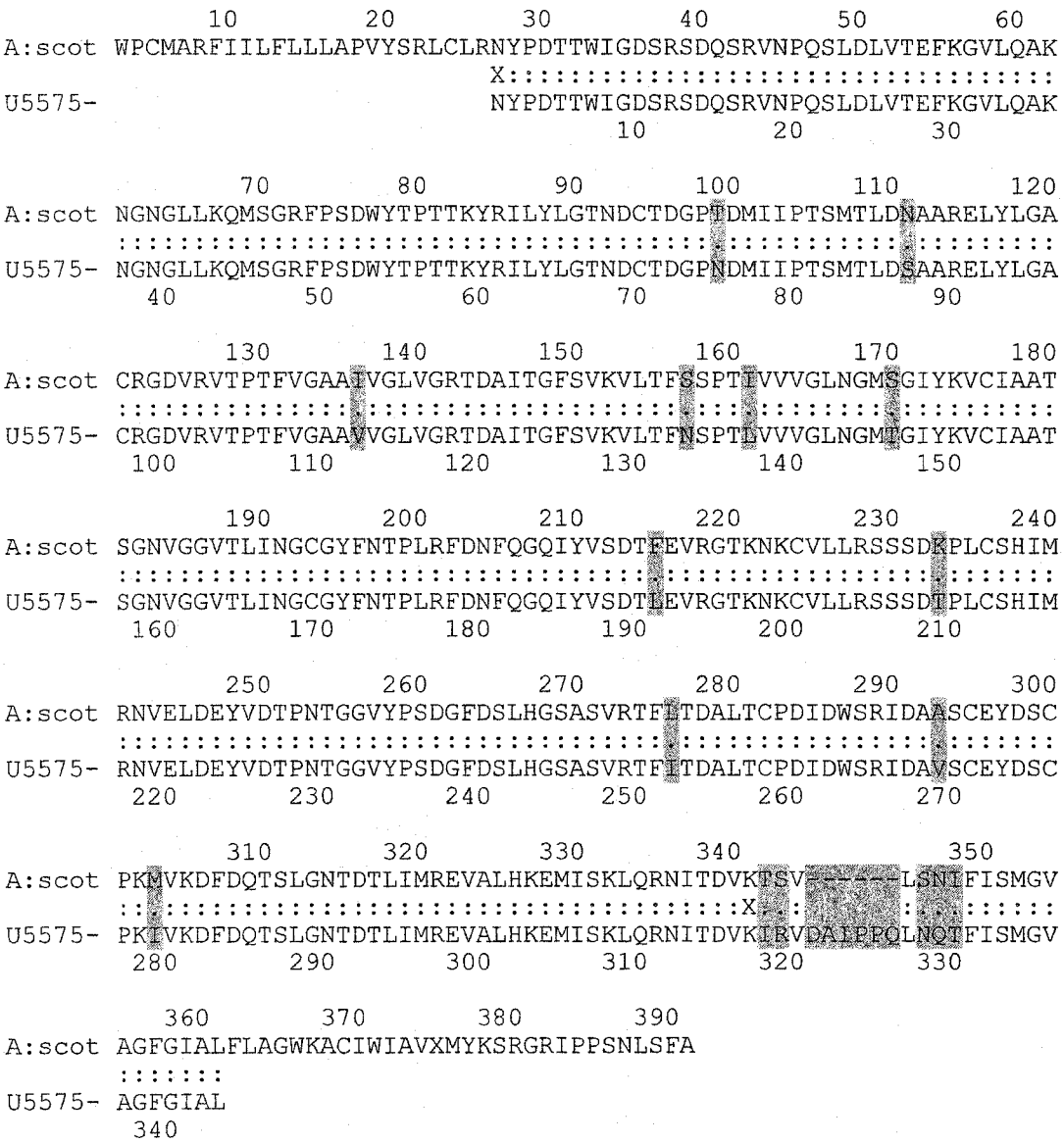


Figure 4: Amino acid homology between ISAV isolates U5575-1 (U5575-) and 390/98 (A:scot) . A indicates a mismatch or deletion. Either an amino acid has been changed (single dot (.)), resulting in a mismatch or a dash (-) indicates that a deletion of an amino acid has occurred. 390/98 has a 6 amino acid deletion from amino acid position 345 to position 350.

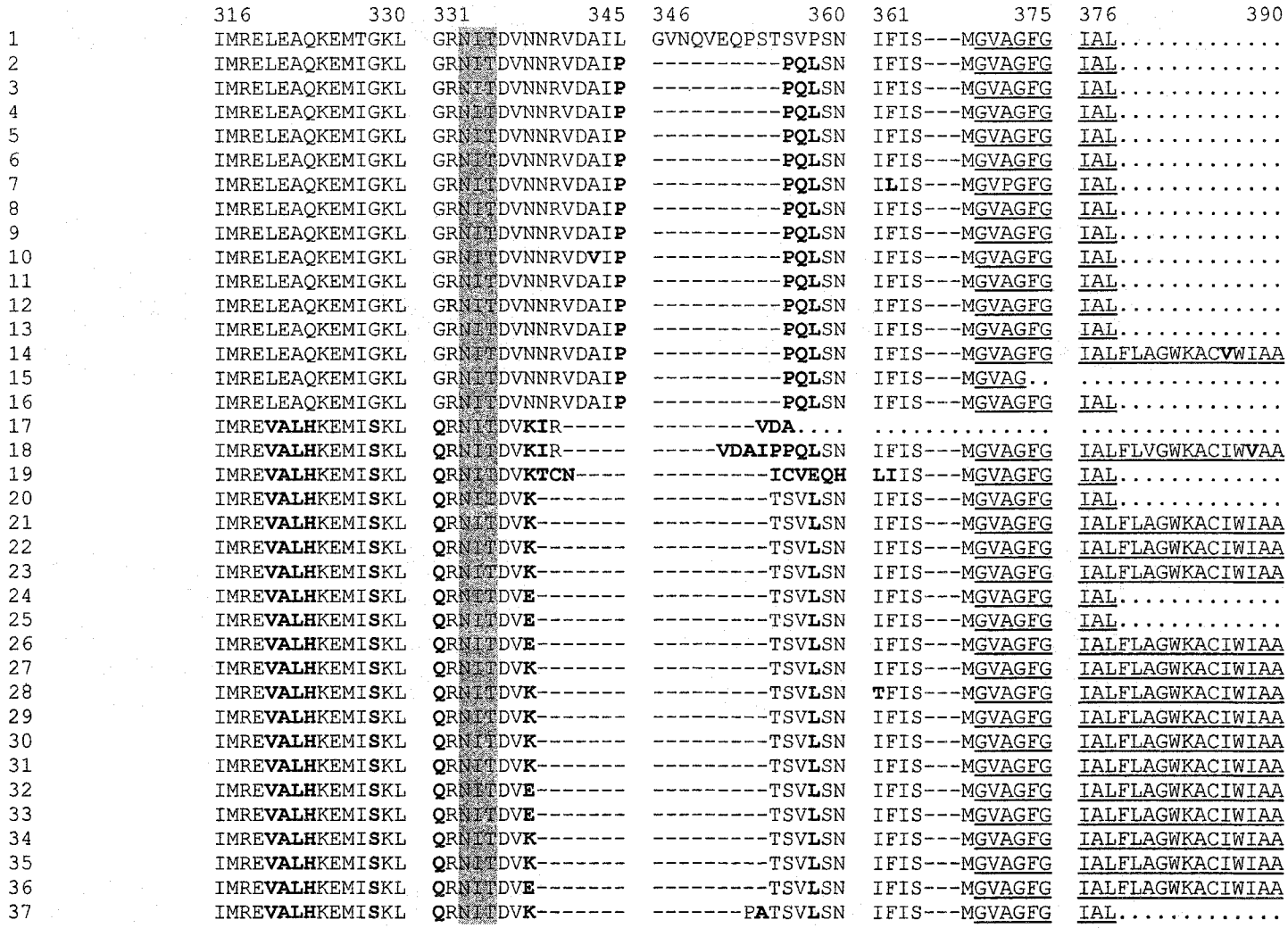
Table 4: Summary of homology of the 62 ISAV isolates analyzed by the FASTA program

	Percentage (%) identity	
Genotype	Nucleotide sequence	Amino acid sequence
European vs European	≥92.1	≥90.7
North American vs North American	≥98.4	≥93.5
European vs North American	≤80.4	≤88.2

unknown) to: Sotra, isolated in 1993 (number 38, from Hordaland county), Eikelandsosen (number 39, from Hordaland county), Selje (number 60, from Sogn-og Fjordane county), Sotra, isolated in 1992 (from Hordaland county, number 40) and to Vestvågøy (number 59, from Nordland county); 810/9/99 to Bremne (number 26, county unknown) and Sørnes (number 36, from Hordaland county); Bremnes to Gulen and Mundheim; Solund (number 48) to Fjaler (number 49) (both from Sogn-og Fjordane county) and Sotra, isolated in 1993 to Sotra, isolated in 1992 and Eikelandsosen (number 39, from Hordaland county), as well as ISAV isolates from New Brunswick, Canada, DFO-1 (number 3) to RPC/NB-980-458-1 (number 4). Scottish isolates 390/98, 832/98 and 912/99 (numbers 20,21 and 22, respectively) were found to be 100% identical at both the nucleotide and amino acid levels (indicating that they maybe the same isolate); ISAV isolates from Norway: Gulen and Mundheim; Sotra, isolated in 1993 and Eikelandsosen; Senja, isolated in 1996 and Senja, isolated in 1998 (numbers 52 and 53, respectively, both from the county Troms) were found to be 100% identical on genome segment 6. Two isolates from New Brunswick, Canada, 'Bay of Fundy 97' and 'Not specified' (numbers 14 and 15, respectively) are also 100% identical on genome segment 6. A map of Norway with the counties from which many ISAV isolates have been collected from is shown in Figure 5.

The deduced amino acid alignment was performed using CLUSTAL W with the default settings (Thompson et al., 1994). Figure 6 illustrates the partial amino acid profiles of the 62 ISAV isolates from both ISAV genotypes (Appendix F). The

Figure 5: Map of Norway, showing the counties from which ISAV isolates have been collected from (modified from Devold et al., 2001).



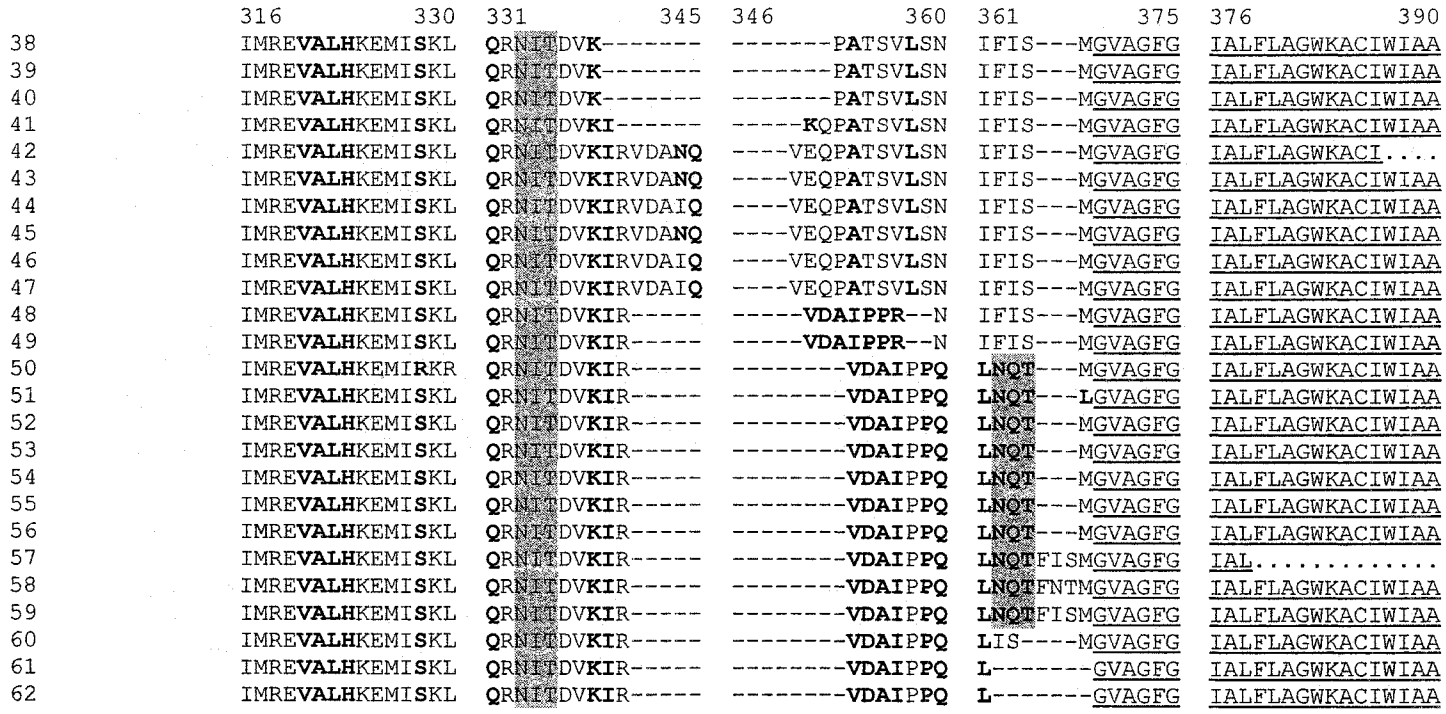


Figure 6: Alignment of partial deduced amino acid sequences of the putative haemagglutinin protein of 62 ISAV isolates demonstrating the hypervariable region (amino acid residues 338-367) in ORF2. Sequences that are not determined are indicated by dots (.) and deletions are indicated by dashes (-). Potential *N*-glycosylation sites are (▒) and a potential transmembrane region is underlined. ISAV identification numbers are referenced as listed in Table 5. Numbers 1-16 are ISAV isolates from New Brunswick, Canada and the lone isolate from Chile. Numbers 17-62 are ISAV isolates from Norway, Scotland and the lone isolate from Nova Scotia, Canada. Amino acid sequence alignment performed using CLUSTALW (Thompson et al., 1994; http://www.ebi.ac.uk/clustalw/index.html).

change of asparagine (N) to serine (S) at position 155 resulted in a loss of a potential glycosylation site, ^{155}NPT^{157} (asparagine, proline, threonine) in the European HA subtype, except isolate Glesvaer (number 19) (Kibenge et al., 2001b). This glycosylation site however, is probably not used (Krossøy et al., 2001a; Feldmann et al., 1988), and since one of the glycosylation sites is located at the cytoplasmic side of the predicted transmembrane region (Kibenge et al., 2001b) at amino acid position ^{400}NLS^{402} (asparagine, leucine, serine) found in the European subtypes, both subtypes share only one *N*-glycosylation site at position ^{333}NIT^{335} (asparaginine, isoleucine, threonine). There is a fourth *N*-glycosylation site at amino acid position ^{362}NQT^{364} (asparagine, glutamine, threonine) found in ten ISAV isolates (numbers 50 to 59) of the European genotype, which is close to the predicted transmembrane region (amino acid positions 368 to 409). There are two major mutation sites between the European and the North American subtypes. At position 320, all the isolates of the North American subtype contain ^{320}LEAQ^{323} (leucine, glutamine, alanine, glutamic acid) yet the isolates of the European subtype contain ^{320}VALH^{323} (valine, alanine, leucine, histidine). The second mutation site is a hypervariable region (HPR) that occurs as an insertion, deletion and amino acid change mix spanning amino acid residues 339 to 367, relative to isolate RPC/NB-980-280-2 (New Brunswick, Canada). Isolate RPC/NB-980-280-2 is depicted as strain number 1 in Figure 6. The one common *N*-glycosylation site occurs between both the major mutation sites. Three isolates, U5575-1 (number 57), Nordfjord (number 58) and Vestvågøy (number 59) have an addition of 3 amino acids at

position 365 to 367 that the other 59 ISAV isolates do not have. Nordfjord (county Sogn og Fjordane) and Vestvågøy (county Nordland) were found to be 96.5% and 97.1%, respectively, identical at the amino acid level to U5575-1.

