Infectious salmon anaemia virus (ISAV):

Evolution, genotyping, reservoirs and transmission.

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LIST OF PAPERS

Paper I

Plarre H, Devold M, Snow M, Nylund A (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. Dis Aquat Org 66: 71 – 79.

Paper II

Nylund A, Plarre H, Karlsen M, Fridell F, Ottem KF, Bratland A, Sæther PA (2007). Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Arch Virol 152: 151 – 179 (E.pub. 2006).

Paper III

Plarre H, Nylund A, Karlsen M, Brevik Ø, Sæther PA, Vike S (submitted). Evolution of ISA virus (ISAV). Arch. Virol.

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INTRODUCTION

History of infectious salmon anaemia (ISA)

The first officially registered outbreak of infectious salmon anaemia (ISA) started in Norway in November 1984 (Thorud and Djupvik, 1988). It was a chronically proceeding disease among Atlantic salmon (*Salmo salar*) parr in a hatchery on the West Coast of Norway (Bremnes). Affected salmon were lethargic with pale gills, fin rot, exophthalmia, and haemorrhages in the anterior eye chamber and abdominal skin. Other clinical signs were a dark, pale or yellowish liver, a dark and swollen spleen, congested intestinal walls, petechia in periviceral fat, ascitic fluid, oedemas, haemorrhages in the swim bladder wall, and muscular haemorrhages. About 80% of the parr in the hatchery died during that winter and spring. Judging from the pathological findings, "Hitra disease" was suspected, however, no bacteria were isolated from diseased fish and oxytetracycline did not reduce mortality. In the following years a disease with similar clinical and pathological signs occurred in smolt and adult salmon in marine farms that had received smolt form this hatchery (Thorud 1991).

The first official registration of ISA came at a very bad time for Norwegian salmon production. One problem was an ongoing discussion of the causes of "Hitra disease". Some insisted it was caused by malnutrition (Poppe et al 1986; Salte et al 1987), while others believed it was a bacterial disease (Holm et al 1985; Egidius et al 1986; Totland et al. 1988) and even managed to culture the bacterium that was later named *Vibrio*

salmonicida (disease: cold water vibriosis). The dispute about the causes of "Hitra disease" goes back to the end of the seventies, and later studies have indicated that the reason for the disagreement was probably that "Hitra disease" encompassed at least three different diseases: cold water vibriosis, cardiomyopathy and ISA. There are several indications of this in articles published in Norwegian (cf. F*risk Fisk* brochure from this period, i.e. published reports from the Norwegian Research council). In some electron microscopic studies of tissues from salmon with "Hitra disease", the authors were not able to detect any bacteria and described pathological changes that later studies have shown to be associated with ISA. Even as late as in 1987 the three diseases were confused. One farm in Northern Norway was given the diagnosis Bremnes syndrome (i.e. ISA), but this was later changed to cardiomyopathy. The reason for the confusion was of course the lack of a safe diagnosis.

The first indication of ISA can probably be found as early as in 1977 (cf: Poppe *et al.* 1983). The authors give a review of haemorrhagic syndrome of Atlantic salmon in Norwegian aquaculture. The first case of haemorrhagic syndrome was registered in September/October 1977 in a fish farm close to Hammerfest, Northern Norway. They were not able to isolate any infective agent and therapy with oxytetracycline did not influence the mortality. The same disease was also registered in 1978 and in 1979 it was found in other areas in Northern Norway. Several explanations were given for this syndrome: stress, environmental stress, genetics, water temperature, malnutrition, virus (VHS, but it was not possible to isolate this virus), bacteria (it was not always possible to isolate bacteria, but in some farms *Vibrio* spp. were found), and physical factors.

Studies of tissues from fish with haemorrhagic syndrome have later revealed the presence of ISA virus particles in some of these tissues (A. Nylund, pers. com.).

ISA was first named "Bremnes syndrome" after the locality of the first official appearance. This was later changed to Salmon anaemia syndrome (SAS). However, protest from an airline company made another change of name necessary and in 1990 ISA (Infectious Salmon Anaemia) was recognised by the Office International des Epizooties.

In the period from the first official outbreak in 1984 until 1991 there was a steady increase in the number of outbreaks of the disease with around 80 in 1990 and 1991 (Figure 1). This increase lead to changes in the structure of salmon farming in Norway. The farmers introduced a system with separate generations at each site and the Norwegian Food Authorities introduced a number of regulatory actions like introduction of zones to combat outbreaks. This lead to a new situation in 1994 with only one official outbreak of ISA (cf. Håstein et al 1999). The number of outbreaks since 1994 up to the present has increased again to around 10 - 15 outbreaks every year. The number of outbreaks does not reflect the increase in the amount of salmon produced in the same period (1994 - 2010), indicating a certain degree of stabilisation.

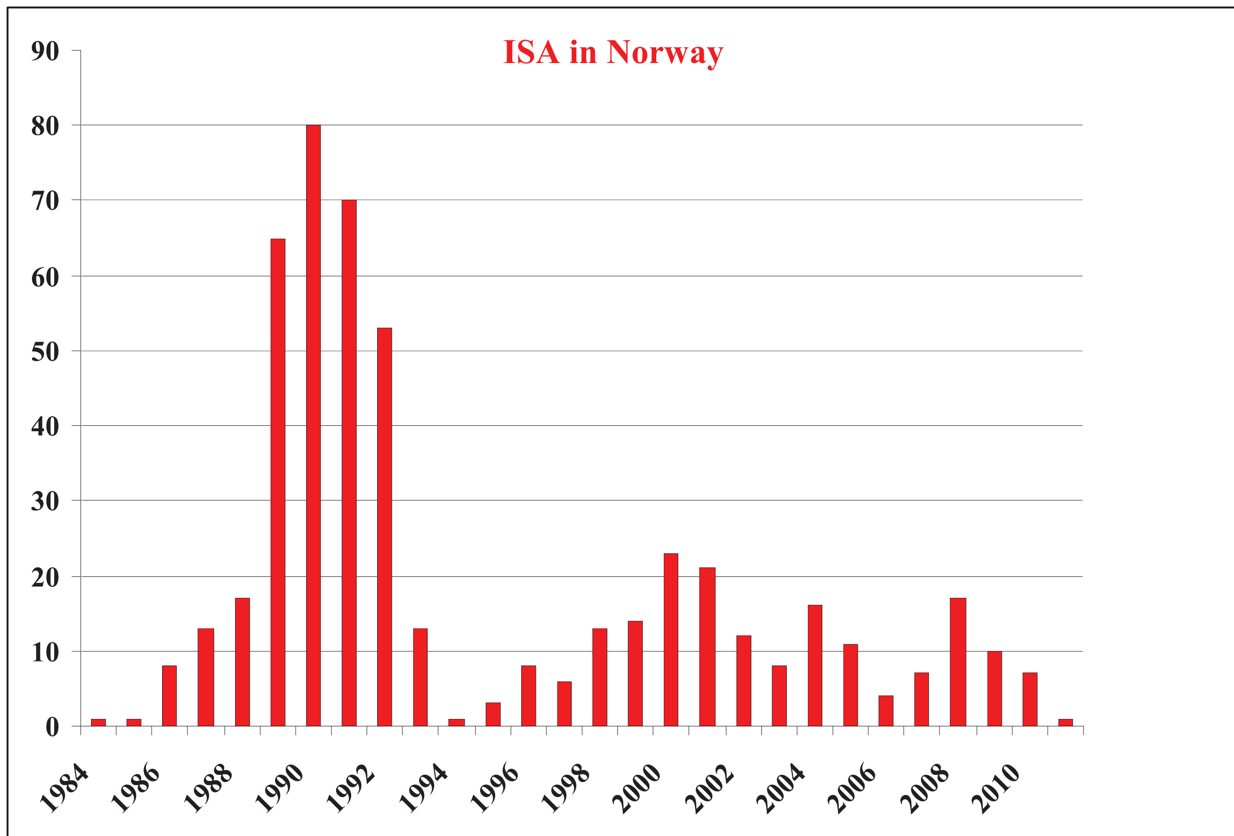


Figure 1. Number of ISA outbreaks each year in the period from 1984 to 2011 (Based on the official record).

For many years Norway was the only country that had Atlantic salmon suffering from ISA, but in 1997 the first official cases of ISA were registered in Canada and a year later in Scotland (Mullins et al 1998; Lovely et al 1999; Rodger et al 1998; Rowley et al 1999; Ritchie et al 2001a). Canada suffered repeated outbreaks of ISA in the years that followed, while in Scotland there was an apparent eradication of ISA in 1999. However, ISA virus was detected in both wild and farmed salmon in Scotland in the following years and in 2008 there were several outbreaks of ISA in the Shetland Islands (Raynard et al 2001; McBeath et al 2009; Murray et al 2010). ISA virus was detected in Chile in 1999 and in 2000 there were outbreaks of ISA in the Faeroe

Islands (Kibenge et al 2001a; Lyngøy 2003). In 2001 the first official outbreaks were registered on the East Coast of the USA and in 2002 ISA virus was detected in Ireland (Bouchard et al 2001). The first large scale outbreaks of ISA in Chile were recorded in 2007, with the majority of outbreaks occurring in region X (Godoy et al 2008; Vike et al 2008; Kibenge et al 2009). The ISA epidemics in Chile continued in 2008 and 2009, leading to a near collapse in the Chilean salmon industry. By 2010 the Chilean authorities seemed to have gotten the ISA epidemic under control, but outbreaks of ISA have also been registered in Chile in 2011.

The first transmission trial with material from salmon suffering from ISA was initiated on Friday 13th, November 1987, and indicated an infectious nature of the disease (Thorud & Djupvik 1988). In the following years several transmission trials were carried out and a pattern has emerged (Thorud 1991; Christie et al 1991, 1993; Dannevig et al 1993, 1994; Nylund et al 1994b). The transmission trials usually resulted in high mortalities where the start and duration of mortality seem to be dependent on genetics (differences in susceptibility between salmon stocks, Nylund et al 1995b), temperature (Thorud & Djupvik 1988), dose (number of virus/gram body weight) (Dannevig et al 1994; Nylund et al 1994b; Nylund et al. 1995c), time of year (yearly variation in hormone levels and immunological status, A. Nylund pers. com.), age (Glover et al 2006, A. Nylund pers. com.), and variation in ISA virus virulence (Kibenge et al 2007b, Mjaaland et al 2005).

The first transmission trials to verify a viral nature of the aetiological agent were carried out by Thorud (1991). In addition to challenge with infective material (liver from a natural outbreak of ISA) the same material was passed through several different pore-size cellulose ester membrane filters (450 nm, 220 nm and 100 nm) and injected into salmon. This study showed that the homogenate was still infective even after filtration through the 100 nm pore-size filter, which excludes any of the bacteria, isolated from farmed Atlantic salmon in Norway, from being the causative agent of ISA. Filtration through the 100 nm pore-size filter indicates an agent of viral nature. In addition, loss of infectivity of the homogenate after ether treatment indicated that the agent was an enveloped virus (Thorud 1991; Christie et al 1993). The study by Thorud also excluded IPN virus and EIBS virus as possible causative agents.

Transmission trials showed that the agent occurred in most tissues (Christie et al. 1991; Thorud 1991; Dannevig et al 1994; Nylund et al 1994b; Totland et al 1996), though some seemed to be more infective than others. In a comparison of tissues from liver, spleen, kidney, plasma, erythrocytes and head kidney leukocytes Dannevig et al. (1994) found the kidney to be most infective. Other studies have shown that mucus from the surface of the salmon and ascitic fluid are nearly as infective as blood (Rolland et al. 1998b; Nylund et al 1994b) and that freeze dried mucus is still infective when dissolved in cell culture media and injected into salmon (A. Nylund pers.com.). In a study of muscle tissue from salmon that were frozen at -20°C and thawed three times the infectivity increased (Thorud & Torgersen 1994). In 1992 it was shown that the

salmon louse *Lepeophtheirus salmonis* could act as a mechanical vector for the ISA virus (Nylund et al 1993a) and in 1993 it was shown that the virus was able to replicate in trout, *Salmo trutta* (Nylund et al 1993a, 1994a, 1995a, Nylund & Jakobsen 1995, Rolland & Nylund 1998a). However, the virus did not kill the trout. The ISA virus was also able to propagate in rainbow trout, *Oncorhynchus mykiss*, without causing any significant mortality (Nylund et al 1997). Later studies have confirmed that rainbow trout and a few other *Oncorhynchus* species are susceptible to ISA virus (Rolland & Winton 2003, MacWilliams et al 2007, Snow et al 2001b).

The only species that develops disease followed by mortalities when infected with the ISA virus is Atlantic salmon, *Salmo salar*. However, several other salmonid species are susceptible to ISA virus and some of these may end up as asymptomatic carriers of the virus (Nylund et al 1994a, b, 1995a, 1997, Nylund and Jacobsen 1995, Devold et al 2000, Snow et al 2001b, Rolland & Winton 2003, MacWilliams et al 2007), eg. *Salmo trutta*, *Salvelinus alpinus* and *Onchorynchus* spp. Mortality among ISA virus challenged rainbow trout has been observed (MacWilliams et al 2007). Among marine species, experimental challenge of herring, *Clupea harengus*, and Atlantic cod, *Gardus morhua*, has shown that these species may support replication of the ISA virus, but they are not able to transmit the virus to salmonid species in challenge trials (Nylund et al 2002, Grove et al 2007). Other marine fish species from the North Atlantic are not able to support replication of the ISA virus (Snow et al 2002, MacLean et al 2003, McClure et al 2004, A. Nylund pers.com). Several wild fish species from the coast of

Chile and Scotland, in areas with outbreaks of ISA, have also been tested and found to be negative for presence of ISA virus (Raynard et al 2001, Gonzales et al 2011). The only fish found to be positive in the study from Chile was escaped Atlantic salmon. In addition, three invertebrate species have also been tested for susceptibility for ISA virus: blue mussel, *Pecten maximus, Lepeophtheirus salmonis*, but none of these were able to sustain replication of the virus (Skår & Mortensen 2007; Nylund et al 1993b). Based on these data, it has been hypothesised that the natural host species for the ISA virus are salmonids in the north Atlantic.