3.3 Interference between ISAV isolates of different CHSE phenotypes

The CHSE-cell line was chosen for the viral neutralization tests because of the apparent antibody enhanced production of CPE in the SHK-1 and TO cell lines (Chapter 1.8). Although some ISAV isolates are unable to propagate on the CHSE-214 cell line (Kibenge et al., 2000) viral interference between the ISAV phenotypes on the CHSE-214 cell line may be used as a neutralization test (Dr. Kibenge, personal communication). However, the degree of interference between the two phenotypes needed to be determined. If neutralization of the CHSE-negative ISAV isolate occurred by addition of antiserum, the interference would be expected to cease and thus allow for the production of CPE by the CHSE-positive ISAV isolate.

ISAV isolates were first propagated on TO and CHSE-214 cell lines to calculate the level of infectivity of each isolate on each of the cell lines. ISAV isolates 7833-1 and U5575-1 were chosen because (a) antisera to both isolates were available and (b) both the phenotype and genotype of the isolates had been previously determined. ISAV isolates 7833-1 and U5575-1 were propagated to high titres in the TO cell line, with CPE occurring at day 6 post infection (p.i.) and were monitored till day 10 p.i. ISAV isolates 7833-1 and NBISA01 propagated to low titres on the CHSE-214 cell line with CPE occurring at 8 days p.i. and were monitored till day 14 p.i. The CPE detected in the TO cell line consisted of cell

rounding and lysis, with a destruction of the whole cell monolayer (Kibenge et al., 2001b; Wergeland and Jakobsen, 2001). The CPE due to ISAV detected in the CHSE-214 cell line also consisted of cell rounding and lysis but was focal (Kibenge et al., 2000; Kibenge et al., 2001a). IPNV was propagated on the CHSE-214 cell line to calculate the infectivity of the virus. The CPE in the CHSE-214 cell line due to IPNV was detected at day 1 p.i. and was complete by day 14 p.i. The CPE due to IPNV consisted of cell rounding and lysis and involved the whole cell monolayer. The viral titres of the ISAV isolates U5575-1, 7833-1 and NBISA01, and IPNV determined in this study are listed in Table 5.

In order to further study the interference between U5575-1 (CHSE-negative phenotype) and 7833-1 (CHSE-positive phenotype), tests involving the two ISAV isolates were performed using virus propagated in the TO cell line. To determine if the interference between the two phenotypes was at the level of the cell surface (i.e. ligand binding) or if it occurred intracellularly (replication inhibition), heat-inactivated U5575-1 was used in combination with 7833-1. To verify that U5575-1 had been completely heat-inactivated, treated samples were inoculated in TO cells. After 10 days p.i., the TO cells that were infected with the neat heat-inactivated ISAV isolate U5575-1 showed no CPE (Table 6). After 14 days p.i. CPE could be detected in the CHSE-214 cells that were infected with the undiluted 7833-1, while no CPE could be detected in the CHSE-214 cells that were infected with the undiluted U5575-1. The flask infected with the 1:1 mixture of 100 $TCID_{50}$ of U5575-1 and 100 $TCID_{50}$ 7833-1 showed no CPE after 14 days p.i. Furthermore no CPE was noted when

Table 5: Titres of the ISAV isolates and the IPNV isolate used in the study of interference between ISAV isolates of different phenotypes

Virus	Isolate	Cell Line	Titer	Final reading of CPE (days p.i.)	Type of CPE	Amount of monolayer destroyed
ISAV	U5575-1	TO	8.16[a]	10	cell rounding and lysis	whole monolayer
ISAV	7833-1	TO	8.5	10	cell rounding and lysis	whole monolayer
ISAV	7833-1	CHSE-214	3.16	14	cell rounding and lysis	focal
ISAV	NBISA01	CHSE-214	3.16	14	cell rounding and lysis	focal
IPNV	U3949.98	CHSE-214	6.32	14	cell rounding and lysis	whole monolayer

[a]Titre expressed as $\log_{10}$ $TCID_{50}$/100 µL

Table 6: Demonstration of interference between ISAV isolates

Strain of virus used	Cell line used	Final reading of CPE (days p.i.)	Presence of CPE	Presence of interference
7833-1	CHSE	38	yes	NA[a]
U5575-1	CHSE	38	no	NA
7833-1 and U5575-1	CHSE	38	no	yes
7833-1 and heat inactivated U5575-1	CHSE	38	no	yes
7833-1 and pH inactivated IPNV	CHSE	14	yes	no
heat inactivated U5575-1	TO	10	no	NA
pH inactivated IPNV	CHSE	14	no	NA

[a]Not applicable

U5575-1 was heat-inactivated in otherwise identical conditions. The maintenance media was replaced in the flasks and CPE was monitored for another 7 days. The flasks were monitored for 38 days (fresh media added every 7 days), however no CPE could be detected in the other flasks (Table 6) indicating that the CHSE-negative ISAV isolate (U5575-1) was able to interfere with the CHSE-positive ISAV isolate (7833-1). Furthermore, the lack of CPE in the flask containing the heat-inactivated 100 $TCID_{50}$ of U5575-1 and 100 $TCID_{50}$ of 7833-1 indicated that the interference was at the cell surface, i.e. that there was competition for a cell surface receptor. To prove that the interference was specific to ISAV, a mixed infection of pH-inactivated IPNV and 100 $TCID_{50}$ of ISAV isolate 7833-1 on one-day old CHSE-214 cells was preformed. To verify that IPNV was completely inactivated, treated samples were inoculated on CHSE-214 cells and were monitored for 14 days (Table 8). As shown in Table 6, the pH-inactivated IPNV did not interfere with 100 $TCID_{50}$ of 7833-1.

To establish the point at which the CHSE-negative ISAV isolate (U5575-1) could no longer interfere with the CHSE-positive ISAV isolates (7833-1 and NBISA01), "checkerboard" titrations were performed. Tables 7 and 8 show the results of the "checkerboard" titrations at day 14 p.i. It was determined that a dilution of 10^{-4} of U5575-1 (1 $TCID_{50}$) interfered with a dilution of 10^{-2} of 7833-1 (100 $TCID_{50}$),and a dilution of 10^{-4} of U5575-1 interfered with a dilution of 10^{-3} of NBISA01 (10 $TCID_{50}$), respectively. These lowest dilution combinations that did not produce CPE were chosen for the viral neutralization tests. The assumption was that if

Table 7: Checkerboard virus titration on CHSE-214 cells using ISAV isolates U5575-1 and **7833-1**.

U5575-1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
7833-1 10^{-1}[a]	-	+	+	+	+	+	+	+
7833-1 10^{-2}	-	-	-	-[b]	+	-	+	+
7833-1 10^{-3}	-	-	-	-	-	-	-	-
7833-1 10^{-4}	-	-	-	-	-	-	-	-
7833-1 10^{-5}	-	-	-	-	-	-	-	-
7833-1 10^{-6}	-	-	-	-	-	-	-	-

Table 8: Checkerboard virus titration on CHSE-214 cells using ISAV isolates U5575-1 and **NBISA01**.

U5575-1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
NBISA01 10^{-1}[a]	+	+	+	+	+	+	+	+
NBISA01 10^{-2}	+	+	+	+	+	-	+	-
NBISA01 10^{-3}	-	-	-	-[b]	+	+	-	-
NBISA01 10^{-4}	-	-	-	-	-	-	-	-
NBISA01 10^{-5}	-	-	-	-	-	-	-	-
NBISA01 10^{-6}	-	-	-	-	-	-	-	-

[a]virus dilution

[b]lowest dilution combination of ISAV isolates indicating interference

+: presence of CPE, therefore no interference

-: lack of CPE, therefore interference

CHSE-negative isolate (U5575-1) was neutralized by ISAV anti-serum, the interference would be neutralized and CPE due to the CHSE-positive isolate (7833-1 or NBISA01) would occur.

3.4 Virus neutralization of ISAV

The virus neutralization tests on CHSE 214 cells are summarized in Table 9. The results of 100 $TCID_{50}$ of 7833-1 and 100 $TCID_{50}$ of NBISA01 are extrapolations of three tests. In one test, CPE was detected in the 1 $TCID_{50}$ of 7833-1 and NBISA01 control wells, therefore too much virus had been added to the monolayers. In a repeat test, too little virus was added (minimal CPE was detected in only one of two control wells which should have contained 100 $TCID_{50}$). However, in the VN test involving the two CHSE phenotypes, the correct amount of both 7833-1 and NBISA01 were used, so combined with the previous two tests, the correct neutralizing capabilities could be determined. Antiserum to 7833-1 had a high titer (>1:1280) for ISAV isolate 7833-1 and a high titer (>1:1280) for ISAV isolate NBISA01 (Kibenge et al., 2001b), both isolates sharing the same HA genotype. The U5575-1 antiserum poorly neutralized both 7833-1 and NBISA01 (1:40). The neutralization tests with the mixed virus preparations of the CHSE-positive and CHSE-negative ISAV isolates showed similar results (Table 9). However, interference could not be detected in the control wells without antiserum. Antiserum to U5575-1 showed the same degree of neutralization of 7833-1 (1:40) but no neutralization to NBISA01. Yet, from the checkerboard titrations a level of 10 $TCID_{50}$ of NBISA01, which was the lowest dilution of virus that had interference

Table 9: Virus neutralization (VN) using rabbit and fish antisera on the CHSE-214 cell line.

		Anti-ISAV serum[a]	
Virus	Isolate(s)	Rabbit	Fish
ISAV	7833-1	>1280[b]	40[b]
ISAV	NBISA01	>1280[b]	40[b]
ISAV	7833-1 and U5575-1	>1280[c]	40[c]
ISAV	NBISA01 and U5575-1	>1280[c]	<10[c]
IPNV	U3949.98	40[b]	<10[b]

[a]Anti-ISAV serum: Rabbit: polyclonal antiserum to ISAV isolate 7833-1 (Kibenge et al., 2000; 2001b). Fish: pooled anti-ISAV sera obtained from experimentally infected rainbow trout using ISAV isolate U5575-1

[b]Virus neutralization titer, expressed as highest dilution of serum to completely neutralize the 100 $TCID_{50}$ of the respective ISAV isolate and IPNV isolate

[c]Virus neutralization titer, expressed as the highest dilution of serum to completely neutralize the ISAV phenotype combinations

with U5575-1, was used (Table 8). The antiserum to 7833-1 had a neutralizing titer of 1:40 against IPNV, while the U5575-1 antiserum did not neutralize IPNV (Table 9).

4.0 DISCUSSION

Through DNA sequence analysis and virus neutralization tests, this study supports the original observation that the two HA genotypes of ISAV correlate to two antigenic types (Kibenge et al., 2001b). The most probable basis for the difference in antigenicity between the two HA genotypes of ISAV are the two mutation sites spanning amino acid positions 320 to 323 and 338 to 367, the latter also known as the highly polymorphic region (HPR). The two mutation sites sandwich a single *N*-glycosylation site that is conserved among all ISAV isolates studied to date. However, virus neutralization tests with monoclonal antibodies (to the mentioned mutation sites) would have to be performed to confirm this observation. A better method to study virus neutralization of U5575-1 and ISAV isolates of the European genotype also needs to be developed before any possible cross-neutralization between the two antigenic groups can be determined.