The first detection of ISA virus in wild salmonids from several locations was registered in Scotland (Raynard et al 2001; Cunningham et al 2002). They found presence of ISA viruses in both Atlantic salmon (parr and adult salmon) and trout (brown trout and sea trout) at different locations in Scotland including a fresh water site distant to areas with marine salmon farms. This is the first evidence that ISA virus can be present in wild juvenile Atlantic salmon and brown trout in fresh water, i.e. before any exposure of these ISA virus hosts to sea water. A study of ISA virus in wild Atlantic salmon and trout, collected in 2001 – 2003, from rivers and a fjord in Sogn og Fjordane county was performed in Norway (Paper I). This paper shows a high prevalence of ISA virus in wild trout and Atlantic salmon in these rivers in 2001 and 2002. We also found three out of 45 sea trout, collected in Nordfjord, positive for ISA virus. These studies from Norway and Scotland show that ISA viruses are relatively

common in wild salmonids and suggest, together with challenge experiments, that these two species are the most likely natural hosts for ISA viruses.

Wild salmonids

One characteristic trait of Atlantic salmon and trout (*Salmo trutta*) is that they live in genetically distinct, small fragmented populations belonging to different river systems along the west coast of Europe and the East coast of North America. Both species spawn and have an early development in fresh water and when they reach the smolt stage, they migrate to sea. However, while the trout stays in the coastal waters, the Atlantic salmon migrates to the high seas, Barents and Norwegian Sea and some are also found in the North Sea. Trout also differ from salmon in that they may return to the rivers during the autumn every year, while salmon usually stay out at sea until they are ready to spawn, which may be 3 - 4 years. Common for both species is that the population density at sea is low compared to the high density found in the river systems during spawning. These two species are probably the main hosts for the ISA virus and it is expected that the ISA virus must have co-evolved with these and adapted to transmission in the fresh water phase, since the population density is highest in the rivers and interactions between individuals are highest during spawning. It has been documented (Paper I) that salmon and trout eat eggs on the spawning grounds during breeding (Figure 2). Hence, if the gonadal products, eggs and sperms, are infected with the virus, it can easily be transmitted to other life stages like fry, parr and smolt when these are feeding on the infected gonadal products.

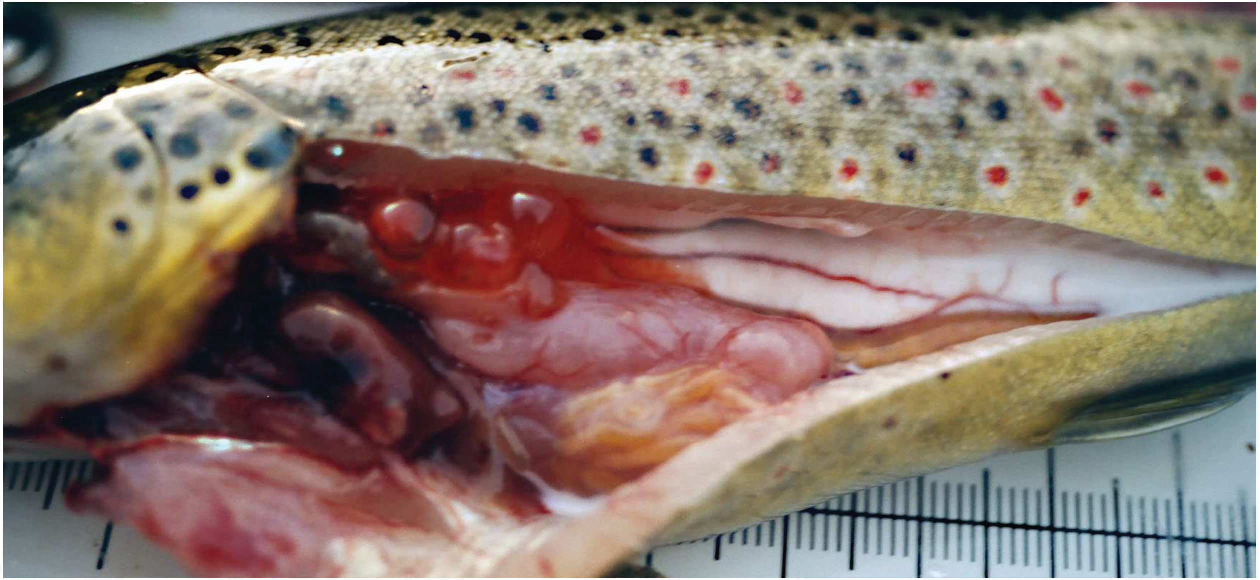


Figure 2. Mature male (*Salmo trutta*) that has been eating eggs during spawning in Bortne River.

Since the populations in fresh water are too small to sustain highly virulent viruses, it is expected that the ISA viruses must consist of low virulent strains in natural populations, or that the fresh water stages of these species are less susceptible or less likely to develop disease compared to the marine stages. It may also be expected that the viruses should be more adapted to the trout, which spends more time in the river systems and coastal areas compared to the Atlantic salmon. It has been shown that even though the ISA virus may replicate in the trout and cause slight disease, it does not cause any mortality (Nylund et al 1993a, 1994a, 1995a, Nylund & Jakobsen 1995). It has also been documented that the production of ISA virus in carrier trout increases during stress and sexual maturation (Nylund et al 1994a, 1995a; Rolland & Nylund 1998a; Devold et al 2000). Both species, salmon and trout, may be infected by ISA

virus in both fresh and sea water and throughout the year. However, it seems that the Atlantic salmon are less likely to develop disease during the period from September to December (A. Nylund pers.com.). This could be an adaptation for protection against the development of ISA after exposure to the virus during spawning. The existence of low virulent or avirulent ISA virus could be another explanation for how the ISA virus is able to survive in wild salmonid populations (Nylund et al 2003). When pathogenic strains develop from the avirulent HPR0 type in the river systems, they will probably soon be lost from the population, if the host dies before the virus is transmitted to a new host.

ISA virus

Several different virus-like particles have been found in salmon suffering from ISA (Thorud et al 1990; Christie et al 1991; Sommer & Mennen 1992), but none of these were identified as the causal agent of the disease. The first pictures of the ISA virus were presented in 1993 (Watanabe et al. 1993) and at the EAFP conference in Brest (September 1993, A Nylund, pers. com.). The virus was seen as early as 7 days after challenge. The morphology of the virion and the target cells have since been dealt with in several studies (Hovland et al 1994; Koren & Nylund 1997; Dannevig et al 1995; Nylund et al. 1995a, 1996; Sommer & Mennen 1997).

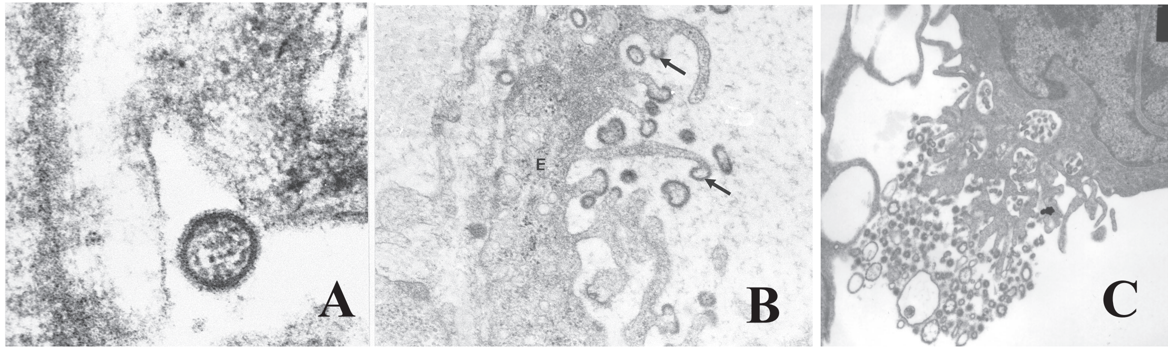


Figure 3. A) ISA virus virion, B) Budding of ISA virions from blood vessel endothelial cells, C) Budding of ISA virions from a polymorphonuclear leukocyte (Nylund et al 1995b, 1996).

The free ISA virus virions are spherical and about 100 nm in diameter, but some variation in size and morphology does occur (Watanabe et al 1993, Hovland et al. 1994; Koren & Nylund 1997, Nylund et al 1995a, 1996). The envelope of the virus is a unit membrane with an electron dense layer, 10 nm thick, on the outer surface (Figure 3). The virions may also be surrounded by an additional electron dense cloud about 50 nm thick. The virions contain electron dense granules, about 10 to 12 nm in diameter, believed to be transverse sections of the helical nucleocapsids. The diameter of negatively stained ISA virus particles ranges from 45 - 140 nm (majority range from 130 – 140 nm) and they are covered with surface projections about 10 nm in length (Dannevig et al 1995). The projections seem to have a knob-like thickening at the distal end. A few, large (up to 700 nm), highly pleomorphic particles could also be found in infected cell culture medium (Dannevig et al 1995).

Budding of the ISA virus has been observed from the surface of endothelial cells lining bloodvessels, endocardial cells and polymorphonuclear leukocytes (Watanabe et al 1993; Hovland et al 1994; Koren & Nylund 1997; Nylund et al. 1995a, 1996) (Figure 3). A few cases of what seems to be budding into intracellular vesicles in the endothelial cells have also been observed. The budding process seemed to start with increasing electron density and thickening of the cell membrane. Areas where this occurred usually protrude from the cell. These protrusions can extend more than 2 µm from the cell surface. Protrusions were not observed when the budding occurred on the abluminal side of the endothelial cells (Nylund et al 1995b).

The host cells seem to be morphological intact despite high budding activity. No viral components can be detected within the host cell cytoplasm until the characteristic electron-dense, crescent-shaped, thickening of the plasma membrane appears. However, destroyed endothelial cells are frequently found in moribund fish, but cells surrounding the blood vessels seem to disintegrate before the destruction of the endothelial cells. Aggregations of what seem to be virus envelopes (i.e. virions not yet matured or completely assembled) are frequently found on the abluminal side of the endothelial cells (Nylund et al 1995b). Large aggregations of viruses surrounded by amorphous substance and cell debris, which seem to be remnants of lysed polymorphonuclear leukocytes, have also been observed (Nylund et al 1995b). Protrusions from the endothelial cells are frequently seen surrounding erythrocytes or protruding into erythrocytes and macrophages can be seen adhering to the endothelial

cells in the salmon suffering from ISA (Nylund et al 1995b, 1996; Koren & Nylund 1997). Erythrocytes, which seemed to be adhered to infected endothelial cells, are frequently found (Figure 4). Erythrophagocytosis can be observed in most tissues of the infected salmon (Figure 4).

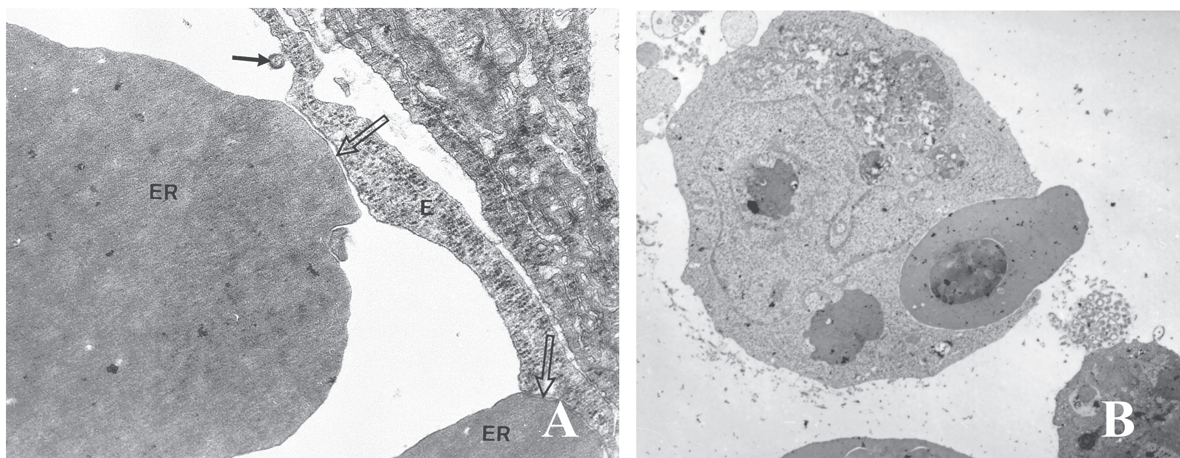


Figure 4. **A**) Erythrocyte attached to a blood vessel endothelial cell expressing ISA virus HE protein on its surface (Nylund et al 1995b). ISA virion budding from the cell surface (solid arrow), attachment area between the erythrocyte (ER) and the blood vessel endothelial cell (open arrow). **B**) Erythrophagocytosis. Macrophage eating erythrocytes with ISA virions attached to the surface.

The ISA virus was suggested as a possible member of the family *Orthomyxoviridae* based on size, morphology, biochemical and physiochemical characteristics, and presence of a segmented RNA genome (Sommer & Mennen 1996; Falk et al 1997; Koren & Nylund 1997; Mjaaland et al 1997). However, it was not until 1999, after analysis of segment 2 (the RNA dependent RNA polymerase, PB1), that the ISA virus was shown to represent a new genus in the family *Orthomyxoviridae* (Krossøy et al

1999). The slow development in the characterization and identification of the causative agent for ISA was partly due to the lack of susceptible cell cultures. The first isolation of the ISA virus in cell culture was obtained in a newly developed culture from salmon head kidney, SHK cells (Dannevig et al 1995), and a year later a similar approach, using head kidney leukocytes, also resulted in isolation of ISA virus (Sommer & Mennen 1996). In 1997 it was shown that the ISA virus could also be cultured in a commercially available established fish cell line, Atlantic salmon cells (AS cells) (Sommer & Mennen 1997). A culture of Atlantic salmon kidney cells (ASK cells) was also established at the University of Bergen and used for isolation of ISA viruses for studies of the virus genome (Devold et al 2000).

The buoyant density of the ISA virus was found to be 1.184 to 1.262 g/cm^3 according to Christie et al (1993), while Falk et al (1997) found the density to be 1.18g/ml in sucrose and CsCL gradients. Several studies have shown that the ISA virus is sensitive to chloroform, freon, heat and low pH (Thorud 1991; Christie et al 1993; Falk et al 1997). The optimal temperature for replication of the ISA virus is according to Falk et al (1997) 15 ºC. The tolerance and survival of the ISA virus, which is of importance both for the epizootic of the disease and for disinfection of infected farms and slaughterhouses, have been dealt with in several studies (Thorud 1991; Christie et al 1993; Nylund et al. 1993a; Torgersen 1993; Nylund et al 1994b; Thorud & Torgersen 1994). The ISA virus may survive for several hours at 20°C (Nylund et al 1993a, 1994b) but the infectivity is lost after exposure to 55°C for one minute (Torgersen

1993). According to the study by Torgersen (1993) the ISA virus is also inactivated by exposure to: a) formic acid at pH < 4.0 for 24 hours, b) sodium hydroxide (NaOH) at pH = 12 for 7 hours or more, c) 0.185% HCHO for 16 hours, d) 8 mg/min/l ozone for 4 - 6 minutes, and e) 20 mg/l sodium hypochlorite for 1 hour. The ISA virus is also inactivated by exposure to organic solvents, ether and chloroform (Christie et al 1993; Thorud 1991). Survival of the ISA virus in active sea water will depend on temperature, biological activty and UV-exposure (A. Nylund pers.com).