It was found that the following isolates from the same geographical area were identical (i.e. 100% sequence identity on genome segment 6) to each other: isolates 390/98, 832/98 and 912/99 from Scotland; isolates Gulen and Mundheim; Sotra (isolated in 1993) and Eikelandsosen; Senja (isolated in 1996) and Senja (isolated in 1998) from Norway; isolates Bay of Fundy 97 and isolate 'Not specified', from New Brunswick, Canada. Homology of the other seven segments from these isolates would have to be performed to conclusively say that these isolates are truly the same. Isolates 390/98 and 832/98 were both recovered in 1998 while 912/99 was recovered in 1999, indicating that this isolate may have persisted for over one

year. Norwegian isolate Gulen was recovered in 1998 from the county Sogn og Fjordane, and Mundheim was isolated in 1998 from the county Hordaland (Figure 5) indicating the possibility that the same isolate was circulating in two separate counties at the same time. Sotra and Eikelandsosen were both isolated in 1993 from Hordaland county, allowing for the possibility that the same isolate was recovered from two separate areas within the same county. Senja (Troms county) was recovered in 1996 and again in 1998, indicating that the same isolate may have persisted in the area for at least two years. The similarities of these ISAV isolates may ultimately reflect the spread of the virus within an affected area and the ability for the virus to survive in the environment. However, as there are only two identities, there is insufficient data to reach such a conclusion. Fish and slaughter plant wastes (Vägsholm et al., 1994), ship movement (Murray et al., 2002), the ability for ISAV to spread from fish to fish (Totland et al., 1996; Rolland et al., 1998) and for the virus to survive in the environment (Nylund et al., 1994a) may be explanations for why an apparently identical isolate could occur in different areas at different time periods. The sequence of isolate 'Not Specified' was deposited in the GenBank database from the very first isolation of ISAV, made in February of 1997, from Atlantic salmon at a site in the Bay of Fundy that the Atlantic Veterinary College (Charlottetown, PE, Canada) had identified as having "haemorrhagic kidney syndrome" (HKS) (Dr. Steve Griffiths, personal communication) and may be the same isolate as 'Bay of Fundy 97' with which it is 100% identical.

It had been originally thought that the antigenic properties of ISA may be

responsible for different pathological responses due to ISAV. However, there is some evidence to support the possibility that the differences in ISA pathology are due to genetic variation among salmon strains rather than the virus. During an outbreak of ISA in Nova Scotia, Canada, diseased fish showed the same pathological lesions evident in diseased fish from New Brunswick, Canada (Dr. Dave Groman, personal communication), and researchers have experimentally infected groups of Norwegian fish stock with a New Brunswick ISAV isolate and a European ISAV isolate and found no difference in the development of the disease nor in the lesions (Dr. Are Nylund, personal communication). These are, however, theories only, and not conclusive evidence.

Infectious salmon anaemia virus has been included into the virus family *Orthomyxoviridae* (Anon., 2001), however, information on the genetic and antigenic variation of ISAV at the present time is not fully known. Therefore, some of the properties associated with antigenic makeup of the influenza viruses have been used to draw potential analogies with ISAV. The genetic and antigenic variation of the influenza viruses are well known. The HA and NA proteins of the influenza viruses occur in 15 and 9 different subtypes, respectively (Klenk et al., 1995; Skehel and Wiley, 2000). The two proteins which are of interest in studying the genetic and antigenic properties of ISAV are those encoded by genome segments 5 and 6. Segment 5 encodes the receptor destroying enzyme (Falk et al., 1997; Clouthier et al., 2002) recently characterized as an acetylesterase (Kristiansen et al., 2002). The equivalent protein in influenza viruses cleaves neuraminic acid residues of

glycoproteins and is designated as neuraminidase. Genome segment 6 of ISAV encodes the HA protein which has also been characterized (Rimstad et al., 2001; Krossøy et al., 2001; Griffiths et al., 2001) and has been the focus of the present study.

The sequence analysis of ISAV isolates U5575-1 and 390/98 showed them to be 95.2% identical at the nucleotide level and 93.6% identical at the amino acid level (Figures 3 and 4). The sequence differences between the two isolates consisted of only single nucleotide mismatches which were evenly spaced throughout the sequence, thus comparable to the sequence variation within genome segments 2 and 8 among various ISAV isolates (Blake et al., 1999). Sixty-two HA sequences from ISAV isolates deposited in the GenBank database (including U5575-1 and 390/98) were compared to each other at the nucleotide and amino acid levels. Two HA genotypes could be identified (Table 4, Appendix E) consistent with an initial analysis of 32 ISAV isolates (Kibenge et al., 2001b). The ISAV isolates from Norway, Scotland, and the one isolate from Nova Scotia, belong to the European genotype, whereas ISAV isolates from New Brunswick and the one isolate from Chile belong to the North American genotype. The two HA genotypes have a nucleotide sequence identity of only $\leq$80.4% and amino acid identity of $\leq$88.2% whereas, within each genotype, the sequence identities were >90.7%. Amino acid sequence identities of the HA protein can range from 25% to 80% for influenza isolates belonging to different subtypes (Nobusawa et al., 1990; Kawaoka et al., 1991; Wright et al., 1995; Shekel and Wiley, 2000). Therefore, for ISAV

based on these 62 isolates, strains belonging to the same genotype (subtype) have at least a 90% similarity to each other at the nucleotide and amino acid levels for the HA protein indicating less variation within a subtype than for influenza virus A.

As seen in the partial amino acid sequence alignment (Figure 6), both genotypes were found to share one *N*-glycosylation site at position ^{333}NIT^{335} (Kibenge et al., 2001b). The two major mutation sites were found between the European and the North American genotypes involving amino acid residues 320 to 323 and an HPR involving amino acid residues 338 to 367. The one common *N*-glycosylation site is found between these two mutation sites. Changes of any kind in the HA amino acid makeup, particularly around the *N*-glycosylation site may change the epiptope site(s) (Wiley and Shekel, 2000). Since all *N*-glycosylation sites are epiptope sites (Tizard, 1996), it is believed that the mutations occurring on either side of the one common *N*-glycosylation site may be the cause behind the antigenic type of the ISAV isolates.

The Nova Scotia isolate, U5575-1, recovered in 1999 is most closely related to Norwegian isolates Nordfjord (isolated in 1999) and Vestvågøy (isolated in 1993) within the European genotype on genome segment 6. The fact that ISAV isolates U5575-1, Nordfjord and Vestvågøy are 96.5% and 97.1%, respectively, identical on the HA protein and that all three isolates contain a unique three amino acid insertion which is absent in the other 59 isolates further supports the suggestion that U5575-1 may be a recent European ancestor in Canada and that the isolate might have

been introduced from Norway (Kibenge et al., 2001b; Ritchie et al., 2001b).

Much speculation has been made about the geographical and host origin of ISAV. Nylund et al. (1995a) suggested that ISA may be an "old" trout disease, after results from an experiment in which freshwater brown trout (from a lake isolated from sea water for over 5000 years) challenged with ISAV did not show any clinical signs of infection. Nylund et al. (1997) found that ISAV replication in rainbow trout was similar to that in brown trout and four explanations were advanced for the non-pathogenic effects of ISAV in rainbow trout and brown trout: (a) that the breeding regime of Atlantic salmon may have made the fish more susceptible, (b) the high concentration of farmed Atlantic salmon along the coast of Norway may have increased the transmission rate of the virus from brown trout, (c) a non-pathogenic virus may have mutated to a pathogenic form, and (d) that ISAV may have been introduced to Norway with the importation of rainbow trout from North America. Krossøy et al. (2001b) has reported that the divergence date between an isolate from Norway and an isolate from New Brunswick, Canada, was around 1900. Rainbow trout were introduced to Europe from North America around this time period, but on the other hand, brown trout were introduced to North America from Europe around the same time period (Krossøy et al., 2001b).

Overall, the ISAV isolates of the European genotype have a greater amino acid deletion sequence in the second major mutation site as compared to the isolates of the North American genotype, (4 to 20 amino acids long vs 3 to 13 amino acids long, respectively) and isolates of the European genotype are more variable

in the amino acid sequence of their HA protein. There are more missense mutations and the HPR has greater variability in the European genotype isolates when compared to the North American genotype isolates. This could be due to many possibilities including: perhaps the virus in Europe is more recent and is still evolving or perhaps it is due to the broader geographical separation of anadromous spawning populations (Dr. Steve Griffiths, personal communication). It has been speculated that the variation in the deletions of the second major mutation site is an indication that ISAV is still evolving (Kibenge et al., 2001b) as changes in the nucleotide make-up affect the amino acid profile. In contrast, Mjaaland et al. (2002) have stated they believe that the more uniform sequence patterns of the North American genotype may be indicative of these isolates having a single, more recent common source. However, the most likely explanation is because most if the isolates analyzed were of the European genotype (46 of European genotype as opposed to 16 of North American genotype). Mjaaland et al. (2002) compared only 6 North American genotype isolates as opposed to 70 isolates of the European genotype. As all the ISAV isolates in the amino acid sequence alignment have deletions relative to RPC/NB-980-280-2, it is possible that this isolate could be an archetypal ISAV isolate (Kibenge et al., 2001b) although it cannot be conclusively proven that ISAV originated in New Brunswick, Canada. There are currently two antigenic types of ISAV in North America (Kibenge et al., 2001b). However, a continuing increase in aquaculture activity may result in the further genetic variation of ISAV and other antigenic types may be identified. The strain variations of the

Norwegian ISAV isolates from 1987 to 2001 reported by Devold et al. (2001) are products of genetic drift since they all belong to the European genotype. The authors attributed these mutation events to reassortment which are known to occur within influenza viruses of the same subtype (Fields et al., 1996). However, Mjaaland et al. (2002) disagreed with the reassortment theory. These authors believe that the patterns of variability in the HPR are due to deletions from a longer donor virus gene which would include both the HPR1 and HPR2 indicated by Devold et al. (2001).

In addition to co-infection and reassortment, it is also possible that a co-infection of the cell by two viruses results in interference such that only one virus is able to replicate (Fields et al., 1996). If one interfering strain can be neutralized with specific antiserum then the replication of the second will be permitted (e.g. Alsono et al., 1999). The CHSE-negative ISAV isolate was able to interfere with CHSE-positive ISAV isolates such that the latter was no longer able to cause CPE (Tables 7 and 8). Heat inactivation of the CHSE-negative phenotype retained the ability to interfere with viral replication suggesting competition for a cell surface ligand rather than inhibition of intracellular replication (Table 6).

For the virus neutralization study, it was hypothesized that the CHSE-214 cell line could be used to further correlate the HA genotype to the antigenic type. It had previously been shown that antisera raised to isolates Back Bay '98, RPC/NB-980 049-1, 7833-1 and RPC/NB-980 028-10 (all of which are of the North American genotype and CHSE positive, except RPC/NB-980 028-10 which is CHSE negative)

were able to neutralize ISAV of the North American genotype in TO cells generally well, but neutralized ISAV isolates of European genotype poorly, indicating the presence of two antigenic groups (Kibenge et al., 2001b). However, TO cells are not sensitive enough to detect low level neutralization. Thus, the virus interference system in the CHSE-214 cell line was investigated in this study to determine if it would allow virus neutralization to be performed on ISAV of the European genotype as the interference at the cell surface was determined to be ISAV specific.

The viral neutralization tests further support Kibenge et al. (2001b). The 7833-1 antiserum had high neutralizing titres to 7833-1 and NBISA01 (both of the North American genotype) whereas the U5575-1 antiserum (European genotype) did not. However, both antisera were raised to the whole virus and not specifically to the HA protein itself. The RDE protein may play a role in the observed interference as this protein allows for the release of newly synthesized virus particles (Kristiansen et al., 2002). Influenza viruses are serotyped based on antibody titre ratios: any ratio >1 indicates the two viruses belong to the same serotype, while the homologous titre ratio is equal to one (Archetti and Horsefall, 1950). The homologous titer of U5575-1 could not be determined, so the antibody ratios and consequently the true serotyping of the isolates, could not be determined. The 7833-1 antiserum had low neutralizing titre against IPNV (1:40), while the U5575-1 antiserum did not neutralize IPNV at all (Table 9). The reason for this is not clear but may be due to cross-reactivity. This phenomenon happens when identical or similar epitopes are found on apparently unrelated antigens (Tizard,

1996).

This study has examined ISAV isolates from Norway, Scotland, Canada, and Chile. Infectious salmon anaemia has also been detected in Maine, USA, and Denmark. As ISAV continues to spread to other parts of the world and as the virus evolves, the HA protein may occur in new antigenic types. Moreover, any possible antigenic variation in the acetylesterase protein (encoded by segment 5) have yet to be explored to fully understand the correlation of the genotypes to the antigenic type of ISAV.

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APPENDIX A

Comparison of the ISAV genome to those of other genera in *Orthomyxoviridae* (modified from Mjaaland et al.,1997)

	Size Kb				
Genome Segment	*Influenzavirus A*	*Influenzavirus B*	*Influenzavirus C*	*Thogotovirus*	*Isavirus*[e]
1	2.34	2.38	2.35	2.3[a]	2.4
2	2.34	2.38	2.35	2.21[b]	2.3
3	2.23	2.30	2.15	1.9	2.2
4	1.74-1.77	1.88	2.07	1.6	2.0
5	1.56	1.84	1.80	1.4[c]	1.7
6	1.41	1.80	1.18	0.956[d]	1.5
7	1.03	1.19	0.93	----	1.3
8	0.89	1.09	*----	----	1.0
Total	13.6	14.5	12.9	10.36	14.4

*---- denotes no segment.

a: Weber et al. (1999); b: Leahy et al. (1997); c: Weber et al. (1996); d: Kochs et al. (2000); e: Anon., 2001

APPENDIX B

Accession numbers of reported ISAV sequence data *---- denotes not given

Country of Origin	Location or identification number and year of isolation	GenBank Accession Number (Genome Segments)							
		1	2	3	4	5	6	7	8
Norway	Eikelandsosen 1987	*---	----	----	----	---	AF364893	----	----
Norway	Eikelandsosen 1989	----	AF262394	----	----	----	----	----	AF262386
Norway	Foya 1997	----	----	----	----	----	AF364897	----	----
Norway	Misund 1999	----	----	----	----	----	AF364896	----	----
Norway	Vestvågøy 1993	----	----	----	----	----	AF364895	----	----
Norway	Lepsoy 2000	----	----	----	----	----	AF364892	----	----
Norway	Fjaler 2000	----	----	----	----	----	AF364890	----	----
Norway	Senja 1996	----	----	----	----	----	AF364889	----	----
Norway	Nordfjord 1999	----	----	----	----	----	AF364888	----	----
Norway	Senja 1998	----	----	----	----	----	AF364887	----	----
Norway	Frøya 1997	----	----	----	----	----	AF364885	----	----
Norway	Solund 2000	----	----	----	----	----	AF364884	----	----
Norway	Dønna 1998	----	----	----	----	----	AF364883	----	----
Norway	Øygarden 2000	----	----	----	----	----	AF364882	----	----
Norway	Blåmannsvik 1998	----	----	----	----	----	AF364881	----	----
Norway	Sørnes 2000	----	----	----	----	----	AF364880	----	----
Norway	Skatestraumen 1999	----	----	----	----	----	AF364878	----	----

Norway	Strandebaum 1998	----	----	----	----	----	AF364877	----	----
Norway	Hennigsvaer 1999	----	----	----	----	----	AF364876	----	----
Norway	Åfjord 1997	----	----	----	----	----	AF364875	----	----
Norway	Naerøy 1998	----	----	----	----	----	AF364874	----	----
Norway	Selje 1995	----	----	----	----	----	AF364873	----	----
Norway	Torgnes 1997	----	----	----	----	----	AF364872	----	----
Norway	Gulen 1998	----	----	----	----	----	AF364871	----	----
Norway	Mendheim 1999	----	----	----	----	----	AF364870	----	----
Norway	Landøy 1996	----	----	----	----	----	AF364869	----	----
Norway	Glesvaer 1990	----	AF262398	----	AF421317	----	AF283998	AF220607 AJ306488	AF262382 AJ012285
Norway	Gullesfjord 1994	----	AF262396	----	----	----	AF302801	----	AF262384
Norway	Varaldsøy 1996	----	AF262397	----	----	----	AF364891	----	AF262388
Norway	Sotra 1991	----	----	----	----	----	AF364894	----	----
Norway	Sotra 1992	----	----	----	----	----	AF364898	----	----
Norway	Sotra 1993	----	AJ002475	----	----	----	AF309075	----	----
Norway	Svolvaer 1996	----	AF262393	----	----	----	----	----	AF262381
Norway	Bremnes 1998	AY168787	AF262391	----	----	----	AF302799	AY044132	AF262385
Norway	Brekke 1998	----	AF262390	----	----	----	----	----	AF262380
Norway	Hitra 1996	----	----	----	----	----	AF364886	----	----
Norway	Hitra 1997	----	----	----	----	----	AF364879	----	----
Norway	Vedoy 1999	----	AF262395	----	----	----	AF302803	----	AF262383

Norway	819/9/99	----	----	----	----	----	AF378180	----	----
Norway	485/9/97	----	----	----	----	----	AF378181	----	----
Norway	835/9/98	----	----	----	----	----	AF378179	----	----
Norway	HI 1992	----	----	----	----	----	AF294882	----	----
Norway	unknown	----	----	----	----	----	----	AF429990	----
Scotland	Loch Nevis 1998	AJ514403	AF262392	----	----	----	AF302802	----	AF262387
Scotland	390/98	----	----	AJ276858	----	AF429988	AJ276859 AF283997	AJ306487	----
Scotland	not stated 1999	----	----	----	----	----	----	----	AJ242016
Scotland	not stated 1999	----	AJ242808	----	----	----	----	----	----
Scotland	1490/98	----	----	----	----	----	AF391126	----	----
Scotland	301/98	----	----	----	----	----	AF388581	----	----
Scotland	912/99	----	----	----	----	----	AF395337	----	----
Scotland	832/98	----	----	----	----	----	AF388582	----	----
Canada	U5575-1	----	----	----	----	----	AF294881	----	----
Canada	NS 7035-1	----	----	----	----	----	----	AF361363	----
Canada	NB 000912-2	----	----	----	----	----	----	AF328627	----
Canada	Not stated	----	----	AF306549	AF306548	----	----	----	----
Canada	Bay of Fundy 1997	----	AF262399	----	----	----	AF302800	----	AF262389
Canada	Back Bay 1998	----	----	----	----	----	AF283995	----	----
Canada	Bliss Harbor 1997	----	AF095254	----	----	----	----	----	AF095255
Canada	RPC/NB-970-877-2 1997	----	----	----	----	----	AF294877	----	AF312316

Canada	RPC/NB-980-280-2 1998	----	----	----	----	----	AF294870	----	AF312317
Canada	RPC/NB-990-681-3	----	----	----	----	----	AF294880	----	----
Canada	HKS-36	----	----	----	----	----	AF294878	----	----
Canada	RPC/NB-990-002-2	----	----	----	----	----	AF294875	----	----
Canada	RPC/NB-990-508-3	----	----	----	----	----	AF294874	----	----
Canada	RPC/NB-458	----	----	----	----	----	AF294873	----	----
Canada	Not stated	----	----	----	----	----	AX083268	----	----
Canada	GA/TO Fish 03	----	----	----	----	----	AF297551	----	----
Canada	RPC/NB-980-049-1	----	----	----	----	----	AF294876	----	----
Canada	DFO-1	----	----	----	----	----	AF294872	----	----
Canada	RPC/NB-028-10	----	----	----	----	----	AF294871	----	----
Canada	NBISA01	----	----	----	----	----	AF283996	----	----
Canada	CCBB	AF404347	AF404346	AF404345	AF404344	AF404343	AF404342	AF404341	AF404340
USA	ME/01	---	---	---	---	AF429986	AY059402	AF429989	----
Chile	7833-1 1999	----	AF287850	----	----	----	AF294879	----	AF312315

APPENDIX C COMMONLY USED BUFFERS

Growth media for CHSE-214 cells

10% FBS
1% 100X antibiotics
in MEM

Growth media for TO cells

10% FBS
1% gentamycin
0.1% L-glutamine
in Hank's MEM

Maintenance media for CHSE-214 cells

5% FBS
1% 100X antibiotics
in MEM

Maintenance media for TO cells

5% FBS
1% gentamycin
0.1% L-glutamine
in Hank's MEM

Solution #1 for mini-preparations

50 mM glucose
10 mM EDTA
25 mM Tris-HCl, pH 8.0

Solution #2 for mini-preparations

0.2 N NaOH
1% SDS

Solution #3 for mini-preparations

3 M potassium
5 M acetate

TBE sequencing buffer

12% Tris, pH 8.3
5% Boric acid
0.3% EDTA

TE buffer

10 mM Tris, pH 8.3
1 mM EDTA

2X YT Broth

16% Bactotryptone
10% Bactoyeast extract
5% NaCl

YT agar plates

0.8% Bactotryptone
0.5% Bactoyeast extract
0.5% NaCl
1.5% agar

APPENDIX D

Amino Acid Notation (Commission on Biochemical nomenclature, 1968)

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

APPENDIX E

Nucleotide and amino acid sequence identities (%) of the haemagglutinin gene of several ISAV isolates. The ISAV strain numbering used is listed in Table 5. The nucleotide sequence identities of the HA genome are illustrated in the upper right half and the amino acid sequence homologies are found in the lower left half of the table.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	x	99.6	99.4	99.4	99.3	99.3	99.3	99.2	99.1	99	99	98.7	98.6	98.9	99.1	99.2	78.9	80.6	78.8	79.2	79.2	79.2
2	95.5	x	99.7	99.7	99.6	99.6	99.4	99.5	99.4	99.2	99.3	99.1	98.9	99.1	99.5	99.6	78.6	79.4	78.5	79.4	79.4	79.4
3	95.2	99.7	x	99.7	99.7	99.6	99.6	99.4	99.5	99.4	99.6	99.2	99.2	99.4	99.8	99.7	78.6	79.4	78.5	79.4	79.4	79.4
4	95.2	99.7	100	x	99.9	99.9	99.7	99.8	99.8	99.7	99.6	99.2	99.2	99.4	99.8	99.7	78.6	79.4	78.5	79.4	79.4	79.4
5	94.9	99.4	99.7	99.7	x	99.8	99.6	99.7	99.6	99.4	99.5	99.1	99.3	99.3	99.7	99.6	78.5	79.3	78.4	79.3	79.3	79.3
6	94.9	99.4	99.7	99.7	99.4	x	99.6	99.7	99.6	99.4	99.5	99.1	99.1	99.3	99.7	99.6	78.5	79.3	78.4	79.3	79.3	79.3
7	94.3	98.8	99.1	99.1	98.8	98.8	x	99.5	99.4	99.2	99.3	98.9	98.9	99.1	99.5	99.4	78.8	79.1	78.2	79.3	79.3	79.3
8	94.9	99.4	99.7	99.7	99.4	99.4	98.8	x	99.7	99.5	99.6	99	99	99.2	99.6	99.5	78.6	79.4	78.5	79.4	79.4	79.4
9	94.3	98.8	99.1	99.1	98.8	98.8	98.3	99.4	x	99.4	99.5	99.3	98.9	99.1	99.5	99.4	78.5	79.4	78.4	79.3	79.3	79.3
10	94.6	99.1	99.4	99.4	99.1	99.1	98.5	99.7	99.1	x	99.3	98.7	98.7	98.9	99.3	99.2	78.2	79	78.1	79.2	79.2	79.2
11	94.1	98.5	98.8	98.8	98.5	98.5	98	99.1	98.5	98.8	x	98.8	98.8	99	99.4	99.3	78.3	79.1	78.2	79.1	79.1	79.1
12	93.5	98	98.3	98.3	98	98	97.4	98	98.5	97.7	97.1	x	98.4	98.6	99	98.9	78.6	79.2	78.3	79.2	79.2	79.2
13	93.5	98	98.3	98.3	98.5	98	97.4	98	97.4	97.7	97.1	96.5	x	98.6	99	98.9	77.9	78.7	77.8	78.7	78.7	79.2
14	95.5	99.4	99.7	99.7	99.4	99.4	98.8	99.4	98.8	99.1	98.5	97.9	97.9	x	100	99.3	79.1	79.2	78.1	79.1	80.1	80.1
15	95.1	99.4	99.7	99.7	99.4	99.4	98.8	99.4	98.8	99.1	98.5	97.9	97.9	100	x	99.2	79.3	79.1	78.1	79.1	79.5	79.5
16	94.9	99.1	99.4	99.4	99.1	99.1	98.5	99.1	98.5	98.8	98.2	97.6	97.6	99.1	99.1	x	79.2	79.2	78.1	79.1	79.1	79.1
17	84.4	83.7	84	84	83.7	83.7	83.1	83.7	83.7	83.4	83.1	83.7	82.2	85.4	85.4	85.4	x	99.1	95.7	98.5	98.5	98.5
18	81.8	85.7	86.1	86.1	85.7	85.7	85.1	85.7	85.7	85.5	85.1	85.7	84.2	85.9	85.8	85.7	99.6	x	97.8	98.3	98.5	98.5
19	77.9	80.8	81	81	80.8	80.8	80.5	80.8	80.8	80.8	80.2	80.8	79.3	85.6	84.5	84.5	92	93.4	x	98	98	98
20	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	84.2	84.3	84.2	96.3	98.1	94.4	x	100	100
21	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	85.3	84.3	84.2	96.3	96.6	94.4	100	x	100
22	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	85.3	84.3	84.2	96.3	96.6	94.4	100	100	x
23	84.1	84	84.3	84	84	84	83.4	84	84	84	83.4	84	82.5	85.1	84	83.9	96.3	96.6	94.4	99.4	99.5	99.5
24	84.1	84	84.3	84.3	84	84	83.4	84	84	84	83.4	84	82.5	83.9	83.7	83.9	95.4	96.7	93.5	99.1	99.1	99.1
25	78.2	78.4	78.4	78.4	78.3	78.3	78.3	78.4	78.3	78.2	78.1	78.2	77.7	78.1	78.2	78.2	97.5	95.2	97.2	99	99	99
26	81.3	83.9	84.2	84.2	83.9	83.9	83.3	83.9	83.9	83.9	83.3	83.9	82.5	85.1	84	83.9	98.1	98.1	93.5	99.1	98.9	98.9
27	81.5	84.2	84.5	84.5	84.2	84.2	83.6	84.2	84.2	84.2	83.6	84.2	82.8	85.3	84.3	87.2	99.3	98.3	94.7	99.7	99.7	99.7
28	81	83.6	83.9	83.9	83.6	83.6	83	83.6	87.1	83.6	83	83.6	82.2	84.8	83.7	83.6	98.4	97.7	93.5	98.1	98.6	98.6