The ISA virus is an enveloped, single stranded, negative strand RNA virus where the genome consists of 8 segments with a total molecular size of approximately 14.5 kb (Mjaaland et al 1997; Clouthier et al 2002). The ISA virus is the only species in the genus *Isavirus*, family *Orthomyxoviridae* (Kawaoka et al 2005). The first sequence, segment eight, was published in 1997 (Mjaaland et al 1997) and in 1999 the sequence of the putative RNA-dependent RNA polymerase, PB1, was published (Krossøy et al 1999). Several studies on the putative haemagglutinin, segment six, of the ISA virus were published in 2001 (Krossøy et al 2001a,b; Devold et al 2001; Rimstad et al 2001; Griffiths et al 2001), and in 2002 the first study presenting the complete genome of ISA virus was available (Clouthier et al 2002). The first six segments contain one open reading frame (ORF) each (Krossøy et al 1999, 2001a,b; Devold et al 2001; Rimstad et al 2001; Ritchie et al 2001b; Griffiths et al 2001; Clouthier et al 2002; Snow et al 2003; Aspehaug et al 2004) while segment seven and eight contain at least two ORFs each (Biering et al 2002; Clouthier et al 2002; Ritchie et al 2002; McBeath et al 2006;

Kibenge et al 2007a; Garcia-Rosado et al 2008). Identification of the ISA virus proteins is still ongoing, but several of the proteins have been identified (Table 1).

The protein coded by segment one in the ISA virus genome contains a nuclear localization signal (and expression of the protein in a cell line exhibited a nuclear localization) and the protein size (ORF of 2169 nt) and amino acid composition is similar to the PB2 protein from othromyxoviruses (Snow et al 2003). The sequence of segment two contains an ORF of 2245 nucleotides coding for a putative protein showing the core polymerase motifs characteristic of all viral RNA-dependent RNA polymerases consistent with the properties of PB1 from members of the *Orthomyxoviridae* (Krossøy et al 1999). Segment three contains an ORF consisting of 1851 nt encoding a predicted protein of 616 amino acids (Ritchie et al 2001b; Snow & Cunningham 2001; Clouthier et al 2002). The amino acid sequence shows no or little homology with nucleoproteins (NP) from members of the *Orthomyxoviridae*, but functional analyses of the protein support the assumption that segment three is coding for a NP (Aspehaug et al 2004; Falk et al 2004; Goic et al 2008). Segment four contains an ORF consisting of 1737 nt. The putative protein (578 amino acids) contains no conserved motifs, but based on the predicted cytoplasmic location and the presence of putative sites of phosphorylation it was suggested to be the acid polymerase, PA, by Ritchie et al (2001b).

Two surface proteins are present on the ISA virions (Falk et al 2004), i.e. the fusion protein, F, and the heamagglutinin-esterase protein, (HE). Segment five contains an ORF consisting of 1332 nt coding for a protein consisting of 444 amino acids (Clouthier et al 2002; Aspehaug et al 2005). Analysis of the function of the protein coded by segment five shows that it is a pH-dependent F protein produced as a precursor protein, F0, that is proteolytically cleaved into F1 and F2 (Aspehaug et al 2005). It has been shown that inserts may occur in the vicinity of a putative cutting site for trypsin extending the ORF and resulting in ISA virus F proteins ranging from 444 to 455 amino acids (Devold et al 2006). The other surface protein is a heamagglutinin-esterase protein (HE) coded by the only ORF in segment six (Devold et al 2001; Griffiths et al 2001; Krossøy et al 2001b; Rimstad et al 2001; Kristiansen et al 2002; Falk et al 2004; Hellebø et al 2004; Müller et al 2010). The length of the ORF may range from 1164 to 1236 nt resulting in HE proteins ranging from 387 to 411 amino acids (**Paper II**). The variation in length is due to variation in a highly polymorphic region (stretching from nucleotide 1006 to 1117 in the ORF) next to the transmembrane region (Devold et al 2001, **Paper II** and **III**). Table 2 gives an overview of HPR observed in ISA viruses.

Table 1. Characterization /identification of the eight RNA segments in the ISA virus genome and the putative proteins coded by the ORFs.

Seg	ORF	Length	Protein		Literature
1	1	2169 nt	PB2	Polymerase	Clouthier et al 2002, Snow et al 2003
2	1	2245 nt	PB1	Polymerase	Krossøy et al 1999, Clouthier et al 2002
3	1	1851 nt	N	Nucleoprotein	Ritchie et al 2001, Falk et al 2004, Aspehaug et al 2004, Clouthier et al 2002, Goic et al 2008
4	1	1737 nt	PA	Polymerase	Ritchie et al 2001, Clouthier et al 2002
5	1	1332 - 1365 nt	F	Fusion	Aspehaug et al 2005, Clouthier et al 2002, Falk et al 2004, Devold et al 2006
6	1	1164 – 1236 nt	HE	Haemagglutinin-esterase	Krossøy et al 2001, Rimstad et al 2001, Devold et al 2001, Falk et al 2004, Kristiansen et al 2002, Clouthier et al 2002, Mikalsen et al 2005 Muller et al 2008, 2010
7	2 (3)	903 nt 526 nt	NS1 NEP (NS3)	Inf-sig-ant. Nuc exp prot ?	Biering et al 2002, Clouthier et al 2002, Ritchie et al 2002, McBeath et al 2006, Kibenge et al 2007, Garcia-Rosado et al 2008
8	2		M S8ORFs	Matrix Inf-sig-ant	Biering et al 2002, Clouthier et al 2002, Falk et al 2004, Garcia-Rosado et al 2008

Table 2. Overview of the different highly polymorphic region (HPR) groups observed in the stalk region of the HE from ISA viruses. St = surface tail, TMR transmembrane region.

HPR	**St**	**HPR**								**TMR**	**strain**
		1-2	**3 - 7**	**8 - 12**	**13 - 17**	**18 - 22**	**23 - 27**	**28 - 32**	**33 - 37**		
Europe											
HPR0a	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**SF83/04**
HPR0b	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPA**N**	SVLSN	IFISM	GVA	**ISA440971**
HPR0a	NIT	DV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**CH28/08**
Chile	NIT	DV	KIRVD	AIPPQ	LNQTF	NT			ISM	GVA	**FJ594284**
HPR9	NIT	DV	KIRVD	AIPPQ	LNQTF	NT			M	GVA	**SF47/99**
Chile	NIT	DV	KIRVD	AIPPQ	LNQTF				ISM	GVA	**FJ594294**
HPR16	NIT	DV	KIRVD	AIPPQ	LNQTF				M	GVA	**T90/04**
HPR19a	NIT	DV	KIRVD	AIPPQ	LNQT**L**					GVA	**SF18/96**
HPR19b	NIT	DV	KIRVD	AIPPQ	LNQTF					GVA	**MR139/08**
HPR3	NIT	DV	KIRVD	AIPPQ	LNQT				FISM	GVA	**N9/93**
HPR2	NIT	DV	KIRVD	AIPPQ	LNQT				M	GVA	**H2/89**
HPR30	NIT	DV	KIRVD	AIPPQ	LNQ				ISM	GVA	**MR118/06**
HPR31	NIT	DV	KIRVD	AIPPQ	LN				IFISM	GVA	**T121/07**
HPR4a	NIT	DV	KIRVD	AIPPQ	L			SN	IFISM	GVA	**T10/93**
HPR4b	NIT	DV	KIRVD	AIPPQ	L			SN	**T**FISM	GVA	**H101/04**
HPR12a	NIT	DV	**G**I**G**VD	AIPPQ	L			N	IFISM	GVA	**N5/98**
HPR12b	NIT	DV	KIRVD	AIPPQ	L			N	IFISM	GVA	**EU625666**
HPR5	NIT	DV	KIRVD	AIPPQ	L				ISM	GVA	**SF14/95**
HPR8	NIT	DV	KIRVD	AIPPQ	L					GVA	**MR46/99**
HPR11a	NIT	DV	KIRVD	AIPP				RN	IFISM	GVA	**SF54/00**
HPR11b	NIT	DV	KIRVD	AIPP				RN	IF**V**SM	GVA	**SF63/01**
HPR6	NIT	DV	KIRVD	AI		QV	EQPAT	SVLSN	IFISM	GVA	**ST25/97**
HPR32	NIT	DV	KIRVD	A		TNQV	EQPAT	SVLSN	IFISM	GVA	**N128/07**
HPR14a	NIT	DV	KIRVD	A		NQV	EQPAT	SVLSN	IFISM	GVA	**ST21/96**
HPR14b	NIT	DV	KIRVD	A		NQV	EQPAT	SVLSN	**T**FISM	GVA	**FM168/10**
HPR14c	NIT	DV	K**T**RVD	A		NQV	EQPAT	SVLSN	**T**FISM	GVA	**FM174/11**
HPR14d	NIT	D**L**	KIRVD	A		NQV	EQPAT	SVLSN	**T**FISM	GVA	**FM174/11a**
HPR33	NIT	DV	KIRVD			DNV	GQPAT	SVLSN	IFISM	GVA	**N127/07**
HPR18	NIT	DV	KIRV				PAT	SVLSN	IFISM	GVA	**R111/05**
HPR17	NIT	DV	KIR			LEV	EQPAT	SVLSN	IFISM	GVA	**MR103/05**
HPR10	NIT	DV	KIK				QPAT	SVLSN	IFISM	GVA	**MR52/00**

Table 2. Continued....

HPR36	NIT	D**L**	K			TF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**CH09/08**
HPR13	NIT	DV	K					EQPA**N**	SVLSN	IFISM	GVA	**ISA440970**
HPR1	NIT	DV	K					PAT	SVLSN	IFISM	GVA	**H1/87**
HPR7a	NIT	DV	K					T	SVLSN	**T**FISM	GVA	**H17/96**
HPR7b	NIT	DV	K					T	SVLSN	IFISM	GVA	**N29/97**
HPR7c	NIT	DV	K					T	SVLSN	I**S**ISM	GVA	**R171/07**
HPR7d	NIT	DV	**R**					T	SVLSN	IFISM	GVA	**Chile***
HPR7e	NIT	DV	K					T	S**AP**SN	IFISM	GVA	**Chile***
HPR7f	NIT	DV	K					T	SV**S**SN	I**S**ISM	GVA	**CH03/08***
HPR7g	NIT	DV	K					T	SVLSN	IFI**Y**M	GVA	**FJ594307***
HPR7h	NIT	DV	K					T	SV**S**SN	IFISM	GVA	**CH03/08***
HPR7i	NIT	DV	K					T	SV**I**SN	I**S**ISM	GVA	**R171/07**
HPR15b	NIT	DV						A T	SVLSN	IFISM	GVA	**FJ594282**
HPR15a	NIT	DV						ET	SVLSN	IFISM	GVA	**H36/98**
HPR34	NIT							EQPAT	SVLSN	IFISM	GVA	**NT134/08**
America												
HPR00	NIT	DV	KIRVD	AIPPQ		LNQTF	NTNQV	EQPST	TVLSN	IFISM	GVA	**USA2004**
HPR00	NIT	DV	KIRVD	AIPPQ		LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA	**AY646059**
HPR20	NIT	DV	NNRVD	AI		L	GVNQV	EQPST	SVPSN	IFISM	GVA	**CCBB**
HPR21	NIT	DV	NNRVD	AIPPQ		L			SN	IFISM	GVA	**Can31/97**

* = ISA viruses from Chile, but of European origin.

Segments seven and eight of the ISA virus both contain two ORFs (Biering et al 2002; Ritchie et al 2002; McBeath et al 2006; Garcia-Rosado et al 2008), and it has been suggested that there could be a third ORF in segment 7 (Kibenge et al 2007a). The first two ORFs of segment seven consist of 903 and 522 nt coding for proteins consisting of 300 and 159 amino acids, respectively, where the latter is a result of a splicing event

that removes a 526 nt intron during the formation of mRNA (Biering et al 2002). Both proteins share the first 22 amino acids. The largest ORF seems to be coding for a non-structural (NS1) protein, probably an interferon-signalling antagonist (Biering et al 2002; McBeath et al 2006; Garcia-Rosado et al 2008), while the second ORF could be coding for a nuclear export protein (NEP) (Kibenge et al 2007a). The first of the two ORFs in segment eight is coding for a major structural protein, probably the matrix (M) protein (Biering et al 2002), while the latter is suggested to be coding for a type I IFN antagonist (Garcia-Rosado et al 2008).

Genotyping and molecular epizootiology

All organisms, including viruses, are related by common ancestry and display a genetic diversity that reflects their evolutionary history. Changes in the virus genome occur when mutations are incorporated into the genome and passed to later generations. By comparing homological characters, homological nucleotide and amino acid sequences and changes in these, it is possible to reconstruct the evolutionary history of existing viruses. All viruses in farmed populations of fish originate from viruses present in wild populations and these viruses have coevolved with their hosts for millions of years and will, in most cases, not cause large mortalities among their natural hosts. This "equilibrium" will change when there are changes in the host, virus populations, or changes in the environment. An increased number of hosts and reduced distance between the hosts could increase the virulence of a well adapted host specific virus. The idea behind fish farming is to be able to produce a high number of individuals in a

smallest possible area (farm with nets or cages) and, in addition, the farmed fish populations will in most cases have a lower genetic variation compared to wild populations. Introduction of viruses into such farmed populations of fish could speed up the evolution (substitution rate) of viruses and virus genotypes with new or different characteristics may emerge.

Tracing the relationships between members of the same virus species will in practice involve the creation of alignments of homological sequences that can be used for reconstruction of the evolutionary history based on a model of evolution. Choice of sequences is important since each gene in a virus genome may evolve at a different speed, i.e. the substitution rate is in most cases expected to be higher for virus surface proteins exposed to the host immune system compared to genes coding for internal virus proteins like nucleoprotein and polymerases that are part of the ribonucleoprotein complex (Webster et al 1992, Wong et al 2011). The ISA virus genome consists of eight segments coding for a minimum of 10 proteins. Two of the major structural proteins are surface proteins, F and HE, coded in segments five and six.

The ISA virus differs from influenza A virus in the coding and function of the surface proteins where the HA, responsible for binding of virions to host cell receptors and for fusion between the virion envelope and the host cell of the influenza A, is coded by segment four, while segment six of the ISA virus is coding for a protein that functions

both as attachment to host cells and freeing the virions from host cell receptors (Krossøy et al 2001b; Hellebø et al 2004). The fusion activity of ISA viruses is provided by a separate surface protein coded by segment five (Aspehaug et al 2005). The segment six of the influenza A virus codes for a protein with neuraminidase activity (cleaves terminal sialic acid from glycoproteins or glycolipids). The HA0 of the influenza A is posttranslationally cleaved into two subunits (HA1 and HA2) and this cleavage is required for infectivity (virus-cell fusion) and is carried out by host-produced trypsin-like proteases. Inserts in front of the cutting site are known to change the virulence of the influenza A virus (Suarez et al 2004). Similar changes can also be seen in the ISA virus where the fusion protein is produced as a precursor protein, F0, which is posttranslationally, cleaved into F1 and F2 and inserts may occur in front of the cutting sites (Devold et al 2006; Markussen et al 2008; Vike et al 2008). However, the effect of an insert in front of the cutting site of the ISA virus F protein remains to be shown. Both the NA protein of influenza A viruses and the HE protein of the ISA virus may have deletions in the stalk region, and it has been shown that the deletion may affect the influenza NA protein's ability to release the virus from the cell (Matrosovich et al 1999). The region in the ISA virus segment six, where the deletions occur, is named the highly polymorphic region (HPR) and all pathogenic ISA viruses have a shorter HPR compared to what is believed to be the avirulent wild-type HPR0 ISA virus. Hence, in both influenza A and ISA viruses, the neuraminidase or esterase activity of the surface proteins seems to be affected by deletions in the stalk region, while inserts in front of the cutting site of the precursor proteins (HA0 and F0) may have an effect on attachment and fusion activity of the respective surface proteins.

The segment six of the ISA virus, the haemagglutinin (HA) gene, was first identified and characterized by Krossøy et al (2001b). They showed that the protein had haemagglutination activity, but unlike influenza virus HA they did not find posttranslational cleaving of the ISA virus HA. This study also revealed a highly polymorphic region (HPR) in the ectodomain close to the transmembrane region. Later the same year several other studies were published supporting the characterization given by Krossøy et al (2001b) (Devold et al 2001; Griffiths et al 2001; Kibenge et al 2001; Rimstad et al 2001). Receptor-destroying enzyme activity of the ISA virus was detected (possibly an acetylesterase) in 1997 (Falk et al 1997), and in 2004 it was shown that the protein coded by segment six carried receptor-destroying activity identified as an acetylesterase (Hellebø et al 2004), i.e. the protein was identified as a hemagglutinin-esterase (HE). The HE protein has been further characterized by Mikalsen et al (2005), and Müller et al (2010). The first phylogenetic analysis using the HE gene from the ISA virus was published by Devold et al (2001). The study included ISA viruses from 37 different salmon farms in Norway, Scotland and Canada and revealed several different variants of the HPR. The same year Kibenge et al (2001b) said that the shorter HPRs seen in European ISA viruses compared to isolates of Canadian origin suggested that the archetypal ISA virus was probably of Canadian origin, inferring a direction of evolution towards shorter HPRs. However, as shown by Devold et al (2001) there is a large variation in the length of HPRs from European ISA virus isolates and no indication of evolution towards shorter HPRs. Devold et al (2001)

suggested that the variation could be a result of recombination, with the recombination site within the HPR, and it was recommended that only the 5`-end flanking region to the HPR should be used in phylogenetic studies (Devold et al 2001).

The longest HPR observed was first obtained from ISA virus in a wild Atlantic salmon (Cunningham et al 2002), and it became obvious that all other HPRs could be derived from this HPR by recombination as suggested by Devold et al (2001). The long HPR was termed HPR0 by Nylund et al (2003) and they suggested that ISA viruses with HPR0 were low virulent wild-type viruses that could mutate into the virulent isolates with shorter HPRs found in farmed populations of Atlantic salmon. Hence, reservoirs of low virulent ISA viruses could be a constant source of new virulent ISA viruses. It was also suggested that the long HPR found by Cunningham et al (2002) could be an ancestral form of the ISA virus HE gene, and all the shorter European HPRs were a result of parallel evolution due to strong functional selection pressure leading to deletions in the HPR (Mjaaland et al 2002). Mjaaland et al (2002) refuted the suggestion by Devold et al (2001) that the short HPRs could be a result of recombination. HPR0 ISA viruses have been found in all areas with farming of Atlantic salmon, Norway (**Paper II** and **III**), Scotland (Cunningham et al 2002; McBeath et al 2009), USA (**Paper II**), Canada (Cook-Versloot et al 2004), Faroe Islands (Christiansen et al 2011), and Chile (Kibenge et al 2009, **Paper III**).

Nylund et al (2003) showed that the substitution rate of the HE gene was relatively low compared to the substitution rate for genes of surface proteins from other members of the *Orthomyxoviridae*, and they suggested that a possible explanation for this could be

that changes in the HPR could result in changes in virulence of ISA viruses. Hence, all HPR shorter than the HPR0 could be "offspring" of wild type, low virulent, HPR0 ISA viruses, i.e. shorter HPRs would represent a shift towards higher virulence (Nylund et al 2003). This would mean that the shorter HPRs are not a result of an evolutionary trend towards shorter HPRs, but a process where the low virulent, wild type, HPR0 ISA viruses may shift to high virulence. A similar mechanism has been described for changes in virulence of influenza A viruses (Zhou et al 2009; Sorrell et al 2010; Wu et al 2010; Jinling et al 2011). Deletions in segment six of ISA viruses or inserts in the HA gene of influenza A viruses do not change the phylogenetic position of the isolates but lead to changes in virulence. This means that the HPR will not contain any phylogenetic information, distantly related ISA viruses may have the same HPR, but that would be a result of similar recombination events changing wild type HPR0 ISA viruses into virulent ISA viruses with a shorter HPR. The shortening of the HPR seems to follow a fixed pattern.

Nylund et al (2003) launched a hypothesis to explain the maintenance of ISA viruses in Norway and the emergence of ISA in Norwegian salmon farms. The hypothesis was based on the assumption that ISA viruses must have existed for a long time in natural populations of salmonids in Norway. The salmonids (*S. salar* and *S. trutta*) live in small fragmented populations and a specific salmonid virus must have co-evolved with the host resulting in a low or avirulent wild type virus (HPR0 ISA viruses). The emergence of ISA in farmed salmon was suggested to result from transmission of wild

type ISA viruses from wild salmonids to populations of farmed salmon. Change in the HPR (from HPR0 to shorter HPRs) would make the ISA viruses visible in large farmed populations of salmon, and at the same time the virus was given the opportunity to spread within these populations. Transport of infected farmed salmon would also help spreading the virus along the Norwegian coast.

In 2005 it was shown that the ISA virus (probably an avirulent HPR0) could be vertically (or transgenerationally) transmitted (A. Nylund pers.com., Multiple authors 2005), and in a large study of transmission of ISA viruses in farmed populations of Atlantic salmon published in 2006 the importance of vertical transmission was given further support by results from molecular epizootics and detection of a high number of positive smolt populations in fresh water (**Paper II**). This study showed the presence of avirulent HPR0 ISA viruses in smolt populations in Norway and in Atlantic salmon brood fish, and in addition, it was shown that virulent ISA viruses from different areas along the Norwegian coast were closely related. The relationship seemed to reflect the origin of the eggs from which the salmon were hatched, i.e. salmon suffering from ISA caused by closely related ISA viruses came from the same brood fish populations. **Paper II** concluded that a limited number of ISA viruses seem to be circulated in the production cycle of farmed Atlantic salmon in Norway, and suggested that there is little or no transmission of ISA virus from wild salmonids to farmed salmon. The low substitution rate observed for the HE gene could be a result of vertical transmission since this type of transmission will put different constraints on the evolution of the ISA

virus genes compared to viruses that are only transmitted horizontally (**Paper II** and **III**).

The fusion (F) protein was first characterized by Aspehaug et al (2005) who showed that the protein coded for by segment five was a pH-dependent F protein produced as a precursor protein, F0, which was proteolytically cleaved into F1 and F2. Comparison of several F protein genes from several different ISA viruses has shown that inserts may occur in the vicinity of the putative cutting site for trypsin, thus extending the ORF and resulting in ISA virus F proteins ranging from 444 to 455 amino acids (Devold et al 2006). The authors suggested that the insert, which showed 100% identity with other parts of the ISA virus genome, must have been inserted as a result of recombination and could possibly influence the virulence of ISA viruses. Inserts in segment five of ISA viruses has later been demonstrated by several authors (Godoy et al 2008; Markussen et al 2008; Vike et al 2008; Kibenge et al 2009). Devold et al (2006) showed that the substitution rate of segment five, as that of segment 6, is low compared to that of influenza virus surface proteins. It was suggested that the low substitution rate of segments five and six could be understood if the variation in the ISA virus genome generated in marine salmon farms were lost at the time of slaughtering. This would mean that variation in the ISA virus genome generated during the sea water phase of farmed salmon is lost when commercial size is reached and that little variation is transmitted horizontally to other farms or wild reservoirs. This was interpreted as support for the hypothesis of vertical (transgenerational) transmission of

ISA virus in farmed brood fish populations (Devold et al 2006, **Paper II**). Phylogenetic analyses of segments five and six have been used by several authors in studies of ISA virus transmission (Devold et al 2001, 2006; **Paper II & III;** Markussen et al 2008; Kibenge et al 2009; Lyngstad et al 2008, 2011).

Phylogenetic analysis using the complete genome or sequences of all eight segments of the ISA virus have been performed, but only a few isolates were included, 12 and 17 respectively (Markussen et al 2008, Cottet et al 2010). These are the only studies that have included segments three (N protein gene) and four (putative PA gene) in a phylogenetic analysis of ISA viruses. The N and PA proteins are both internal proteins where the former coats the viral RNA and is involved in the nuclear export of the nucleocapsids during ISA virus replication. The putative PA protein is believed to be the acid polymerase and involved in the replication of the ISA virus genome. They are both important for ISA virus replication and their evolution should reflect this.

The combination of transmission of viruses with isolation of different host populations will contribute to the evolutionary divergence generating different virus gene pools. The barriers may be due to infrequent likelihood for transmission connected to partial geographical isolation, behaviour of the host, or that the virus may use several host species. The population structure and the behaviour of the natural hosts (*S. salar* and *S. trutta*) for the ISA virus suggest that a large variation in the number of virus genotypes

could have been maintained along the Norwegian coast reflecting the genetic distinctness of the host species populations. It is less likely that interspecies transmission of ISA virus between *S. salar* and *S. trutta* should have resulted in any differences in the surface protein genes (HE and F) within river systems, but the internal protein genes (PA and NP) may have adapted to the two respective hosts. Hence, the surface protein genes should, to a larger extent, reflect the variation in the different wild populations, while changes in the internal protein genes may, to a larger extent, be a result of adaptation to host species and show less variation. However, farming of Atlantic salmon during the last 30 years must have had a strong influence on the evolution of the ISA virus and the maintenance of virus gene pools. It is well documented that movement of embryos and fish have resulted in movement of ISA viruses along the Norwegian coast, but also as far as from Norway to Chile (Devold et al 2006, **Paper II & III**, Vike et al 2008, Kibenge et al 2009). This has resulted in a situation where the same ISA virus may be found as far apart as in contemporary ISA outbreaks in Northern Norway and Chile (**Paper III**). Chile has now imposed a ban on import of embryos from other salmon producing countries which should isolate the Chilean ISA viruses and should lead to the evolution of a separate gene pool.

Virulence

The virulence and host range of influenza A (family *Orthomyxoviridae*) are believed to be controlled by all genes in the genome (Webster et al 1992; Brown 2000). However, the surface proteins are believed to play a key role (Webster et al 1992). ISA virus HE proteins have a stalk region of variable length (Devold et al 2001; Krossøy et al 2001b;

Rimstad et al 2001; Griffiths et al 2001; **Paper II; Paper III**) and a variable stalk region is also present in the neuraminidase protein of influenza A viruses (Zhou et al 2009; Sorrell et al 2010; Wu et al 2010; Jinling et al 2011). The stalk region of the influenza viruses is known to play a role in determination of virulence. The longest stalk observed in ISA virus HE is called HPR0 and viruses of this type can not be cultured in any of the fish cell cultures tested and they do not cause any pathology or mortality in infected Atlantic salmon. Hence, it has been suggested that the HPR of the HE protein could be an important virulence factor for the ISA viruses (Nylund et al 2003; Kibenge et al 2007b; Markussen et al 2008; Mjaaland et al 2002; Ritchie et al 2008; **Paper II; Paper III**).

Inserts in front of the cutting site of the precursor protein F0 could also influence the virulence of ISA viruses (Devold et al 2006; Kibenge et al 2007b; Markussen et al 2008). As a comparison it has been shown that inserts in the HA protein of influenza A, in front of the cutting site for HA0, could increase the virulence (Hirst et al 2004; Suarez et al 2004). Hence, the HPR of segment six (HE gene) and inserts in segment five (F gene) may both be important virulence factors for ISA viruses.

Little is known about the possible importance of variation in the other six segments of ISA viruses, and one reason for this is the lack of information about the genetic variation in these segments. However, based on knowledge from other

Orthomyxoviruses there is every reason to believe that they play a part in the determination of virulence (Webster et al 1992; Brown 2000).

Virulence of viruses is not only dependent on the virus genome, but also, to a large extent, on the host and host factors. ISA viruses are able to multiply in both salmon and trout in the North Atlantic, but all isolates tested have only been able to cause mortality in salmon (Nylund et al 1994a,b, 1995a,b,c, 1997; Nylund and Jacobsen 1995; Devold et al 2000). The host factors responsible for this difference have not yet been identified.

Risk factors and transmission of ISA virus

The first official outbreak of ISA was registered at a smolt production site in 1984 in western Norway (Thorud & Djupvik 1988). The next year the disease was observed at marine sites that had received smolt from this hatchery (Thorud 1991), and in 1990 ISA (about 80 cases) was present in all counties with production of Atlantic salmon with the highest number of cases in western Norway (Jarp & Karlsen 1997). Most cases of ISA occur in sea water production of Atlantic salmon, during spring and late autumn, i.e. in periods with temperatures between 10 and 15 ºC.