Continued

23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
79.2	78.8	82.2	78.6	79	78.9	79.2	79.2	78.9	78.7	78.7	79.4	78.6	78.4	78.7	78.9	78.9	78.9	79.1	78.4	79.2	78.8	79
79.4	79	82.2	78.8	79.2	79.1	79.4	79.4	79.1	78.9	78.9	79.6	78.8	78.6	78.5	79.3	79.3	79.3	78.9	77.8	79	78.3	78.3
79.4	79	82.5	78.8	79.2	79.1	79.4	79.4	79.1	79.1	78.9	79.7	79	78.6	78.5	79.3	79.3	79.3	79	77.7	78.5	78.5	78.9
79.4	79	82.5	78.8	79.2	79.1	79.3	79.4	79.1	78.9	78.9	79.7	79.4	79.4	78.5	79.3	79.3	79.3	78.8	77.7	79	78.5	78.9
79.3	78.9	82.2	78.7	79.1	79	79.2	79.3	79	78.8	78.8	79.6	78.7	78.5	78.4	79.2	79.2	79.2	78.9	77.6	78.9	78.4	78.8
79.3	78.9	82.2	78.7	79.1	79	79.3	79.2	79	78.8	78.8	79.5	78.7	78.5	78.4	79.2	79.2	79.2	78.8	77.6	78.9	78.5	78.7
79.3	78.9	81.6	78.7	79.1	79	79.4	79.3	79	78.8	78.8	79.5	78.7	78.5	78.2	79.2	78.4	79.2	78.7	77.9	79	78.4	78.8
79.4	79	82.2	78.8	79.2	80	79.4	79.3	79.2	78.9	78.9	78.2	78.8	78.6	78.5	79.3	79.3	79.3	78.7	77.7	79.1	78.5	78.9
79.3	78.9	82.2	79.7	80.1	78.9	79.2	80.3	80	80	79.8	79.1	80.4	80.3	78.4	80.2	80.2	80.2	79.7	77.7	79.5	79.2	79.2
79.2	78.8	82.2	78.6	79	78.8	79.2	79.2	78.9	78.7	78.7	79.5	78.6	78.4	78.1	79.1	79.1	79.1	79	77.3	78.8	78.7	78.6
79.1	78.7	81.6	78.5	78.9	78.9	79.1	79.1	78.8	78.6	78.6	79.4	78.5	78.3	78.2	79	79	79	78.6	77.4	78.8	78.2	78.6
79.2	78.8	82.2	78.6	79	79.2	79.2	79.2	78.9	78.7	78.7	79.5	78.6	78.4	78.3	79.1	79.1	79.1	78.8	77.7	78.9	78.3	78.7
78.7	78.2	80.8	78.1	78.5	78.7	78.6	78.6	78.4	78.2	78.2	79	78.1	77.9	78	78.8	78.8	78.8	78.3	77	78.4	77.8	78.2
80.1	78.6	82.2	79.6	80.1	77.9	80.2	80.2	79.9	79.3	78.9	80.3	79.7	79.4	78.8	80.1	80.1	80.1	80	77.6	79.4	79.3	79.2
79.5	78.7	81.9	79	79.5	79.3	79.6	79.6	79.7	79.1	78.7	79.8	79.1	78.8	78.9	79.5	79.5	79.5	79	77.9	79	78.7	78.8
79.1	78.7	82.2	78.6	79.1	78.1	79.2	79.2	79.7	79.1	78.7	79.1	78.7	79.2	78.9	78.1	78.1	79.1	78.7	77.4	78.2	78.3	78
98.5	*98.1*	*93.7*	*98*	*98.3*	*97.9*	*98.6*	*98.1*	*98.8*	*98*	*98.1*	*98.9*	*99.1*	*98.9*	*98.7*	*98.9*	*98.9*	*98.9*	*97.7*	*99.6*	*99.7*	*98.7*	*98.7*
98.5	*98.4*	*97.8*	*98.3*	*98.3*	*98*	*97.7*	*98.2*	*98.7*	*98.3*	*98.4*	*97.3*	*98.5*	*98.3*	*98.8*	*99*	*99*	*99*	*98*	*96.5*	*96.9*	*97.8*	*97.8*
98	*97.7*	*92.1*	*97.3*	*97.8*	*97.2*	*96.5*	*97.5*	*95.5*	*97.4*	*97.4*	*98*	*97*	*96.1*	*98.4*	*98.5*	*98.9*	*98.5*	*97.3*	*94.8*	*98.1*	*96.5*	*96.5*
99.8	*99.6*	*97.3*	*99.5*	*99.6*	*99.4*	*98.7*	*99.5*	*98.3*	*99.6*	*99.6*	*97.9*	*98.9*	*98.4*	*98.8*	*99*	*99*	*99*	*99.3*	*97.8*	*98.4*	*98.8*	*98.8*
99.8	*99.6*	*97.3*	*99.4*	*99.6*	*99.3*	*98.8*	*99.4*	*98.4*	*99.4*	*99.5*	*98*	*98.9*	*98.5*	*98.8*	*98.9*	*98.9*	*98.8*	*99.2*	*98.1*	*98.5*	*98.9*	*98.9*
99.8	*99.6*	*96.7*	*99.4*	*99.6*	*99.3*	*98.8*	*99.4*	*98.4*	*99.4*	*99.5*	*98*	*98.9*	*98.5*	*98.8*	*98.9*	*98.9*	*98.8*	*99.2*	*98.1*	*98.5*	*98.9*	*98.9*
x	*99.4*	*96.7*	*99.3*	*99.6*	*99.2*	*98.8*	*99.4*	*98.4*	*99.3*	*99.3*	*98*	*98.9*	*98.5*	*98.8*	*98.9*	*98.8*	*98.8*	*99*	*98.1*	*98.5*	*98.9*	*98.9*
98.5	x	*98.2*	*99.9*	*99.2*	*99.4*	*98.3*	*99.1*	*98*	*99.8*	*100*	*97.5*	*98.5*	*98.3*	*98.4*	*98.6*	*98.6*	*98.6*	*99.3*	*97.7*	*98.1*	*98.5*	*98.5*
98.8	*97.4*	x	*99.3*	*98.6*	*98.8*	*97.7*	*98.5*	*97.4*	*99.2*	*99.4*	*96.9*	*97.9*	*97.7*	*95.9*	*98*	*98*	*98*	*98.7*	*97.2*	*97.5*	*97.8*	*97.8*
98.4	*100*	*98.2*	x	*99.2*	*99.3*	*98.3*	*99.1*	*98*	*99.6*	*99.9*	*97.7*	*98.5*	*98.3*	*98.3*	*98.6*	*98.6*	*98.5*	*97.2*	*97.6*	*98.1*	*98.4*	*98.4*
99.7	*98.8*	*97*	*97.7*	x	*99.2*	*98.8*	*99.5*	*98.4*	*99.3*	*99.4*	*98.1*	*99*	*98.5*	*98.6*	*98.8*	*99*	*99*	*99.1*	*98.1*	*98.5*	*98.9*	*98.9*
98.1	*99.1*	*97.3*	*99.2*	*97.3*	x	*99*	*98.9*	*99.3*	*99.4*	*99.4*	*97.8*	*99.3*	*99.3*	*98.2*	*98.5*	*98.5*	*98.4*	*98.3*	*97.5*	*98*	*98.4*	*97.8*

Continued

46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
78.7	78.7	78.9	79	78.5	78.6	79.1	79.1	79.1	79.1	79.1	78.1	79	79	79.1	78.7	79
78.4	78.4	79.4	79.5	78.3	78.6	79.2	79.2	79.2	79.2	79.2	78.4	78	79.5	79.4	78	79.4
78.3	78.4	79.4	79.5	78.3	78.8	79.2	79.4	79.2	79.2	79.2	78.5	79.3	79.5	79.4	78	79.4
78.4	78.4	79.4	79.5	78.3	78.6	79.2	79.2	79.1	79.2	79.2	78.5	79.3	79.5	79.4	78.9	79.4
78.3	78.3	79.3	79.4	78.2	78.5	79.1	78.6	79.2	79.1	79.1	78.4	79.2	79.4	79.3	77.9	79.3
78.4	78.4	79.2	79.4	78.2	78.4	78.8	79.1	79.1	78.6	78.6	78.4	79.1	79.3	79.2	77.9	79.2
78.3	78.3	79.1	79.2	78.1	78.5	79.1	79.2	78.6	79.1	79.1	78.2	79	79.2	79.3	79	79.3
78.4	78.4	79	79.2	78.3	78.6	79.1	79.2	79.1	79.2	79.2	78.5	79.3	79.5	79.4	78	79.3
79.3	79.3	80.2	80.4	78.2	79.6	79.9	80.3	79.9	80	80	78.4	78.9	80.4	80.2	79.8	80.3
78.6	78.6	79	79.1	78	78.5	78.8	78.8	78.8	78.8	78.8	78.2	79	79.2	79.1	77.8	78
78.1	78.1	79.1	79.2	78	78.3	78.9	78.9	78.9	78.9	78.9	78.2	79	79.2	79.1	77.7	79.1
78.2	78.2	79.2	79.3	78.1	78.4	79	79	79	79	79	78.4	79.1	79.3	79.2	77.8	79.2
77.7	77.7	78.9	78.8	77.6	77.9	78.5	78.5	78.5	78.5	78.5	77.8	78.6	78.8	78.7	77.3	78.6
79.2	79.2	80.1	80.2	79.1	79.5	79.9	79.9	79.9	79.9	79.9	77.9	80.2	80.4	80	78.8	79
78.6	78.6	79.2	79.3	78.6	79	79.4	79.4	79.4	79.4	79.4	77.9	79.1	79.3	79.6	78.2	79.5
78.2	78.2	79.2	79.3	78.2	78.6	79	79	79	79	79	78.1	79.1	79.3	79.2	77.8	78
99.7	*98.8*	*99.3*	*99*	*95.2*	*98.6*	*98.9*	*98.1*	*99*	*98.8*	*98.2*	*95.7*	*98.8*	*99.1*	*99.1*	*98.1*	*98.9*
96.7	*97.9*	*97.1*	*98.4*	*97.9*	*98.3*	*98.6*	*98.2*	*98.8*	*98.7*	*98.1*	*95.3*	*97.3*	*98.4*	*99.3*	*98.4*	*98*
94.9	*96.6*	*96.6*	*96.2*	*95.7*	*95.5*	*95.9*	*97.5*	*96.1*	*95.5*	*95*	*93.1*	*98*	*98.3*	*96.1*	*97.4*	*98.1*
98.2	*98.9*	*98.7*	*98.5*	*96.2*	*98.9*	*98.9*	*99.5*	*99.1*	*98.3*	*97.7*	*95.2*	*98.1*	*99.2*	*97.8*	*99.6*	*99*
98.3	*99*	*98.8*	*98.6*	*96.4*	*99*	*98.9*	*99.3*	*99.1*	*98.4*	*97.8*	*92.9*	*98.1*	*99.3*	*97.9*	*99.5*	*99.1*
98.3	*99*	*98.8*	*98.6*	*96.4*	*99*	*98.9*	*99.3*	*99.1*	*98.4*	*97.8*	*92.9*	*98.1*	*99.3*	*97.9*	*99.5*	*99.1*
98.3	*99*	*98.8*	*98.6*	*96.4*	*98.8*	*99.5*	*99.3*	*99.1*	*98.4*	*97.8*	*92.9*	*98*	*99.3*	*97.9*	*99.3*	*99.1*
97.9	*98.5*	*98.3*	*98.4*	*95.9*	*98.9*	*98.5*	*99.3*	*98.7*	*98*	*97.4*	*94.8*	*98.1*	*98.8*	*97.5*	*100*	*98.6*
97.3	*97.9*	*97.6*	*97.8*	*95.3*	*97.6*	*97.9*	*98.5*	*98.1*	*97.4*	*96.8*	*94.2*	*97.5*	*98.2*	*96.9*	*99.4*	*99.1*
97.9	*98.5*	*98.3*	*98.4*	*97.3*	*96.7*	*98.4*	*99*	*98.6*	*98*	*97.4*	*92.5*	*98.1*	*98.8*	*97.6*	*99.9*	*99.5*
98.3	*99*	*98.8*	*98.6*	*97.4*	*95.4*	*98.9*	*99.5*	*99.1*	*98.4*	*97.8*	*92.9*	*98*	*99.3*	*97.2*	*99.4*	*98.5*
98.5	*98.3*	*98*	*98.1*	*96.9*	*96.7*	*98.2*	*98.4*	*97.7*	*97.7*	*97.3*	*94.4*	*98.4*	*98.6*	*97.6*	*98.2*	*98.1*

Continued

29	81.8	84.5	84.8	84.8	84.5	83	83.9	83.9	87.8	84.5	83.9	84.5	83	85.6	84.5	84.5	*99*	*98.1*	*94.4*	*98.6*	*99.2*	*99.2*
30	81.8	84.5	84.8	84.8	84.5	83	83.9	83.9	87.8	84.5	83.9	84.5	83	85.6	84.5	84.5	*99*	*98.1*	*94.4*	*98.4*	*98.9*	*98.9*
31	81	83.6	83.9	83.9	83.6	83.6	83	83.6	86.7	83.6	83	83.6	82.2	84.5	83.7	83.6	*98.1*	*97.7*	*93.5*	*98.4*	*98.6*	*98.6*
32	81.3	83.9	84.2	84.2	83.9	82.5	83.3	83.9	87.1	83.9	83.3	83.9	82.5	85.1	84	83.9	*99*	*98.1*	*93.5*	*98.4*	*98.9*	*98.9*
33	81.3	83.9	84.2	84.2	83.9	83.9	83.3	83.9	87.1	83.9	83.3	83.9	82.5	85.1	84	83.9	*98.1*	*98.1*	*93.5*	*98.4*	*98.9*	*98.9*
34	81.5	84.2	84.2	84.5	84.2	82.7	83.6	84.2	88.2	84.2	83.6	84.5	82.7	85.9	84.8	84.2	*98.7*	*98.1*	*93.8*	*98.6*	*98.9*	*98.9*
35	81.3	83.9	84.2	84.2	83.9	83.9	83.3	83.9	87.1	83.9	83.3	83.9	82.5	84.8	84	83.9	*98.4*	*98.1*	*93.8*	*98.6*	*98.9*	*98.9*
36	81.3	82.4	84.2	84.2	83.9	82.5	83.3	83.9	87.1	83.9	83.3	83.9	82.5	85.1	84	83.9	*98.1*	*98.1*	*93.2*	*98.1*	*98.6*	*98.6*
37	81.6	83.7	84	84	83.7	83.7	83.1	83.7	83.7	84	83.1	83.7	82.2	85.6	83.4	83.6	*96.6*	*96.4*	*93.8*	*98.5*	*98.5*	*98.5*
38	81.8	83.9	84.2	84.2	83.9	83.9	83.3	83.9	83.9	84.2	83.3	83.9	82.5	80.1	79.5	78.1	*100*	*96.3*	*94.4*	*99.1*	*98.7*	*98.7*
39	81.8	83.9	84.2	84.2	83.9	82.5	83.3	83.9	87.5	84.2	83.3	83.9	82.5	84.8	84	83.9	*100*	*98.5*	*98.3*	*98.4*	*98.7*	*98.7*
40	81.8	83.9	84.2	84.2	83.9	82.7	83.3	83.9	87.5	83.9	83.3	83.9	82.7	84.8	84	83.9	*100*	*98.5*	*93.5*	*98.4*	*98.7*	*98.7*
41	81.5	83.6	83.9	83.9	83.6	83.6	83	83.6	87.5	83.3	83	83.6	82.2	85.3	84.3	83.6	*98.4*	*98.5*	*93.2*	*96.9*	*97.4*	*97.4*
42	83.5	82.7	83	83	82.7	82.7	82.1	82.7	82.7	83	82.1	82.7	81.2	83.1	83	83	*99.3*	*96.9*	*92.1*	*97.7*	*95.7*	*95.7*
43	84.1	83.6	83.9	83.9	83.6	82.2	83	83.6	87.8	83.6	83	83.6	82.2	85.3	84.2	83.6	*99.6*	*98.5*	*92.2*	*95.7*	*96.2*	*96.2*
44	84.1	84.1	84.2	84.2	83.9	82.5	83.3	83.9	87.5	83.6	83.3	83.9	82.5	85	84	83.3	*99.6*	*98.5*	*92.5*	*95.7*	*96.2*	*96.2*
45	83.5	83.8	83.6	83.6	83.3	81.9	82.8	82.8	87.5	83.3	82.8	83.3	81.9	85	84	83.3	*99.3*	*98.1*	*91.7*	*95.4*	*95.9*	*95.9*
46	84.1	83.9	84.2	84.2	83.9	82.5	83.3	83.3	87.1	83.6	83.3	83.9	82.5	85	84	83.9	*99.3*	*98.5*	*92.2*	*97.5*	*95.9*	*95.9*
47	84.1	83.9	84.2	84.2	83.9	82.5	83.3	83.3	87.1	83.6	83.3	83.9	82.5	85	84	83.9	*99.3*	*98.1*	*92.2*	*95.4*	*95.9*	*95.9*
48	81.8	84.8	85.1	85.1	84.8	83.3	84.2	84.8	86.7	84.5	84.2	84.8	83.3	85.9	84.8	84.8	*99.3*	*98.1*	*93.2*	*96.7*	*96.9*	*96.9*
49	81.8	84.8	85.1	85.1	84.8	83.3	84.2	84.8	87.6	84.5	84.2	84.8	83.3	85.9	84.8	84.8	*99.3*	*98.5*	*91.8*	*96.3*	*96.9*	*96.9*
50	83.9	83.7	84	84	83.7	83.7	83.4	83.7	83.7	83.4	83.1	83.7	82.2	84.8	83.7	83.6	*92.7*	*95.5*	*95.6*	*93.8*	*94.1*	*94.1*
51	80.1	83.9	84.2	84.2	83.9	82.5	83.6	83.9	87.1	83.6	83.3	83.9	82.5	85.1	84	83.9	*99*	*98.1*	*93.5*	*96*	*95.8*	*95.8*
52	81	84.8	85.1	85.1	84.8	83.3	84.5	84.8	87.5	84.5	84.2	84.8	83.3	85.6	84.8	84.8	*99.6*	*98.1*	*93.5*	*95.7*	*96.2*	*96.2*
53	81	84.8	85.1	85.1	84.8	83.3	84.5	84.5	87.5	84.5	84.2	84.8	83.3	85.6	84.5	84.8	*99.6*	*98.1*	*93.5*	*95.8*	*95.8*	*95.8*
54	80.7	84.5	84.8	84.8	84.5	83	84.2	84.2	87.5	84.2	83.9	84.5	83	85.6	84.5	84.5	*99.3*	*97.7*	*93.2*	*95.5*	*95.8*	*95.8*
55	80.7	84.5	84.8	84.8	84.5	83	84.2	84.2	87.5	84.2	83.9	84.5	83	85.6	84.4	84.4	*99.3*	*97.7*	*93.2*	*95.5*	*95.6*	*95.6*
56	80.4	84.2	84.5	84.5	84.2	82.7	83.9	84.2	87.1	83.9	83.6	84.2	82.7	85.3	84.3	84.2	*98.7*	*97*	*92.6*	*94.8*	*95.3*	*95.3*