It has been thoroughly documented that the ISA virus can be transmitted horizontally between individuals of Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and trout (*Salmo trutta*) kept in tanks or sea cages (Thorud & Djupvik 1988; Thorud 1991; Nylund et al 1993b, 1994a, 1995a, 1995c, 1997; Nylund & Jakobsen 1995; Dannevig et al 1994; Thorud & Torgersen 1994; Rolland & Nylund 1998a; Devold et al 2000; Griffiths & Melville 2000; Jones & Groman 2001; Snow et al 2001b,c; MacWilliams et al 2007), and in 1992 it was shown that the salmon louse (*Lepeophtheirus salmonis*) could play a role as a mechanical vector for transmission of ISA virus (Nylund et al 1992, 1993b; Rolland & Nylund 1998b). No other organisms have been shown to play a role in ISA virus transmission. The ISA virus may replicate in herring (*Clupea harengus*) and cod (*Gadus morhua*), but these species are not able to transmit the virus to Atlantic salmon during cohabitation (Nylund et al 2002; Grove et al 2007).

The ISA virus is present in mucus, blood and urine from positive Atlantic salmon (Thorud 1991; Nylund et al 1994b; Totland et al 1996; Rolland & Nylund 1998b) and these “fluids” may play an important role in the horizontal transmission between individuals. The distance the ISA virus can be transmitted passively through sea water will depend on the survival time of the virus and the dilution effect during dispersion. The virus will survive for a longer time at low temperatures (like 5 ºC) compared to high temperatures (15 ºC), but the survival time is even more dependent on biological activity in the sea water and exposure to UV light (Henrik Duesund pers. com.). In a

pilot study of the survival of ISA virus in active sea water the survival time was less than 48 hours (A. Nylund pers. com.).

Most cases of ISA occur about one year after sea transfer of Atlantic salmon, but the disease may occur in hatcheries and as early as one month after transfer to sea (Jarp & Karlsen 1997; Nylund et al 1999). Several studies have looked at possible ISA risk factors in marine production of Atlantic salmon, and the overall conclusion has been that the ISA virus is mainly transmitted from infected salmon in farms and slaughterhouses to clean sites through sea water (Vågsholm et al 1994; Jarp & Karlsen 1997; Hammell & Dohoo 2005; McClure et al 2005; Gustafson et al 2007a; Mardones et al 2009; Murray et al 2010). Scheel et al (2007) developed a stochastic space-time model to quantify risk factors for the transmission of ISA virus in Norway (period 2002 to 2005) and they found that seaway distance and local contact networks were factors that affected the transmission between sites. However, there was a large unidentified component of risk other than transmission of virus from infected sites (Scheel et al 2007). A similar result was obtained by Aldrin et al (2010) who found that seaway distance between farms was important for transmission of ISA (period 2003 to 2007), but with a dominance (70 to 80 %) of a non-defined transmission pathway for this disease.

Another important risk factor for introduction of ISA into a sea site is suggested to be purchase of smolts from several fresh water hatcheries (Vågsholm et al 1994; Jarp & Karlsen 1997). It was suggested that the risk was not due to transport of smolt with undiagnosed ISA, but that the smolt was infected during transportation (Jarp & Karlsen 1997). According to Vågsholm et al (1994) the risk factors are not consistent with a hypothesis that ISA has a ubiquitous marine reservoir, and they suggested that rapid sanitary slaughter of primary cases of ISA outbreaks could limit the spread of ISA in a district. In a study of ISA outbreaks in Scotland in 1998 to 1999 it was suggested that the spread of ISA virus was linked to well boat traffic through shipment of live fish (Murray et al 2002). The pattern of spread did not support a natural diffusive expansion of the ISA virus epizootic. The sources for the ISA viruses carried by well boats were believed to be processing plants and adjacent harvesting stations (Murray et al 2002). In a study of spatial and non-spatial risk factors connected to transmission of ISA virus between cages at salmon production sites a diffuse distribution of virus exposure throughout infected sites was observed i.e. limited relationship between cage adjacency and timing of disease (Gustafson et al 2007b). The authors suggested that disease manifestation could be related with host-susceptibility factors.

A method for detection of ISA virus in sea water (able to detect 5.5 viruses/ml) was developed and tested by Løvdal & Enger (2002). Using this method they were able to detect the virus inside cages with outbreaks of ISA, but not in samples collected downstream from the ISA cages (Løvdal & Enger 2002). McClure et al (2004b) tested

the prevalence of ISA virus in farms with and without outbreaks of ISA in the New Brunswick area in the period 2000 to 2002 using RT PCR. Samples were taken from moribund salmon and healthy salmon from cages with outbreaks of ISA, from healthy salmon in a non-outbreak cage on an outbreak farm, from healthy salmon in the non-outbreak neighbour farm, and from healthy salmon from non-outbreak farms distant from outbreak farms. The prevalence of ISA virus was highest in the salmon from cages in the outbreak farms, but ISA virus positive salmon were also found in the two latter groups.

These data are slightly contradictory where some studies indicate horizontal transmission over relatively long distances, while other studies suggest limited transmission between neighbouring cages. One explanation for this could be the fact that ISA virus can be present in salmon populations without causing outbreak of ISA and, hence, spreading of the virus to new sites or cages is not detected, but it also means that the source for the virus causing an ISA outbreak may not be detected. An important fact is that in none of the above mentioned studies were the ISA viruses identified, genotyped, and most of these studies seem to rest on the assumption that salmon infected with ISA virus will eventually develop ISA. Nor have any of these studies discussed the possible importance of avirulent ISA viruses in the spread of the virus and eventual development of ISA (cf: Nylund et al 2003, **Paper II & III**).

In a recent modelling study of transmission of ISA the phylogenetic relationships between virus isolates was combined with space–time data on disease occurrences (Aldrin et al 2011). The study period was 2003 – 2009 with the major emphasis on outbreaks of ISA in a small area in Troms County in 2007 – 2009. The authors found that the risk of infection decreases with increasing distance between sites and that the source of infection in nearly 50 % of the cases is most likely to be ISA viruses closely related to viruses from outbreaks in neighbouring farms (Aldrin et al 2011). The remaining half of the farms included in the study was infected with ISA virus from an unknown source.

The high number of ISA cases in the period 1987 – 1990 made it necessary for Norwegian salmon farmers to introduce measures to reduce the impact of ISA on Norwegian salmon culture. The salmon farmers changed their production from two or more generations at each site to only one generation of Atlantic salmon per site starting in 1990 – 1991. In addition, the authorities introduced a ban on use of sea water in hatcheries and a ban on moving fish already stocked into sea water in 1989, regulations on transport of salmonids in 1990, regulations on disinfection of waste water/offal from fish slaughterhouses, and regulations on disinfection of intake water to hatcheries in 1991 (Håstein et al 1999). After this the official number of ISA cases dropped to only one in 1993, and has since stabilised with about 10 to 20 cases every year. Use of oil vaccines (against bacterial diseases in the production of Atlantic salmon) was also introduced in the early 90s and it has been observed that the oil

adjuvant may give a certain protection against ISA virus. It is difficult to know which of these measures implemented were most important for the reduction in the number of ISA outbreaks from close to 100 every year to as few cases as 10 to 20 every year, however, separation of generations must have played a major role preventing horizontal transmission and a continuous production of virulent ISA viruses at infected sites (cf Gustafson et al 2007a).

The first study of vertical transmission of ISA virus was published by Melville & Griffiths (1999). They collected eggs from Atlantic salmon with ISA virus positive ovarian fluids, and the eggs and resulting offspring (alevins and parr) were tested for virus by cell culture and RT PCR. Healthy parr were also injected with homogenates from eyed eggs. All samples were negative for ISA virus and the homogenates did not induce any mortality in the parr. However, as pointed out by the authors, the detection limit for the RT PCR is not known and the amount of virus could be below the detection limit for the methods used (Melville & Griffiths 1999). The most sensitive method they employed was a PCR using FA-3/RA-3 primers (Devold et al 2000), but this assay will not detect ISA virus in carriers (**Paper I**). In addition they decreased the sensitivity of the RT PCR assays by pooling samples from individual fish. The only method that is sensitive enough to detect carriers of ISA virus is real time RT PCR used on individual samples (pooling of samples will also destroy the sensitivity of real time assays). It should also be added that this study was performed before the scientific

community was aware of the existence of avirulent ISA viruses which can not be cultured in cell cultures.

The first indication of possible vertical or transgenerational transmission of ISA virus came in 2004 (A. Nylund pers. com). Later that year a Norwegian Broodfish company, AS Bolaks (SalmoBreed AS), experienced an outbreak of ISA on brood fish of Atlantic salmon kept in Hordaland County (Multiple authors 2005). Offspring (eggs, embryos and fry) from the ISA virus positive fish were screened, with the help of real time RT PCR (cf. **Paper I**), for presence of ISA virus and the results showed that virus genome could be detected in all stages of development suggesting vertical transmission of the ISA virus, but the fry were killed and it was not verified if the infected fish would develop ISA at a later stage (Multiple authors 2005). Embryos of Atlantic salmon, produced in Norway, have for many years been transported to Chile and in 2007 a large epizootic of ISA started in the Chilean salmon industry. The virus and the disease had previously only been detected in countries around the North Atlantic. The causative ISA virus was identified as a Norwegian genotype and the presence of a Norwegian ISA virus in Chile, a country without natural populations of salmonids, was interpreted as evidence of vertical transmission (Vike et al 2008).

Genotyping of ISA viruses from outbreaks of ISA in Norway has also given support to a hypothesis suggesting that vertical/transgenerational transmission of ISA virus could

be of importance for the dissemination of the virus in Norwegian salmon production (**Paper II**). However, two other studies using genotyping as a tool for understanding the transmission of ISA virus (Lyngstad et al 2008, 2011) did not find any evidence of vertical transmission. But these studies did not include any information about the origin of the salmon (brood fish) at the different production sites suffering from ISA and they included material from a limited time period only. In the latter study it is stated that shared broodfish company was excluded from the analysis, because there are so few broodfish companies in Norway (Lyngstad et al 2011). Lyngstad et al (2008, 2011) arrived at the conclusion that seaway distance between salmon farms is the only trustworthy explanation for the genetic similarity between ISA viruses isolates collected during outbreaks of ISA in Norway.

AIMS OF STUDY

The goal of this project was to characterize the partial genome from a large selection of ISA viruses to gain knowledge about the evolution of this virus and a better understanding of the maintenance and dissemination of ISA viruses in farmed populations of Atlantic salmon.

Specific aims for this study were:

- Mapping of the variation in the nucleotide sequences of the genome segments coding for the two surface proteins, F and HE protein, and the two internal proteins, N and PA protein, constituting a part of the ribonucleoprotein complex of ISA viruses.
- Mapping of important mechanisms (mutations, recombination, and reassortment) for the evolution of ISA viruses in farmed populations of Atlantic salmon.
- Mapping of virulence indicators in the ISA virus genome.
- Establishing a genotyping system for ISA viruses.
- Using the genotyping system to gain understanding of the maintenance and dissemination of ISA viruses.

SUMMARY OF PAPERS

Paper I

Plarre H, Devold M, Snow M, Nylund A (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. Dis Aquat Org 66: 71 – 79.

Studies of infectious salmon anaemia virus (ISA virus), an important pathogen of farmed salmon in Norway, Scotland, the Faeroe Islands, Ireland, Canada, USA and Chile, suggest that natural reservoirs for this virus can be found on both sides of the north Atlantic. Based on existing information about ISA virus it is believed that it is maintained in wild populations of trout and salmon in Europe. It has further been suggested that the ISA virus is transmitted between wild hosts mainly during their freshwater spawning phase in rivers, and that wild salmonids, mainly trout, are possible carriers of benign wild-type variants of ISA virus. Change in virulence is probably a result of deletions of amino acid segments from the highly polymorphic region (HPR) of benign wild-type isolates after transmission to farmed salmon. Hence, it has been suggested that the frequency of new outbreaks of ISA in farmed salmon could partly reflect natural variation in the prevalence of ISA virus in wild populations of salmonids.

The aims of the present study were to screen for ISA virus in wild salmonids during spawning in rivers and to determine the pathogenicity of resultant isolates from wild fish. Tissues from wild salmonids were screened by RT-PCR and Real Time PCR. The prevalence of ISA virus in wild trout (*Salmo trutta*) varied from 62 to 100% between tested rivers in 2001. The prevalence dropped in 2002 ranging from 13 to 36 % in the same rivers and only 6% in 2003. All ISA virus were non-pathogenic when injected into disease-free Atlantic salmon, but were capable of propagation as indicated by subsequent viral recovery. However, non-pathogenic ISA virus has also been found in farmed salmon where prevalences as high as 60% have been registered but with no mortalities occurring. Based on the results of the present and other studies it must be concluded that vital information about the importance of wild and man-made reservoirs for the emergence of ISA in salmon farming is still lacking. This information can only be gained by further screening of possible reservoirs combined with the development of a molecular tool for typing virulence and geographical origin of the virus isolates.

Paper II

Nylund A, Plarre H, Karlsen M, Fridell F, Ottem KF, Bratland A, Sæther PA (2007). Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Arch Virol 152: 151 – 179 (E.pub. 2006).

In the present study 24 smolt production sites were screened for the presence of infectious salmon anaemia virus (ISAV) with the help of a specific real time RT PCR assay, and 22 of these sites had smolts that were positive. If these smolt production sites are representative for the prevalence of ISAV in Norwegian smolts then most marine production sites must be considered to be positive for ISAV. In addition, 92 European ISAV isolates have been genotyped based on the hemagglutinin-esterase gene (HE), and the distribution pattern of these analysed. This pattern has been coupled to information about the origin of smolt, eggs and broodfish in those cases where it has been possible to obtain such information, and with information about ISAV in neighbouring farms. The pattern suggests that an important transmission route for the ISAV could be that the salmon farming industry in Norway is circulating some of the isolates in the production cycle, i.e. some sort of vertical or transgenerational transmission may occur. It has also been shown that avirluent ISAV isolates are fairly common in Norwegian farmed salmon. Based on this it is hypothesized that the change from avirulent to virulent ISAV isolates is a stochastic event dependent on the replication frequency of the virus and the time available for changes in a highly polymorphic region (HPR) of the HE gene to occur. This, and the possibility that only avirluent ISAV isolates are vertical transmitted, may explain why ISA most often occurs at marine sites and why no more than about 15 farms get ISA every year in Norway.

Paper III

Plarre H, Nylund A, Karlsen M, Brevik Ø, Sæther PA, Vike S (submitted). Evolution of Infectious Salmon Anaemia virus (ISA virus). Arch. Virol.

Infectious salmon anaemia virus, ISA virus (*Isavirus, Orthomyxoviridae*), emerged in Norwegian salmon culture in the mid-80s. The genome consists of eight segments coding for at least 10 proteins. ISA viruses show a lot of similarities with influenza A viruses, but differ in many important aspects like number of hosts, host population structure, and transmission. The only known hosts and reservoirs for ISA viruses are salmonids found in countries surrounding the North Atlantic. In this study four different segments in the genome of about 100 ISA viruses have been sequenced in an attempt to understand the evolution of ISA viruses and how these viruses are maintained in and transmitted between populations of farmed Atlantic salmon. The four gene segments are coding for the nucleoprotein (NP), the putative acid polymerase (PA), the fusion protein (F) and the haemagglutinin-esterase (HE).

Analyses of these four genes show that the substitution rates of the internal proteins (NP and PA) are lower compared to the two surface proteins (F and HE). All four segments are evolving at a lower rate compared to similar genes in influenza A viruses. The ISA virus populations consist of avirulent viruses and pathogenic strains with variable virulence in Atlantic salmon. Recombination resulting in inserts close to the proteolytic-cleavage site of the precursor F0 protein and deletions in the stalk region of the HE protein seem to be responsible for the transition from avirulent ISA viruses into

pathogenic strains. It is also shown that reassortment is a frequent event among the dominating ISA viruses in farmed Atlantic salmon.

The pattern that is obtained after phylogenetic analyses of the four gene segments from ISA viruses, suggests that the variation is limited to a few distinct clades and that no major changes have occurred in the ISA virus population in Norway since the first viruses were isolated. Calculation of the time of most recent common ancestor (TMRCA) suggests that the Norwegian ISA viruses separated from the European subtype found in North America between 1932 and 1959. The TMRCA data also suggest that the ISA viruses in Chile were transmitted from Norway in the period from 1995 to 2007, depending on which of the four genes are used in the analysis.

GENERAL DISCUSSION

Host species for ISA viruses

Two major approaches, experimental challenges and screening of wild species, have been used in the search for possible host species for ISA viruses in the North Atlantic. The challenge experiments have shown that naturally occurring salmonids, Atlantic salmon (*Salmo salar*), trout (*S. trutta*) and Arctic Char (*Salvelinus alpinus*), in countries around the north Atlantic can be infected with the virus and may sustain virus replication (Nylund et al 1994a,b, 1995a,b,c; Nylund and Jacobsen 1995; Devold et al 2000; Snow et al 2001a; Rolland & Nylund 1998a). However, while Atlantic salmon and trout can become carriers of ISA viruses the char seem to be able of viral clearance (Snow et al 2001a). Several species of pacific salmon, steelhead trout/rainbow trout (*Oncorhychus mykiss*), coho salmon (*O. kisutch*), chum (*O. keta*) and Chinook (*O. tschawytscha*), have also been tested in challenge experiments as possible hosts for ISA viruses (Nylund et al 1997; Snow et al 2001a; Rolland & Winton 2003; MacWilliams et al 2007). None of these species are naturally occurring in the North Atlantic, but rainbow trout have been introduced several times to countries around the North Atlantic. Some of these *Oncorhynchus* species can sustain replication of the ISA virus and the rainbow tout may even show signs of disease and some of these will end up as carriers (Nylund et al 1997; Snow et al 2001a; Rolland & Winton 2003; MacWilliams et al 2007). During screening of wild salmonids (Atlantic salmon and trout) ISA viruses have been detected in both species in Scotland and Norway in both fresh and sea water (Raynard et al 2001, **Paper I**). Hence, there is both experimental

and empirical support for the hypothesis that wild salmonids in the North Atlantic are the natural hosts for the ISA viruses (cf Nylund et al 2003).

Several other fish species in the marine environment and most often in connection with areas with farming of Atlantic salmon or areas experiencing outbreaks of ISA have been tested for presence of ISA viruses (table 3). All marine fish tested have been negative for presence of ISA virus with the exception of two Pollock (*Pollachius virens*) collected from inside a cage with ISA-diseased Atlantic salmon. It was not possible to culture the virus from these two specimens and the PCR product could not be sequenced (MacLean et al 2003). MacLean et al (2003) also found a pool of tissues (including gill tissues) from five Atlantic cod (*Gadus morhua*), taken from a well boat containing salmon exhibiting clinical ISA, positive for ISA virus using a cell culture. However, all five specimens were negative for ISA virus when they were tested by RT PCR. Both the ISA virus RT PCR positive Pollock and the isolation of ISA virus from Atlantic cod were obtained from populations kept together with Atlantic salmon suffering from outbreaks of ISA. The chances for contamination from the ISA virus positive Atlantic salmon must be extremely high under such conditions and the positive results obtained by MacLean et al (2003) are, in my opinion, best explained as detection of ISA virus contamination of the samples. The contamination hypothesis is also supported by a study performed by Snow et al (2002) who found, after experimental challenges, that *P. virens* was resistant to ISA virus infections and unable to support ISA virus replication. The same result, *P. virens* being resistant to ISA virus

infection, was obtained by McClure et al (2004a) who looked at 93 specimen cohabitating with Atlantic salmon suffering from ISA. All 93 were negative for ISA virus RNA. In a challenge experiment on Atlantic cod (weight = 6 gram) Grove et al (2007) found indication of ISA virus replication and detected virus RNA in the brain of 6 out of 8 Atlantic cod as late as 45 days post challenge (after intraperitoneal injection of ISA virus). The cod showed no signs of clinical disease and no mortality was observed. Atlantic cod cohabitating with Atlantic salmon suffering from ISA (cohabitation challenge) remained negative for presence of ISA virus RNA throughout the experimental period of 74 days (Grove et al 2007). No attempt was made to isolate the possible ISA virus from the brain tissues or inject it into susceptible salmon, which makes it impossible to decide if the virus was replicating in the brain or if the RNA in the brain was a result of selective retention. Based on the result from this study it can not be concluded that the ISA virus is able to replicate in Atlantic cod. The fact that cod cohabitating with salmon suffering from ISA, remained negative with respect to presence of ISA virus strongly suggests that cod is not a potential reservoir species for this virus.

Experimental challenge of herring, *Clupea harengus*, has shown that this species may support replication of the ISA virus after bath challenge, but they were not able to transmit the virus to Atlantic salmon (Nylund et al 2002). However, when tissue samples, taken from herring 26 days post challenge (bath challenge), were homogenized, sterile filtered, and injected into salmon all the challenged individuals

ended up as RT PCR positive for ISA virus and one salmon died showing clinical signs of ISA (Nylund et al 2002). This study shows that the ISA virus is able to infect herring and this species is also able to sustain replication of the virus, but the ISA virus was not transmitted from infected herring to Atlantic salmon. It was also shown that the herring is able of viral clearance 42 day after a bath challenge (Nylund et al 2002). Based on this study it can not be excluded that the wild herring could play a role as a vector for ISA viruses in the marine environment. This could be of importance during outbreaks of ISA in salmon farms in areas with dense populations of herring. However, screening of wild herring for presence of ISA virus, including spawning individuals, has not resulted in any positive samples (table 3). Hence, there are no studies suggesting that herring may constitute a reservoir host for ISA viruses in the marine environment.

Other marine fish species (*Scophthalmus maximus, Anguilla anguilla, Ctenolabris rupestris, Labrus berggylta, Pollachius virens*) have also been challenged with ISA virus, but it has not been possible to show replication of the ISA virus in these species (Kvenseth 1997; Snow et al 2002; MacLean et al 2002; McClure et al 2004a; A. Nylund pers.com). Several wild fish species from the coast of Chile, in areas with outbreaks of ISA, have also been tested and found to be negative for presence of ISA virus (Gonzales et al 2011). The only fish found to be positive in the study from Chile was escaped Atlantic salmon. In addition, three invertebrate species have also been

Table 3. Wild marine fish species tested for presence of ISA virus.

Species	N	Pos	Authors
Scyliorhinus canicula	4	0	Raynard et al 2001
Raja clavata	35	0	Raynard et al 2001
Clupea harengus	1037	0	Raynard et al 2001 MacLean et al 2003, Pers. obs.
Sprattus sprattus	192	0	Raynard et al 2001, Pers. obs.
Ciliata mustela	21	0	Raynard et al 2001
Melanogrammus aeglefinus	321	0	Raynard et al 2001 MacLean et al 2003
Merlangius merlangus	152	0	Raynard et al 2001
Trisopterus esmarki Norway	229	0	Raynard et al 2001
Trisopterus minutus	79	0	Raynard et al 2001
Merluccius merluccius	86	0	Raynard et al 2001
Lophius piscatorius	1	0	Raynard et al 2001
Pleuronectes platessa	48	0	Raynard et al 2001
Solea solea	30	0	Raynard et al 2001
Zeus faber	1	0	Raynard et al 2001
Eutrigla gurnardus	18	0	Raynard et al 2001
Aspitrigula cuculus	30	0	Raynard et al 2001
Myoxocephalus scorpius	2	0	Raynard et al 2001
Agonus cataphractus	1	0	Raynard et al 2001
Callionymus lyra	6	0	Raynard et al 2001
Pomatoschistus microps	2	0	Raynard et al 2001
Lepidorhombus whiffiagonis	25	0	Raynard et al 2001
Zeugopterus punctatus	1	0	Raynard et al 2001
Hippoglossoides	21	0	Raynard et al 2001
Limanda limanda Common	20	0	Raynard et al 2001
Micromesistius poutassou	40	0	Raynard et al 2001
Pollachius virens	232	2[a]	McClure et al 2004a, MacLean et al 2003
*Alosa pseudoharengus**	1059	0	MacLean et al 2003
*Anguilla rostrata**	297	0	MacLean et al 2003
Gadus morhua	235	0[b]	MacLean et al 2003
Pseudupleurinectes	259	0	MacLean et al 2003
Scomber scombrus	211	0	MacLean et al 2003
Cyclopterus lumpus	26	0	MacLean et al 2003
Alosa sapidissima	3	0	MacLean et al 2003
Hippoglossus hippoglossus	2	0	MacLean et al 2003

* = occur in both fresh and sea water.

a = Two positives (by RT PCR) Pollock collected inside a cage with ISA diseased salmon, but it was not possible to culture or sequence the ISA virus (MacLean et al 2003).

b = ISA virus was cultured from a pool of tissues (including gill tissues) from five Atlantic cod taken from a well boat containing salmon exhibiting clinical ISA. However, all five specimens were negative when tested by RT PCR.

Table 4. ISA virus in wild salmonids from selected rivers and fjords in Norway (unpublished). This study is based on testing of gill tissues from Atlantic salmon and trout using real time RT PCR (**Paper I**).

River / fjord	*Salmo salar*	*Salmo trutta*	Year
Møre og Romsdal county			
Eira	0/6 (0.0 %)	0/4 (0.0 %)	2008
Eira	0/31 – (0.0 %)	-	2009
Sogn og Fjordane county			
Nordgulen	-	**1/33 (3.0 %)**	2005
Nordgulen	-	0/30 (0.0 %)	2006
Nordgulen	-	**1/30 - (3.3 %)**	2009
Brekke	-	**6/63 (10.3 %)**	2004
Brekke	-	0/35 (0.0 %)	2005
Brekke	-	0/30 (0.0 %)	2006
Brekke	-	0/30 - (0.0 %)	2009
Hordaland county			
Vosso	0/29 – (0.0 %)	-	2009
Vosso	0/30 – (0.0 %)	-	2010
Øystese (fjord)	**1/4**	0/11 – (0.0 %)	2009
Eidfjord	**1/15 – (6.7 %)**	-	2006
Etnefjord	-	0/54 – (0.0)	2003/2005
Etne-elva	0/21 – (0.0 %)	-	2009
Dale-elva	0/30 – (0.0 %)	-	2009
Rogaland county			
Lyse	0/19 – (0.0 %)	0/11 – (0.0 %)	2007
Lyse	**1/19 – (5.3 %)**	0/10 – (0.0 %)	2008
Lyse	0/13 – (0.0 %)	**1/17 - (5.9 %)**	2009
Lyse	**1/21 - (4.8 %)**	0/18 – (0.0 %)	2010
Lyse	0/13 – (0.0 %)	0/15 – (0.0 %)	2011
Vestbøelva	-	**2/10 – (20.0 %)***	2009
Denmark			
Skjern	0/18 – (0.0 %)	-	2010

* Segment six of the ISA virus has been sequenced from one individual. This is the first ISA virus from wild salmonids that does not have an HPR0 stalk region in the HE protein.

tested for susceptibility to ISA virus: blue mussel (*Mytilus edulis*), *Pecten maximus, Lepeophtheirus salmonis*, but none of these were able to sustain replication of the virus (Skår & Mortensen 2007; Nylund et al 1993a). Based on the existing data about

susceptibility to and presence of ISA virus in wild fish it seems as if the only natural hosts for this virus in the North Atlantic are Atlantic salmon (*S. salar*) and trout (*S. trutta*) (cf. **Paper I**; Raynard et al 2001; table 4).

ISA virus evolution

To fully understand the mechanisms that cause variation and evolution of ISA viruses it is necessary with basic knowledge of the structure and function of the ISA virus genome. The genome consists of eight segments of single stranded RNA which encode 10 or 11 polypeptides (Mjaaland et al 1997; Clouthier et al 2002; Kibenge et al 2007a). All proteins of ISA viruses have not yet been identified, but existing knowledge is presented in Table 1. In addition to the two surface proteins (HE and F) the encoded polypeptides include the same three polymerase (PB1, PB2 and possibly PA) proteins as seen in influenza A viruses, a nucleoprotein (NP), a matrix protein (M), two interferon antagonists (NS1 & NS2) and nuclear export protein (NEP). Segment seven encodes two proteins (NS1 & NEP), but the presence of a third protein (NS3) encoded by this segment has been suggested by Kibenge et al (2007a). However, this has yet to be confirmed by other studies.

The surface protein, HE, is responsible for binding the virions to residues (sialic acid) on the surface of the host cell and releasing the virions from infected cells through cleavage of the host cell residues, while the other surface protein, F, provides fusion of

the virion envelope and the host cell membrane. The three polymerase proteins and the NP constitute the ISA virus replication machinery, the ribonucleoprotein complex (RNP). The major focus of the present study is on segments three, four, five and six, encoding two surface proteins (HE & F) and two internal proteins (NP & PA). The evolution of these four proteins is compared with proteins with a similar function in influenza A viruses.