Continued

99.2	98.5	96.7	98.6	98.5	98.6	x	99.9	99.1	99.2	99.2	98.7	99	99	98.5	95.5	98.7	98.7	98.5	98.1	98.2	98.8	98
98.9	98.5	96.7	98.4	98.5	98.4	99.7	x	99	99.1	99.1	97.8	98.7	99.1	98.5	98.7	98.7	98.6	98.5	97.7	98.2	98.8	98
98.1	99.4	97.6	99.2	97.3	99.2	98.4	98.1	x	99.7	99.7	98.5	99.5	99.3	98.4	96.4	98.7	98.7	98.4	95.4	98.7	97.9	98.6
98.4	100	98.2	100	97.7	99.4	98.6	98.4	99.2	x	100	98.6	99.4	99.6	98.4	98.7	98.7	98.6	98.4	97.6	98.2	98.7	98
98.4	100	98.2	100	97.7	99.4	98.6	98.4	99.2	100	x	98.6	99.4	99.6	98.4	98.7	98.7	98.6	98.4	97.7	98.2	98.7	98
98.4	99.7	97.9	99.4	98.1	98.1	98.9	98.6	97.9	98.1	98.1	x	98.4	98.4	97.9	99.4	99.4	98.1	99.6	95.2	95.6	96.5	99.2
98.4	99.7	97.9	99.4	97.7	99.4	98.6	98.4	99.7	99.4	99.4	98.1	x	99.6	98.1	98.1	98.5	98.4	98.1	98.9	97.8	98.4	95.4
98.1	100	98.2	96.9	97.7	99.2	98.4	98.6	98.9	99.7	99.7	97.9	99.2	x	98.3	97.3	98.5	98.4	98.3	95.6	98.1	98.6	97.9
98.5	97.6	97.8	97.6	98.8	97.3	98.5	98.5	97.6	97.6	97.6	97.9	97.9	97.6	x	99.8	99.8	99.8	98.4	98.3	98.7	99	98.5
98.7	98.2	96.4	99.4	98.1	98.1	98.9	98.7	98.4	98.1	98.1	98.4	98.7	97.9	99.4	x	100	99.9	98.5	94.6	95.3	95.6	95.6
98.7	98.4	96.4	98.1	98.9	98.1	98.9	98.7	98.4	98.1	98.1	98.4	98.7	97.9	94.4	100	x	99.9	98.7	94.6	95.3	95.6	95.1
98.7	98.2	96.5	98.1	98.9	98.1	98.9	98.7	98.4	98.1	98.1	98.4	98.7	97.9	99.4	100	100	x	98.7	94.6	95.3	95.6	95.1
97.4	96.7	95	96.9	98.1	96.9	98.9	97.6	96.9	96.9	96.9	97.6	96.9	96.9	97.9	98.2	98.2	98.3	x	97.3	96.1	95.1	95.9
95.7	94.7	93.2	96.9	97.1	96.2	96.9	96.9	96.2	96.9	96.9	97.7	96.9	96.9	95.9	96.5	97.7	97.7	96.9	x	99.6	98.2	98.2
96.2	95.1	93.4	95.4	98.5	95.4	96.2	95.9	95.2	95.4	95.4	96.7	95.4	95.2	96.2	96.7	96.7	96.7	97.2	97.7	x	98.5	99.8
96.2	95.4	93.6	95.9	98.5	95.9	96.4	96.2	95.7	95.9	95.9	95.9	95.9	95.7	96.2	96.9	96.9	96.9	96.9	96.9	98.9	x	98.5
95.9	94.8	93.1	95.23	98.1	95.2	95.9	95.7	94.9	95.2	95.2	96.4	95.2	94.9	95.9	96.4	96.4	96.4	96.9	96.9	99.7	98.7	x
95.9	95.1	93.4	95.7	98.5	95.7	96.2	95.9	95.4	95.7	95.7	95.7	95.7	95.4	95.9	96.7	96.7	96.7	96.7	96.9	98.7	99.7	98.4
95.9	95.1	93.4	95.7	98.1	95.7	96.4	96.2	95.4	95.7	95.7	95.7	95.7	95.4	95.9	96.7	96.7	96.7	96.7	96.2	98.7	99.7	98.4
96.9	96.4	94.7	96.9	98.1	96.9	97.1	96.9	96.6	96.9	96.9	96.6	96.9	96.6	96.1	96.9	96.9	96.9	96.6	96.9	96.2	96.9	95.9
96.9	96.4	94.7	96.9	98.1	96.9	97.1	96.9	96.6	96.9	96.9	96.6	96.9	96.6	96.1	96.9	96.9	96.6	96.6	96.9	96.2	96.9	96.9
94.1	93.2	91.5	94.1	95	94	94.5	94.3	93.7	94	94	94	94	93.7	93.5	94.3	99.4	94.3	94	92.6	93.2	93.7	92.9
95.7	95.8	94.1	96.3	97.7	96.3	95.6	95.3	96.1	96.3	96.3	95	96.3	96.1	94.4	95.3	95.3	95.3	95.1	96.9	94.2	94.9	93.9
96.2	95	93.2	95.6	98.1	95.6	96.1	95.8	95	95.3	95.3	95.6	95.3	95	93.2	95.8	95.8	95.8	95.6	96.9	94.7	95.2	94.4
95.8	95	93.2	95.3	98.1	95.6	96.1	95.8	95	95.3	95.3	95.6	95.3	95	92.5	95.8	95.8	95.8	95.6	96.9	94.7	95.2	94.4
95.8	95	93.2	95.6	98.1	95.8	96.1	95.8	95.3	95.6	95.3	95.6	95.6	95.3	95	95.8	98.5	95.8	95.6	96.9	94.7	95.2	94.4
95.6	95	93.2	95.3	98.1	94.4	95.6	95	95.3	95.3	97.6	95.3	95.3	95.6	95	95.6	95.6	98.1	95	96.9	94.9	94.2	94.7
95.3	94.4	92.6	95	97.3	95.3	95.6	95.3	94.8	95	95	95	95	94.8	94.4	95.3	95.3	95.9	95.1	96.2	94.2	94.7	93.9

Continued

98.7	98.7	95.8	98.3	95.3	96.7	98.8	98.8	98.1	98.1	97.7	95.2	98.8	99	97.7	97.8	96.2
98.7	98.5	98.2	98.3	97.3	98.5	98.8	98.8	98.1	98.1	97.7	95.1	98.8	99	97.6	97.7	97.9
98.1	98.4	98.2	98.4	99	98.5	98.7	98.7	98	98	97.6	96	98.7	98.9	97.5	98.1	98.6
98.6	98.6	98.3	98.5	97.2	99	98.7	98.7	98.1	98.1	97.7	94.7	98.7	98.9	97.7	98.2	97.7
98.6	98.6	98.4	98.5	98.1	99	98.7	98.7	98.1	98.1	97.7	94.8	98.7	98.9	97.7	98.2	97.8
96.4	98.1	98.4	98.5	98.1	99	99.7	99.7	98.3	98.3	97.9	94.2	99.3	98.6	97.6	99.3	97.8
98.3	98.8	98.4	98.1	97.6	98.1	98.4	98.4	97.9	97.9	97.3	95.2	97.9	98.6	97.5	97.9	96.3
98.5	98.3	97.2	98.4	97.5	96.2	98.6	98.6	98	98	97.6	95.2	98	98.8	97.5	98	95.3
99.1	98.9	96.9	97	95.3	96.2	96.6	96.7	96	96	95.4	93.4	99.2	99.4	96.8	99.1	98.1
95.1	95.7	98.7	97.4	95.7	98.6	97	98.7	97.1	96.5	95.9	93.6	99.4	99.7	97.2	98.7	98
95.7	95.5	97.3	97.4	95.6	98.9	97	97.1	96.5	96.4	96.1	95.5	99.5	99.7	97.4	99.4	99.1
95.7	95.5	98.3	97.3	98	98.9	96.9	97	96.4	96.3	98.6	95.5	99.4	99.7	97.1	99.4	96
95.2	95	98	97.3	96.9	98.9	96.4	96.5	96.3	96.2	95.9	94.4	98.5	97.2	98.8	98.5	96.3
99.6	98.3	95.4	95.7	94.7	98.9	95.6	97.7	95.6	95.4	94.8	95.1	94.9	98.6	95.6	97.7	98.6
98.6	98.4	98	96.3	95.4	98.7	96.1	96.1	96	96	95.6	95.6	98.8	99	96.2	95.5	98.4
99.7	99.7	96.2	96	95.4	96	96.7	96.7	96.3	96.3	95.7	95.3	99.5	99.5	96.4	95.9	99.4
98.4	98.3	96.1	96.1	95.4	96.8	95.9	95.9	95.8	95.8	95.4	95.3	98.6	98.8	96	95.3	98.4
x	99.6	96	96.1	95.3	95.8	96.6	96.8	96.2	96.2	95.6	95.4	99.6	99.6	96.3	96	95.4
99.4	x	96	95.9	95.4	96.7	96.6	96.6	96	96.2	95.6	95.4	99.4	99.4	96.3	95.8	98.3
96.7	96.7	x	99	97.2	95.5	97.7	98	98	98.3	98.2	93.9	98.2	98.5	98.9	99.1	99
96.7	96.7	100	x	97.6	97.8	97.9	98.1	98.4	98.3	98.1	95.2	98.2	98.6	99	99.2	99.1
93.4	93.4	95.8	95.8	x	96.6	97.9	97.3	98.1	99.1	95.3	94.8	98	98.3	98.3	97.3	95.8
94.7	94.7	97.4	97.4	97.1	x	99.2	97.4	98.5	98.4	98.1	95.4	99.3	99.5	98.5	99.8	98.1
94.9	94.9	97.4	97.4	97.4	98.4	x	100	98.9	98.8	98.4	96	99.8	99.6	98.7	99.3	99.2
94.9	94.9	97.4	97.4	97.4	98.4	100	x	99.8	98.8	98.6	95.1	98.4	99.8	98.7	99.5	98.4
94.9	94.9	97.4	97.4	98.4	98.7	98.9	98.9	x	99.9	99.6	96	98.9	99.1	99.2	98.8	98.9
94.7	95.8	97.1	97.1	98.4	98.7	98.7	98.7	99.7	x	99.5	96	98.9	99.1	99.1	98.8	98.3
94.4	94.4	96.9	96.9	97.9	98.1	98.4	98.4	99.4	99.2	x	95.4	98.2	98.5	98.8	98.2	97.4

Continued

57	81	84.5	84.5	84.5	84.3	84.3	83.7	84.3	82.2	84	83.7	84.5	82.8	84.2	84	84.2	*93.4*	*95.4*	*90.7*	*93.6*	*93.6*
58	81.3	84.8	85.1	85.1	84.8	83.3	84.2	84.8	87.5	84.5	84.2	84.8	83.3	85.9	84.8	84.8	*99.3*	*98.1*	*92.4*	*95.1*	*95.6*
59	81.8	85.4	85.7	85.7	85.4	83.9	84.8	85.4	87.5	85.1	84.8	85.4	83.9	86.4	85.4	85.4	*100*	*98.5*	*98.5*	*96.1*	*96.6*
60	81.5	85.1	85.4	85.4	85.1	83.6	84.8	85.1	87.5	84.8	84.5	85.1	83.6	86.1	85.1	85.1	*100*	*98.5*	*94.4*	*96.6*	*97.1*
61	80.4	83.9	84.2	84.2	83.9	82.5	83.6	83.9	87.1	83.6	83.3	83.9	82.5	85.1	84	83.9	*99*	*98.1*	*92.9*	*96.7*	*96.8*
62	80.7	84.2	84.5	84.5	84.2	82.7	83.9	84.2	87.5	83.9	83.6	84.5	82.7	85.3	84.3	84.2	*99.3*	*98.1*	*93.2*	*96*	*96.6*