The ISA viruses, like the other members of the family *Orthomyxoviridae*, lack "proofreading" mechanisms and are therefore unable to repair errors occurring during the replication process, which may lead to substantial genetic diversity and result in a high substitution rate. The segmented genome of ISA viruses also allows for exchange of RNA segments between genotypically different ISA viruses resulting in generation of new genotypes/strains, a process termed "shift" in the evolution of influenza viruses. In addition to mutations and reassortment causing evolutionary change in ISA virus populations, the evolution of the ISA virus genome is also influenced by recombination events (cf. Devold et al 2001, 2006; **Paper II** and **III**). Recombination is considered as a very rare event in the evolution of influenza A viruses, while it seems to occur more frequently in the evolution of ISA viruses (Forrest & Webster 2010; Devold et al 2001, 2006; **Paper II** and **III**). Another important difference between influenza A viruses and ISA viruses is the number and variation of host species. Influenza A viruses have been found in a large number of aquatic bird species and domestic poultry, and in humans, pigs, horses, dogs, cats, mink, seals and whales, while only two closely related

fish species (*Salmo salar* and *S. trutta*) are recognized as natural hosts for the ISA viruses (Raynard et al 2001; Nylund et al 2003; MacLean et al 2003; **Paper I;** see discussion above). The difference in number of hosts and the sizes of the host populations should have a large influence on the evolution of these two viruses where the genetic variation maintained in ISA virus populations is expected to be much smaller compared to that of influenza A viruses.

Antigenic drift, caused by point mutations selected for because of increased genetic fitness, is considered as a continuous process in influenza A viruses (Forrest & Webster 2010; Nelson & Holmes 2007). Accumulation of such changes is expected to be most pronounced in the surface proteins (HA and NA) of influenza A viruses at the antibody binding sites, while the changes in internal proteins should occur at a lower rate (Webster et al 1992). However, the substitution rate of influenza A viruses is also influenced by reassortment (shift), which may speed up the substitution rate of internal proteins like NP and PA (Wong et al 2011). Reassortment occurs when genotypically different viruses infect the same host cell and an exchange of RNA segments occurs, resulting in a new combination of gene segments. For influenza A viruses this may occur in avian reservoir species or any of the other hosts resulting in a virus to which the host population is immunologically naive (Nelson & Holmes 2007). For the ISA virus the situation is different with only two known host species, *S. salar* and *S. trutta*, in the north Atlantic area, and with very small fragmented wild populations of these two species. The highest number of hosts for the ISA viruses is found as farmed

Atlantic salmon, which means that the host populations' structure for ISA viruses are very different from that of influenza A viruses. Farmed Atlantic salmon are slaughtered if experiencing an outbreak of ISA or when the commercial size is reached, meaning that all variation accumulated in ISA virus populations during the production of the Atlantic salmon is lost unless the virus is transmitted to neighbouring salmon populations (horizontal transmission) or to the offspring of Atlantic salmon brood fish (transgenerational/vertical transmission). The difference in population size and life expectancy of the hosts for ISA viruses and influenza A viruses should influence the substitution rates and the survival of new genotypes generated as a result of reassortment and mutations.

The substitution rates will also reflect differences in methodology and number of virus sequences used for analysis (Table 5). The substitution rate for the ISA virus HE gene ranges from 1.13×10^{-3} per site per year, N = 13 sequences (Kibenge et al 2007b) to 6.0×10^{-6}, N = 54 sequences (Devold et al 2006). The substitution rates for this gene obtained in **Paper II** and by Devold et al (2006) are nearly identical while the substitution rate for the HE gene obtained in **Paper III** is significantly higher, but lower than the rate given by Kibenge et al (2007b). The number of sequences used in **Paper II** and **III** in the calculation of the substitution rate for the HE gene is nearly the same, but the time span for the collection of the viruses differs with six years and two different methodologies have been used, which may have influenced the results. The relatively large differences in substitution rates between influenza A viruses and ISA

viruses could be explained by many factors (population size, transmission routes, number of host species, etc) where one explanation could be that the ISA viruses are "old" viruses in salmonid hosts which evolve more slowly compared to influenza A viruses that may change host from birds to humans and evolve more rapidly to evade host immunity and achieve efficient transmission in new host species (cf. Nelson & Holmes 2007).

Table 5. Substitution rates per site per year of four ISA virus genome segments. N = number of sequences included in the analysis.

N	Year span	Seg. 3	Seg. 4	Seg. 5	Seg. 6	Publication
54	1987-2004	-	-	8.60×10^{-5}	6.10×10^{-6}	Devold et al 2006
93	1987-2005	-	-	-	0.70×10^{-5}	**Paper II**
13	1997-2004			0.67×10^{-3}	1.13×10^{-3}	Kibenge et al 2007b
96	1987-2011	1.9×10^{-4}	2.2×10^{-4}	3.9×10^{-4}	3.5×10^{-4}	**Paper III**

Recombination can occur as non-homologous recombination between two different RNA segments or as homologous recombination involving template switching while the polymerase is copying the RNA. Both types of recombination may occur in influenza A and ISA viruses, but the latter type of recombination will be more difficult to document. Non-homologous recombination does occur between the HA and NP genes of influenza A viruses and is thought to be responsible for outbreaks of some types of avian influenza (Suarez et al 2004; Perdue & Suarez 2000; Hirst et al 2004). This type of recombination has also been observed in the F protein gene of ISA viruses where inserts are introduced ahead of the trypsin cutting site of the precursor F protein, F0 (Devold et al 2006). The inserts have so far been obtained from the N, F and PB1

proteins of the ISA virus genome. It is believed that the inserts in the F protein influence the virulence of ISA viruses (Devold et al 2006; Godoy et al 2008; Markussen et al 2008; Vike et al 2008; **Paper III**). Evidence of homologous recombination in influenza A viruses has not yet been definitely shown according to Nelson & Holmes (2007), but it has recently been demonstrated that intragenic, homologous, recombination plays a role in driving the evolution of influenza A viruses (He et al 2008, 2009). In studies of ISA viruses it has also been suggested that the variation seen in the stalk region of the HE protein is a result of homologous recombination (Devold et al 2001; **Paper II**). A major argument for the hypothesis of recombination in the stalk region (HPR) of the ISA virus HE proteins is the deletion pattern observed in this region of the protein (Devold et al 2001; **Paper II & III;** Table 2).

Studies of influenza A have shown that the N proteins evolve into host-specific lineages and that the evolutionary rates may vary among the lineages (Gorman et al 1990a, 1991; Xu et al 2011). The substitution rate of ISA virus N proteins is relatively low compared to that of influenza A viruses, and the number of variable positions in the nucleotide and putative amino acid sequences is significantly lower compared to that of the surface proteins from the same viruses (Table 6, **Paper III**). The PA nucleotide and amino acid sequences have an even lower number of variable positions, but a slightly higher substitution rate compared to the N protein genes from these ISA viruses. All ISA virus sequences of the NP and PA have been obtained from farmed

Atlantic salmon (**Paper III**), which means that we are probably only looking at two proteins already adapted to this host. The phylogenetic analysis of the N proteins shows poor resolution with only a few well supported clades and no indications of more than one distinct lineage (cf. Xu et al 2011; **Paper III**). The pattern obtained after analysis of the PA protein gene is different from that of the N protein gene where the former has evolved into two separate lineages in European farmed Atlantic salmon (**Paper III**). It could be that this is a result of reassortment between Atlantic salmon ISA viruses and trout ISA viruses where a trout ISA virus segment 4 (PA) has been introduced into the former. Another possibility is, of course, that one of the PA lineages has been introduced from an Atlantic salmon ISA virus from a different geographical area in Europe. It can not be excluded, but I find it less likely that one of the lineages comes from an unidentified marine reservoir or from herring.

Table 6. Percentage of variable positions in the nucleotide and amino acid sequence alignments of the nucleoprotein (N), acid polymerase (PA), fusion protein (F) and the hemagglutinin-esterase (HE) protein from 96 ISA viruses (based on **Paper III**).

	NP	**PA**	**F**	**HE**
Nucleotides	12.7	11.7	15.1	16.9
Amino acids	11.8	9.3	15.1	16.2
	Internal proteins		**Surface proteins**	

Virulence

In their natural reservoir, influenza A viruses are largely benign and cause asymptomatic infections (Forrest & Webster 2010), and the same observation is typical for a large number of ISA viruses from wild and farmed Atlantic salmon in fresh and sea water (Cunningham et al 2002, **Paper II, Paper III**, A. Nylund pers.com). In avian species lethal influenza A causes systemic infections, and the major change in these viruses is the acquisition of basic amino acids in the cleavage site of the HA (cf. Forrest & Webster 2010). High pathogenicity of influenza A in mammals is associated with polygenic traits and specific mutations have been identified in the two polymerases, PB1 and PB2, and in the NS, HA (basic amino acid inserted) and NA (deletions in the stalk region). Hence, the virulence of influenza A viruses is polygenetic. Even though it has yet to be documented there is every reason to believe that the virulence of ISA viruses is also polygenetic.

Like the influenza A viruses the ISA viruses show some of the same characteristics in the surface proteins: a) inserts of amino acids ahead of the cutting site of the HA0 (influenza A) and F0 (ISA viruses), and b) a variable length of the stalk region of the other surface protein, neuraminidase, NA (influenza A) and haemagglutinin-esterase, HE (ISA viruses). It is well documented in the literature of influenza A viruses that these characteristics play a role in the determination of virulence (Matrosovich et al 1999; Hirst et al 2004; Cheung & Poon 2007; Zhou et al 2009; Wu et al 2010; Sorrell et al 2010; Spackman et al 2010; Li et al 2011). It is also acknowledged that ISA

viruses with a full length stalk region, HPR0, in the HE protein are of low (avirulent) virulence, while all ISA viruses, detected so far, with a shorter HPR have the ability to cause disease in Atlantic salmon (Nylund et al 2003; Kibenge et al 2007b; Markussen et al 2008; Mjaaland et al 2002; Ritchie et al 2008; Christiansen et al 2011; **Paper II** & **III**). The ISA viruses with shorter HPRs can be cultured in available cell cultures, while the HPR0 ISA viruses can only be cultured in salmonid hosts (McBeath et al 2011; A. Nylund, pers. com.). However, it has been shown that the HPR0 HE protein is fully functional in terms of both receptor-binding and receptor-destroying activity (McBeath et al 2011), which raises a question about the role of the HPR in determination of virulence of ISA viruses. In a study of cells transfected with F protein Aspehaug et al (2005) found that the fusion activity was much more efficient in cells co-expressing the HE and F proteins. This led them to suggest that the ISA virus HE protein could be required in the fusion process (Aspehaug et al 2005). A comparison of F proteins from avirulent (low virulent) HPR0 ISA viruses shows that they all share the same residue at position 266 (Q^{266}) while all pathogenic ISA viruses have L^{266} at this position or an insert close to this residue (**Paper III**). The ISA viruses with an insert close to the putative trypsin cleavage sites all have a Q or H at position 266 (**Paper III**). Only one low virulent ISA virus deviating from this pattern, with proline (P^{266}) at this position, has been found (Kibenge et al 2007b). The fact that all avirulent HPR0 ISA viruses, detected so far, have a unique amino acid (Q) at position 266, only shared by virulent ISA viruses with an insert close to this residue, makes it impossible to decide, at the present, if the HPR0 is an important factor for virulence or if the virulence is determined by the residue at position 266 in the F protein, or if a

combination of these two markers determines if ISA viruses are of low or high virulence.

The study published by McBeath et al (2011) clearly shows that the HPR0 stalk region of ISA viruses does not affect the functional activity of the HE protein. Hence, if the fusion activity of the ISA virus F protein is dependent on the HE protein stalk length then this may explain what seems to be a coupling between HPR0 in the HE protein and the residue Q^{266} in the F protein. This hypothesis requires that a change in one of these characters requires a complementary change in the other. Another explanation for what seems to be a coupling between the two characters could be that the sampling size is small and not a real representation of naturally occurring combinations of these characters. Low virulent (or avirulent) ISA viruses could exist that have a short HPR and Q^{266} in the F protein or *vice versa*, however, the latter combination is probably less likely to occur compared to the former. It should be added that while Kibenge et al (2007b), Johnsen et al (2008), and Ritchie et al (2008) consider the HPR as an important virulence factor based on field data and challenge experiments, Cottet et al (2010) hypothesized that the HPR is not by itself a virulence factor, but that virulence is the consequence of multiple factors rather than a specific change in only one segment. They suggest that the HPR should be discarded as a virulence marker (Cottet et al 2010). It is not difficult to agree with the assumption that virulence is a consequence of multiple factors including the host and all eight gene segments of the ISA virus, however, it is still a fact that all HPR0 ISA viruses with a Q^{266} residue in the

F protein are of low (avirulent) virulence. Whether it is the residue in the F protein (Q^{266}) or the HPR in the HE protein, or a combination of both, that is responsible for the virulence of these ISA viruses remains to be shown.

It is also known that glycosylation sites in the surface proteins may have an influence on the virulence of influenza viruses by affecting the level of binding to the host cells receptor, i.e. influencing structure and function of the surface proteins (Lebarbenchon & Stallknecht 2011; Matrosovich et al 1999; Zaraket et al 2009). The NetNGlyc 1.0 and the NetOGlyc 3.1 servers were used to identify the N-Glycosylation sites and for predictions of type GalNAc O-glycosylation sites in the F and HE proteins from ISA viruses (http://www.cbs.dtu.dk/services/NetOGlyc/ and http://www.cbs.dtu.dk/services/NetNGlyc/) (Julenius et al 2005). The predictions of N-glycosylation sites are done only on the Asn-Xaa-Ser/Thr sequons, including the Asn-Pro-Ser/Thr. This software identified one O-glycosylation and two possible N-Glycosylation sites in the HE protein from European ISA viruses (Table 7). The position of the O-glycosylation site was at residue T^{125} and this site was present in all European ISA viruses. Two putative N-Glycosylation sites were identified in the surface part of the HE protein of the European ISA viruses, but only one ($^{333}NIT^{335}$) of these was present in all viruses with two exceptions: one virus from Norway, Vir28 (Ac. no. DQ785257), and one from Chile, VT11282007-042, (Ac.no. EU625679), which both lack this glycosylation site. The putative amino acid sequence of the HE from Vir28 lacks N-Glycosylation sites completely, while the Chilean ISA virus has

one in position ^{154}NSS^{156}. The ^{154}NSS^{156} residue is shared with the majority of the other Chilean ISA viruses, and ISA viruses belonging to the North American genotype have a putative glycosylation site in nearly the same position, ^{155}NPT^{157} (Krossøy et al 2001; Kibenge et al 2001b). Both Krossøy et al and Kibenge et al suggest that this N-Glycosylation site may not be used. The other N-Glycosylation site (^{349}NQT^{351}) of the European ISA viruses was located in the stalk region of the HE protein and, hence, not present in all viruses. However, this is considered as a weak glycosylation sequon and probably not in use.