Continued

93.6	*92.7*	*91*	*92.7*	*95.9*	*93*	*94.2*	*94.2*	*92.7*	*92.7*	*92.7*	*93.3*	*93*	*95*	*93*	*93.5*	*93.6*	*93.6*	*95.3*	*93.9*	*93.1*	*93.4*	*92.8*
95.6	*94.7*	*93*	*95.3*	*98.1*	*95.8*	*95.8*	*95.6*	*95.1*	*95.3*	*95.3*	*95.3*	*95.3*	*95.1*	*94.7*	*95.6*	*95.6*	*95.6*	*95.6*	*96.2*	*95.2*	*96.2*	*94.9*
96.6	*95.6*	*93.8*	*96.1*	*98.9*	*96.6*	*96.9*	*96.6*	*95.8*	*96.1*	*96.1*	*96.4*	*96.1*	*95.8*	*95.9*	*96.6*	*96.6*	*96.6*	*96.6*	*97.7*	*96.2*	*96.7*	*95.9*
97.1	*96.1*	*94.3*	*96.6*	*98.9*	*96.8*	*97.4*	*97.1*	*96.3*	*96.6*	*96.6*	*96.8*	*96.6*	*96.3*	*96.4*	*97.1*	*97.1*	*97.1*	*96.4*	*97.7*	*95.7*	*96.2*	*95.4*
96.3	*97*	*95.2*	*97.3*	*97.7*	*97.3*	*96.6*	*96.3*	*97.1*	*97.3*	*97.3*	*99.3*	*97.9*	*98*	*94.6*	*95.5*	*95.5*	*99.4*	*98.5*	*96.9*	*94.4*	*95.2*	*94.1*
96.6	*95.8*	*94*	*96.3*	*98.1*	*96.3*	*96.8*	*96.6*	*96*	*96.3*	*96.3*	*96.3*	*96.3*	*96*	*94.9*	*95.8*	*95.8*	*95.8*	*95.3*	*96.2*	*94.6*	*95.7*	*94.4*

Continued

93.4	*93.4*	*95*	*92.7*	*94.2*	*95*	*96.5*	*96.5*	*95.6*	*95.6*	*95*	**x**	*95.6*	*96.1*	*96.1*	*98.4*	*95.9*
95.9	*95.9*	*97.4*	*97.4*	*96.6*	*97.9*	*98.7*	*98.7*	*98.2*	*97.9*	*98.5*	*96.5*	**x**	*99.6*	*99.2*	*99.5*	*98.4*
96.4	*96.4*	*98.2*	*98.2*	*97.1*	*98.2*	*98.7*	*98.7*	*98.7*	*98.4*	*98.2*	*97.1*	*98.9*	**x**	*99.4*	*99.7*	*97.9*
95.9	*95.9*	*98.4*	*98.4*	*97.1*	*98.1*	*98.7*	*98.7*	*98.7*	*98.4*	*98.1*	*95.9*	*97.9*	*98.9*	**x**	*99.1*	*99.2*
94.9	*94.9*	*97.4*	*97.4*	*96.3*	*98.9*	*97.6*	*97.6*	*97.9*	*97.6*	*97.4*	*94.2*	*97.1*	*97.4*	*98.4*	**x**	*97.2*
95.4	*98.6*	*97.4*	*97.4*	*96.3*	*97.9*	*98.4*	*98.4*	*97.9*	*97.9*	*97.4*	*95*	*98.2*	*97.6*	*98.7*	*98.9*	**x**

APPENDIX F

	1 15	16 30	31 45	46 60	
1	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
2	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
3	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
4	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
5	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
6	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
7	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
8	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
9	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
10	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
11	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
12	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
13	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	39
14	....IILFLLLAPVY	SRLCLRN**H**PDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	56
15	.....	SRLCLRN**H**PDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILQAKNGN	45
16	.....	.....NYPDTTWIG	DSRSDQSRVNQQSLD	LVTNFKGILRAKNGN	39
17	.....	.....NYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	39
18	.....	.....W**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	33
19	.....	.....NYPDTTRIG	DSPSDQSRMN**P**QSLD	LVT**E**FKG**V**LQAKNGN	39
20	.....	.....NYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	39
21	MARFIILFLLLAPVY	SRLCLRNYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	60
22	MARFIILFLLLAPVY	SRLCLRNYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	60
23	MARFIILFLLLAPVY	SRLCLRNYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	60
24	.....	.....NYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	39
25	.....	.....NYP**E**TTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	39
26	MARFIILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	60
27	....IILFLLLAPVY	SRLCLRNYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
28	....IILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT-FKG**V**LQAKNGN	55
29	....IILFLLLAPVY	SRLCLRNYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
30	....IILFLLLAPVY	SRLCLRNYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
31	....IILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSKVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
32	....IILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
33	....IILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
34	....IILFLLLAPVY	SRLCLRN**H**PDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
35	....IILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
36	....IILFLLLAPVY	SRLCLRNYPDTTW**M**G	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	56
37	.....	.....NYPDTTWIG	DSRSDQSRVN**P**QSLD	LVT**E**FKG**V**LQAKNGN	39

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                1            15  16           30  31           45  46           60
38              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
39              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
40              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
41              ....IILFLLLAPVY  SRLCLRNHPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
42              ..............  ............IG  DSRSDQSRVSPQSLD  LVAEFKGVLQAKNGN 32
43              ....IILFLLLAPVY  SRLCLRNHPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
44              ....IILFLLLAPVY  SRLCLRNYPDTTWLG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
45              ....IILFLLLAPVY  SRLCLRNHPDTTWIG  DSRSDQSRVNPHSLD  LVTEFKGVLQAKNGN 56
46              ....IILFLLLAPVY  SRLCLRNYPDTTWLG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
47              ....IILFLLLAPVY  SRLCLRNYPDTTWLG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
48              ....IILFLLLAPVY  SRLCLRNYPDTTWMG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
49              ....IILFLLLAPVY  SRLCLRNYPDTTWMG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
50              MARFIILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 60
51              ....IILFLLLAPVY  SRLCLRNYPDTTWMG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
52              ....IILFLLLAPGY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
53              ....IILFLLLAPGY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
54              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
55              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
56              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVIEFKGVLQAKNGN 56
57              ..............  ......NYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 39
58              ....IILFLLLAPVY  SRLCLRNYPDTTWLG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
59              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
60              ....IILFLLLAPVY  SRLCLRNYPDTTWIG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
61              ....IILFLLLAPVY  SRLCLRNYPDTTWMG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
62              ....IILFLLLAPVY  SRLCLRNYPDTTWLG  DSRSDQSRVNPQSLD  LVTEFKGVLQAKNGN 56
```

	61 75	76 90	91 105	106 120	
1	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LDKVARDLYLGACRG	99
2	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LDKVARDLYLGACRG	99
3	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
4	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
5	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
6	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
7	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSVT	LD**N**VARDLYLGACRG	99
8	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
9	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
10	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
11	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LN**N**VARDLYLGACRG	99
12	GLMKQMSGRFPSNWY	QPTTKYRILYIGTND	CTEGPND**M**IIPTSMT	LD**N**VARDLYLGACRG	99
13	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
14	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	116
15	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	105
16	GLMKQMSGRFPSDWY	QPTTKYRILYIGTND	CTEGPNDVIIPTSMT	LD**N**VARDLYLGACRG	99
17	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	99
18	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	93
19	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	99
20	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	99
21	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	120
22	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	120
23	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	120
24	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	99
25	GL**L**KQMSGRFPSDWY	**T**PTTKYRMVYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	99
26	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	120
27	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
28	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	115
29	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
30	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
31	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
32	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
33	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
34	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
35	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
36	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
37	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	99

	61 75	76 90	91 105	106 120	
38	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
39	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
40	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
41	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPNSMT	LD**NA**AR**E**LYLGACRG	116
42	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	92
43	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
44	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
45	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
46	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
47	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
48	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
49	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
50	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	120
51	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
52	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GPND**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
53	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GPND**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
54	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
55	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
56	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
57	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GPND**M**IIPTSMT	LD**SA**AR**E**LYLGACRG	99
58	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GPND**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
59	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
60	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
61	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GP**T**D**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116
62	GL**L**KQMSGRFPSDWY	**T**PTTKYRILYLGTND	CT**D**GPND**M**IIPTSMT	LD**NA**AR**E**LYLGACRG	116

	121 135	136 150	151 165	166 180	
1	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
2	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
3	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
4	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
5	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
6	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCI**GG**SSGN	159
7	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
8	DVRVTPTFVGAAELG	LIGRTDALTEFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
9	DVRVTPTFVGAAELG	LIGRTDALTEFSVKV	LTFNNPTIVV**F**GLNG	MSGIYKVCIAASSGN	159
10	DVRVTPTFVGAAELG	LIGRTDALTEFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
11	DVRVTPTFVGAAELG	LIGRTDALTEFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
12	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVV**F**GLNG	M**P**GIYKVCIAASSGN	159
13	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCI**GG**SSGN	159
14	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	176
15	DVRVTPTFVGAAELG	LIGRTDALTGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	165
16	DVRVTPTFVGAAELG	LIGRTDA**S**TGFSVKV	LTFNNPTIVVVGLNG	MSGIYKVCIAASSGN	159
17	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	159
18	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	153
19	DVRVTPTFVGAA**IV**G	L**V**GRTNAITGFSVKV	LTF**S**NPTIVVVGLNG	MSGIYKVCIAA**T**SGN	159
20	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	159
21	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	180
22	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	180
23	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	180
24	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	159
25	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	159
26	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	180
27	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
28	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	175
29	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTFN**S**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
30	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTFN**S**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
31	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
32	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
33	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
34	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
35	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
36	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
37	DVRVTPTFVGAA**IV**G	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVLVGLNG	MSGIYKVCIAA**T**SGN	159

	121 135	136 150	151 165	166 180	
38	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
39	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
40	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
41	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
42	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	152
43	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
44	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
45	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
46	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
47	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**RS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
48	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
49	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
50	DVRVTPTFVGAA**I**VG	L**V**GRTDA**V**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	180
51	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
52	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
53	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
54	DVRVTPTFVGAA**I**VG	L**V**GRTDA**V**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
55	DVRVTPTFVGAA**I**VG	L**V**GRTDA**V**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
56	DVRVTPTFVGAA**I**AG	L**V**GRTDA**V**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
57	DVRVTPTFVGAA**V**VG	L**V**GRTDA**I**TGFSVKV	LTFN**S**PT**L**VVVGLNG	M**T**GIYKVCIAA**T**SGN	159
58	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
59	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
60	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
61	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176
62	DVRVTPTFVGAA**I**VG	L**V**GRTDA**I**TGFSVKV	LTF**SS**PTIVVVGLNG	MSGIYKVCIAA**T**SGN	176

	181 195	196 210	211 225	226 240	
1	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
2	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
3	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
4	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
5	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKR**H**I	219
6	VGGVNLVNGCGYFSA	P**V**RFDNFKGQIYVSD	TFEVRGTKNKCVILR	S**Y**SNAPLCTHIKR**H**I	219
7	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
8	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
9	VGGVNLVNGCGYFSA	PLRFDNFQGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
10	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
11	VSGVNLVNGCGYFIA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
12	VGGVNLVNGCGYFSA	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**T**LR	SSSNAPLCTHIKRNI	219
13	VGGVNLVNGCGYFSA	P**V**RFDNFKGQIYVSD	TFEVRGTKNKCVILR	S**Y**SNAPLCTHIKR**H**I	219
14	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	236
15	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	225
16	VGGVNLVNGCGYFSA	PLRFDNFKGQIYVSD	TFEVRGTKNKCVILR	SSSNAPLCTHIKRNI	219
17	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	219
18	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	213
19	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVGAPKNKCV**L**IR	SSS**DT**PLC**S**HI**M**RN**V**	219
20	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	219
21	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	240
22	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	240
23	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	240
24	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	219
25	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGAKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	219
26	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	240
27	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
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30	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
31	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	236
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33	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	236
34	VGGV**TLI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**NT**PLC**S**HI**M**RN**V**	236
35	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	236
36	VGGV**ALI**NGCGYF**NT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	236
37	VGGV**TLI**NGCGYF**DT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	219

	181 195	196 210	211 225	226 240	
38	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
39	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
40	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
41	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
42	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	212
43	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
44	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
45	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
46	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
47	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
48	VGGV**T**L**I**NGCGY**FNT**	PLRFDDF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
49	VGGV**T**L**I**NGCGY**FNT**	PLRFDDF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
50	VGGV**K**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	**P**SS**ET**PLC**S**HI**M**RN**V**	240
51	VGGV**A**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	236
52	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
53	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
54	VGGV**K**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
55	VGGV**K**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
56	VGGV**K**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
57	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	T**L**EVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	219
58	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
59	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
60	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236
61	VGGV**A**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DK**PLC**S**HI**M**RN**V**	236
62	VGGV**T**L**I**NGCGY**FNT**	PLRFDNF**Q**GQIYVSD	TFEVRGTKNKCV**L**LR	SSS**DT**PLC**S**HI**M**RN**V**	236

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2	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
3	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
4	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
5	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
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7	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
8	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
9	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
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11	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
12	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
13	ELDEYVDTPHTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
14	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	296
15	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	285
16	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**ASIR	TFLTEALTCPGVDWD	RIDAASCEYDSCPKL	279
17	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	279
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24	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	279
25	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTSP**DI**DW**S**	RIDAASCEYDSCPK**M**	279
26	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	300
27	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
28	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	295
29	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
30	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
31	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
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34	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
35	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
36	ELDEYVDTPNTGGV	YPSDGFDSLHG**S**AS**V**R	TFLT**D**ALTCP**DI**DW**S**	RIDAASCEYDSCPK**M**	296
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	241 255	256 270	271 285	286 300	
38	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
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40	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
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42	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	272
43	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
44	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
45	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
46	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKT	296
47	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
48	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
49	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
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52	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
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55	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
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57	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFITDALTCPDIDWS	RIDAVSCEYDSCPKI	279
58	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
59	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
60	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
61	ELDEYVDTPNTGGV	YPSDGFDSLHGSASVR	TFLTDALTCPDIDWS	RIDAASCEYDSCPKM	296
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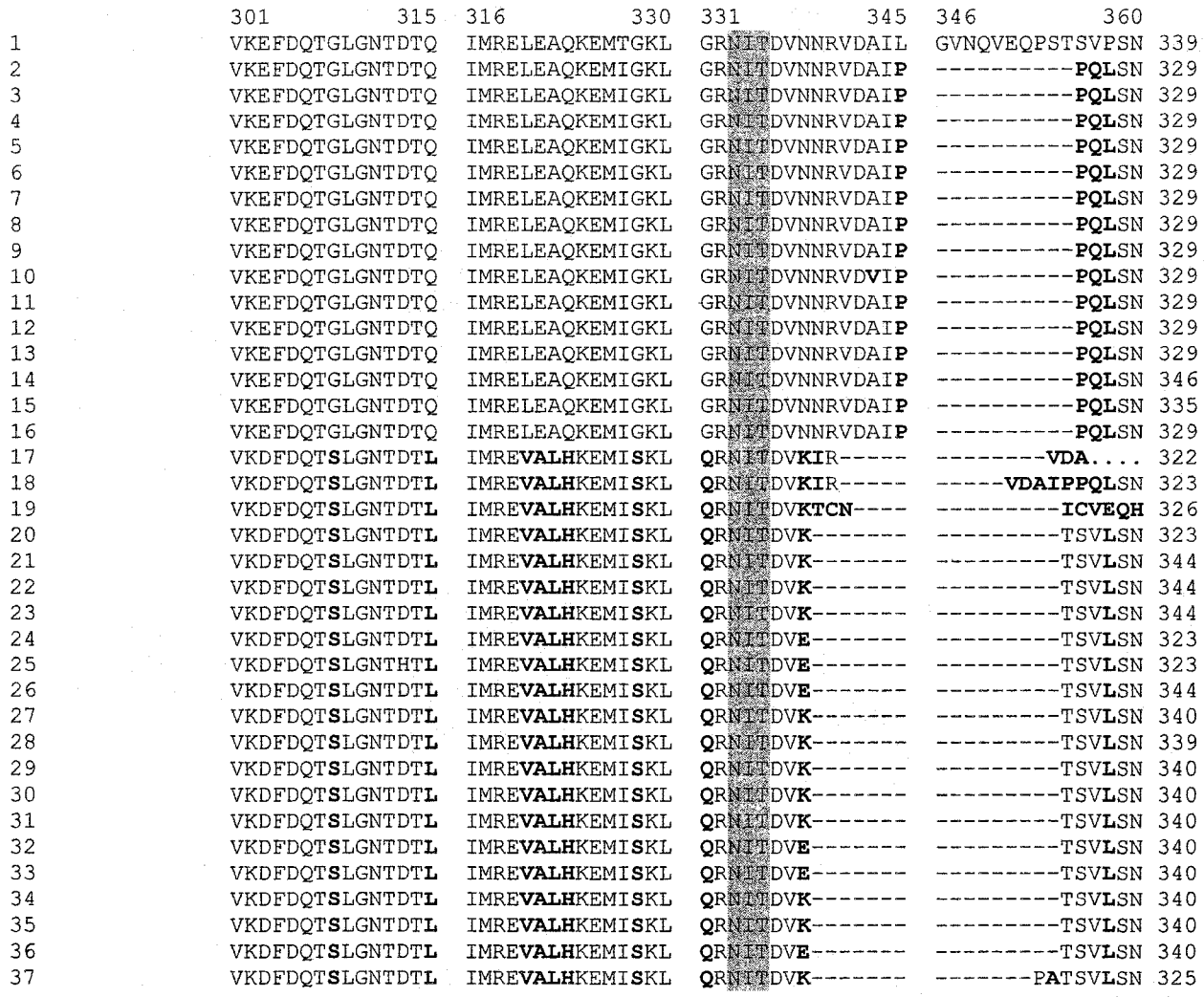

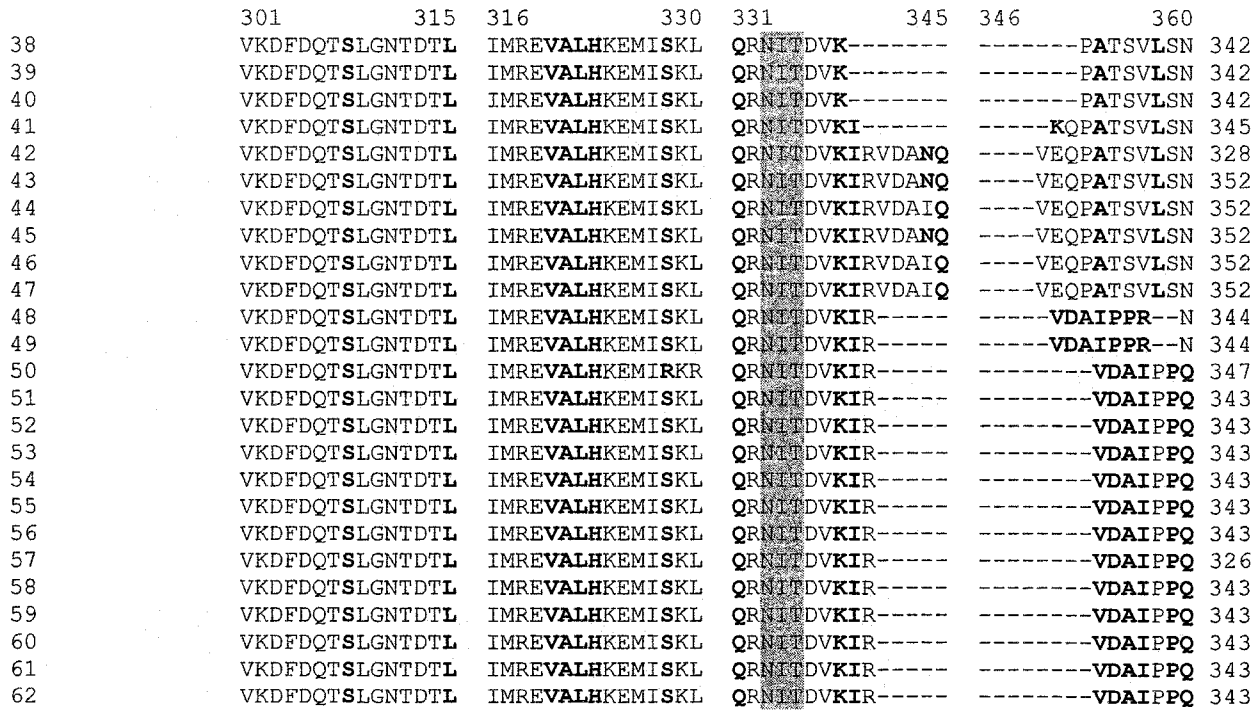

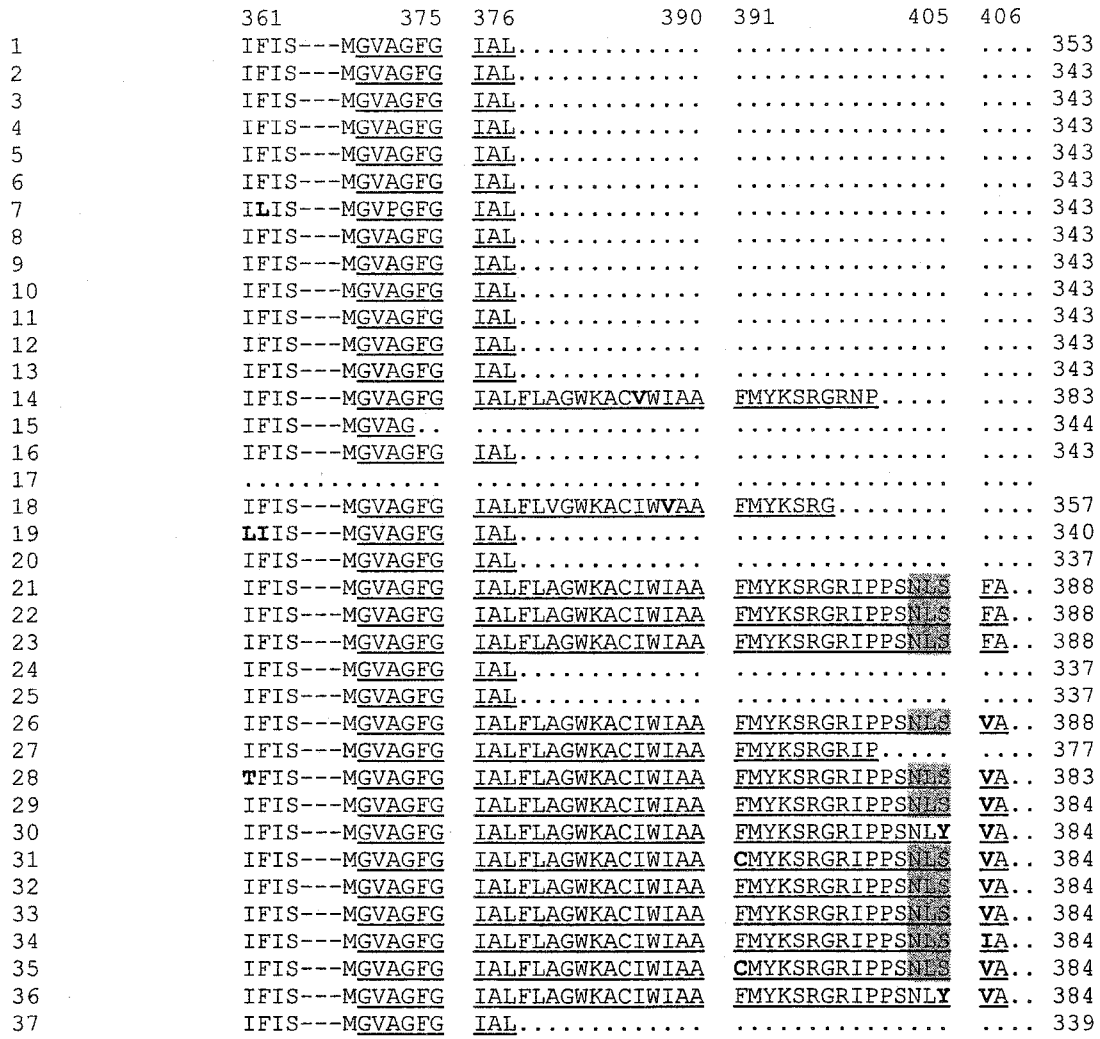

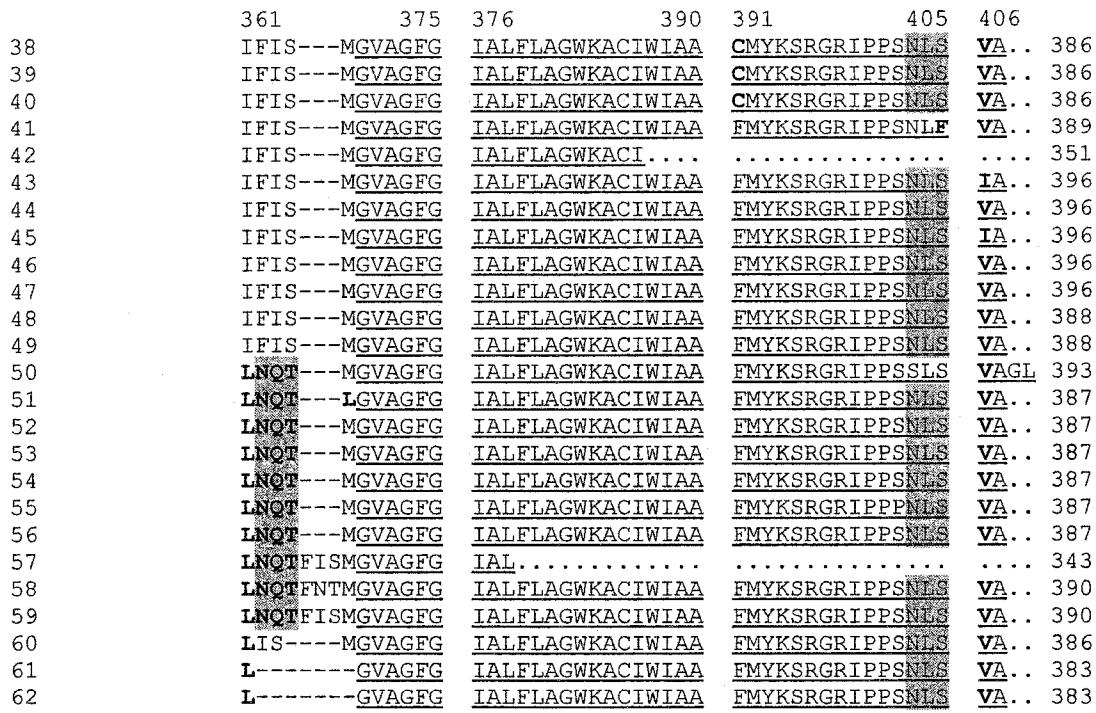

Alignment of complete deduced amino acid sequences of the putative haemagglutinin protein of 62 ISAV isolates demonstrating the hypervariable region (amino acid residues 339-354) in ORF2. Sequences that are not determined are indicated by dots (.), amino acid changes relative to ISAV isolate RPC/NB-980-280-2 (1) are in bold and deletions are indicated by dashes (-). Potential *N*-glycosylation sites are highlighted ( ) and a potential transmembrane region is underlined. ISAV identification numbers are referenced as listed in Table 5. Numbers 1-16 are ISAV isolates from New Brunswick, Canada and the lone isolate from Chile. Numbers 17-62 are ISAV isolates from Norway, Scotland and the lone isolate from Nova Scotia, Canada. Amino acid sequence alignment performed using CLUSTALW (Thompson et al., 1994; http://www.ebi.ac.uk/clustalw/index.html).