Table 7. O-glycosylation and N-Glycosylation sites in the F and HE protein from European genotype of ISA viruses (cf. Julenius et al 2005).

Protein	Protein	N-Glycosylation, aa no			O-glycosylation, aa no	
F	Position	24 - 26	110 - 112	359 - 361	48	50
	Sequence	NPT++	NLT+++	NIS+	T	T
HE	Position	154 - 156	333 - 335	349 - 351	125	
	Sequence	NSS+	NIT ++	NQT-	T	

Two and three O-glycosylation and N-glycosylation sites were identified in the F proteins from European ISA viruses, respectively (Table 7). The NetOGlyc 3.1 server identified two O-glycosylation sites (T^{48} and T^{50}) in the F proteins of ISA viruses belonging to the European genotype, while the two sites given in an earlier publication are T^{52} and T^{64} (Aspehaug et al 2005). All four of these putative O-glycosylation sites are highly conserved with three exceptions, H93/04, N29/97, and N32/98. The first lacks a glycosylation site in position 48, while the latter two (N29/97, N32/98) lack a

glycosylation site in position 52. It remains to be shown which of these four putative O-glycosylation sites are functional. All three putative N-glycosylation sites detected are present in both the European and in the North American ISA virus genotypes. The conservation of ^{110}NLT^{112} and ^{359}NIS^{361} suggests, according to Cottet el at (2010), that these two sites are indispensable for the function of the fusion protein. The first putative N-glycosylation site (with a proline, P^{25}), ^{24}NPT^{26}, is also shared by all members of the European genotype, but was not considered as a functional site by Aspehaug et al (2005) and Cottet et al (2010). The glycosylation sites in the F and HE proteins from ISA viruses belonging to the European genotype are conserved among both avirulent (HPR0 ISA viruses) and virulent ISA viruses with only a few possible exceptions (see above).

The three polymerase proteins (PB1, PB2, PA) and the NP constitute the ISA virus replication machinery, the ribonucleoprotein complex (RNP), and single amino acid substitutions in these proteins may lead to an increased virulence in influenza A viruses (Forrest & Webster 2010; Cheung & Poon 2007). This may also be the case for ISA viruses, but the PA and NP from avirulent ISA viruses do not differ from any of the virulent isolates (**Paper III**), and the information about PB1 and PB2 is limited to a few isolates only, giving no solid bases for any conclusions. However, it has been suggested that mutations in the RNP of ISA viruses from Chile could be an adaptation to a new environment allowing the wide distribution observed (Cottet et al 2010). One such substitution is the I^{67} – T^{67} in the NP of the Chilean ISA viruses. However, the

change of residue 67 in the NP is also found in avirulent ISA viruses from Chile and in a virulent ISA virus (isolate FM168/10) from Norway (**Paper III**), hence, this is not a unique adaptation for spreading of virulent ISA virus along the Chilean coast as suggested by Cottet et al (2010). The two residues S^{105} and S^{558} in the NP of ISAV901 from Chile are unique substitutions found in no other ISA viruses including other viruses from Chile. The only unique amino acid substitutions (T^{204}, A^{328}) in the PA protein from Chile, mentioned by Cottet et al (2010), are found in the isolate ISAV901, but are not present in any of the other ISA viruses from Chile (cf. **Paper III**). Two of the substitutions in the PA protein (L^{292}, E^{471}) mentioned by Cottet et al are commonly occurring in European ISA viruses and the residue R^{531} is also present in avirulent ISA viruses from Chile and a virulent ISA virus isolate (FM168/10) from Norway (cf. **Paper III**). Hence, segment three (NP gene) and segment four (PA gene) of ISA viruses from Chile provide no evidence for unique substitutions in these segments adapting the virus to new environments explaining the wide distribution of these along the Chilean coast.

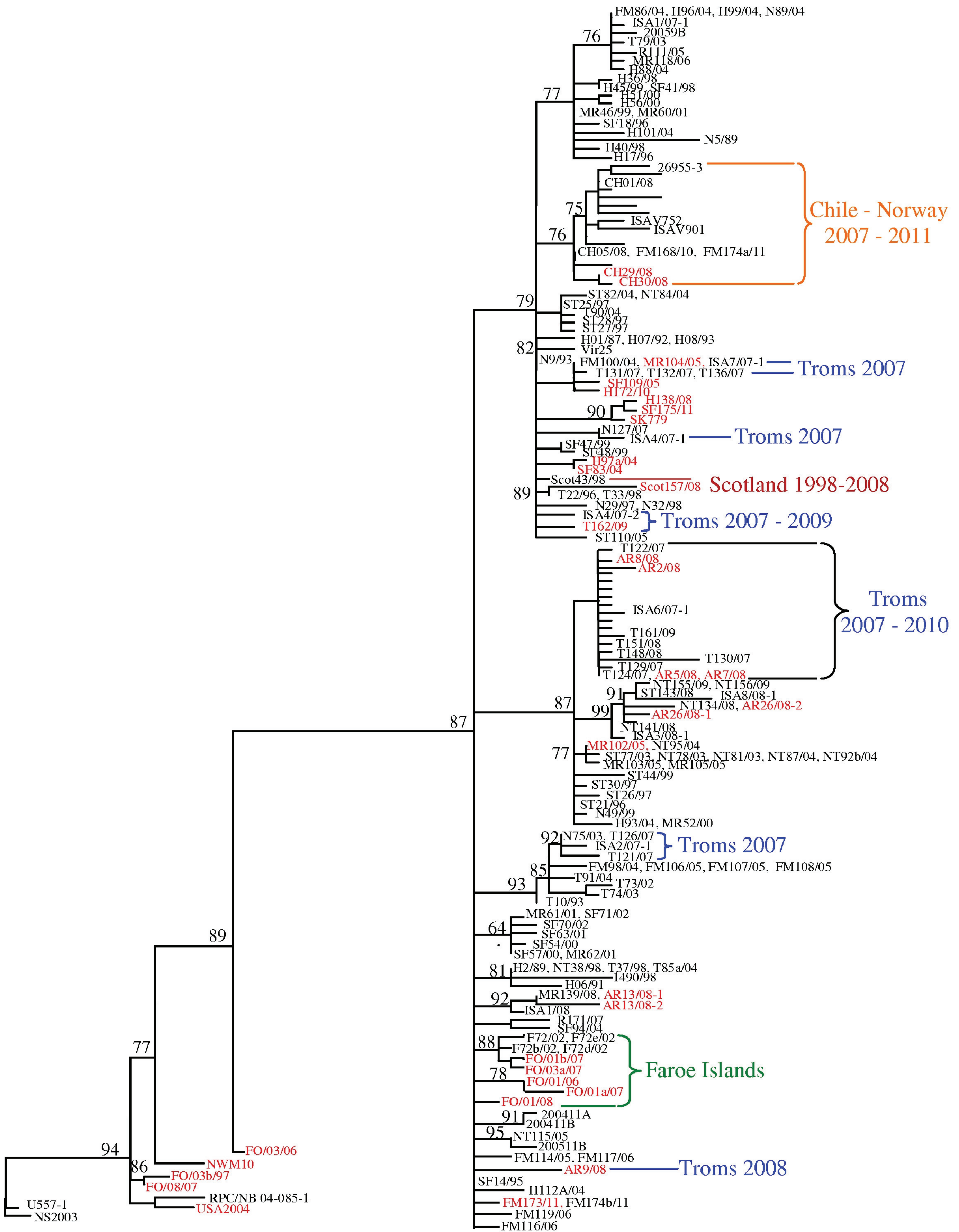
Molecular epizootiology

Viruses are best characterized and identified by sequencing of their respective genomes. Single stranded RNA viruses have a relatively high mutation rate, resulting in high substitution rates, due to lack of enzymes for proofreading, and hence, information from such genomes can be used to identify and follow the spreading of specific virus isolates (Gorman et al 1990a, 1990b, 1991; Subbarao & Shaw 2000;

Ikonen et al 2005; Bahl et al 2009; McHardy & Adams 2009; Zaraket et al 2009; Fourment et al 2010; Bataille et al 2011; Lebarbenchon & Stallknecht 2011; Xu et al 2011). For most RNA viruses the largest variation is found in genes coding for virus surface proteins, and such genes have been used in several studies of phylogeny and molecular epizootiology of fish viruses (Einer-Jensen et al 2004, 2005a,b; Kurath et al 2003; Snow et al 2004; Thiery et al 2004; Nylund et al 2008; Karlsen et al 2006; Duesund et al 2010). In phylogenetic studies of ISA viruses the main targets have been the two surface protein genes, F and HE (segments five and six) and have included ISA viruses from most salmon producing countries around the North Atlantic and from Chile (**Paper II & III;** Devold et al 2001, 2006; Krossøy et al 2001a; Nylund et al 2003; Vike et al 2008; Kibenge et al 2009; Cottet et al 2010; Christiansen et al 2011; Lyngstad et al 2011). These studies show that the best resolution between ISA virus isolates is obtained by using the surface protein genes (F and HE) in the phylogenetic analysis, but they also show that reassortment of the gene segments of ISA viruses are frequent events among the dominating isolates in farmed Atlantic salmon (**Paper III**). Hence, a safe identification of the isolates depends on sequencing of a large part of the genome. Another requirement is that as many ISA virus isolates as possible should be included in the phylogenetic analysis to make sure that the correct affinities of the isolates in question are identified. Figure 5 gives an example of a phylogeny based on the majority of variable HE gene sequences available in the GenBank. This figure also shows that the outbreaks of ISA in Troms in the period 2007 – 2010 were caused by several different ISA viruses, i.e. ISA viruses belonging to six different strains, which

show that the epizootic was not caused by the introduction of a single virus spreading horizontally between farms (cf. **Paper III**).

Figure 5. Phylogenetic tree showing the relationship between ISA viruses from Europe and North America based on 942 nucleotides that code for the HE protein excluding the highly polymorphic region (HPR), the transmembrane region and the cytoplasmic tail region. The best-fitting nucleotide substitution model was used during maximum likelihood analysis and the tree was bootstrapped (50 000 quartet puzzling steps) in TREE_PUZZLE. The scale bar shows the number of nucleotide substitutions as a proportion of branch lengths. Information about the sequences can be found in Appendix A.


Chile - Norway
2007 - 2011
Troms 2007
Troms 2007
Scotland 1998-2008
Troms 2007 - 2009
Troms
2007 - 2010
Troms 2007
Faroe Islands
Troms 2008
0.01

Reservoirs and transmission of ISA virus

The only wild species that seem to be able to sustain replication of ISA viruses in Norway are the salmonids, *Salmo salar* (Atlantic salmon) and *Salmo trutta* (Trout). The virus has been found in these species in Scotland and Norway and positives have also been found in both fresh and sea water (Raynard et al 2001, **Paper I**). The prevalence of ISA virus in trout in a few selected rivers in western Norway (Sogn og Fjordane) was relatively high in 2001 – 2002 (**Paper I**). In the years since 2003, however, it has only been possible to find a few ISA virus positive salmonids in rivers and fjords on the west coast of Norway (Table 4). Only a few outbreaks of ISA in farmed Atlantic salmon have occurred in this area in the same period. This raises the question about which reservoir for ISA viruses is most important in the present situation in Norway. Is it wild salmonids or farmed Atlantic salmon? Based on the population size of wild salmonids compared to the number of farmed Atlantic salmon it is obvious that farmed salmon has the largest potential as reservoir for this virus. The relatively low prevalence of ISA virus in wild salmonids, the low population sizes, the behaviour of these species in sea water, and the fact that all those found positive so far are carrying avirulent ISA viruses, suggest that wild salmonids are not important as reservoir for ISA virus and for transmitting this virus to farmed Atlantic salmon.

The major reservoirs for ISA viruses seem to be farmed Atlantic salmon in fresh and sea water (**Paper II & III**). The majority of smolt production sites tested contain smolt positive for ISA viruses (**Paper II**) and the virus is also very often present in Atlantic

salmon in the marine production phase without causing any outbreaks of ISA (**Paper II** and **III,** Nylund et al 2011, A. Nylund pers. com). Sequencing of ISA virus from Atlantic salmon kept in farms with no outbreaks of ISA will in most cases result in identification of HPR0 ISA viruses. These avirulent ISA viruses seem to be dominating in farmed Atlantic salmon and can be found in farms in all parts of Norway. Based on the population size and distribution of farmed Atlantic salmon along the Norwegian coast it is relatively safe to assume that they constitute the major reservoir for this virus in Norway. Even if herring may sustain replication of ISA virus after experimental challenge it has not been possible to detect the virus in wild herring (see Table 3), and no evidence exists to suggest that this species may play a role as reservoir for ISA viruses.

It is obvious from challenge experiments and empirical data from the field that ISA viruses can be horizontally transmitted between individuals within a farm site during an outbreak of ISA. However, the distance of transmission is more difficult to document, and in several farms it has been observed that if salmon in a cage suffering outbreak of ISA are slaughtered, transmission to neighbouring cages can be prevented. Still, when several outbreaks of ISA occur in the same area it is not uncommon to find nearly identical ISA viruses in Atlantic salmon in neighbouring farms (Lyngstad et al 2011, **Paper III**). The hypothesis promoted by the Norwegian Food Authorities, to explain several local outbreaks of ISA, is that all spreading of ISA virus is due to horizontal transmission in the marine production phase of Atlantic salmon (cf.

Lyngstad et al 2011), and that the origin of "new" ISA viruses comes from unidentified marine reservoirs. That ISA viruses can be transmitted horizontally between farms in a small area when escaped infected salmon are present is not very controversial (cf. Lyngstad et al 2011, **Paper III**). However, the studies of horizontal transmission based on genotyping of the ISA virus are few and,so far, it has not been possible to detect any natural marine reservoirs for ISA viruses in such areas. One area where possible horizontal transmission has been studied is in northern Norway, southern Troms, in the time period from 2007 - 2010 (Lyngstad et al 2011, **Paper III**). It is well documented that in the majority of salmon farms suffering outbreaks of ISA the salmon were infected with nearly identical ISA viruses. However, all these farms had also received salmon that originated from the same brood fish company (**Paper III**). In addition, to get the complete picture of the situation in this area, one will also have to include all ISA virus isolates detected in all farms during the mentioned period. A complete overview of the ISA outbreaks in this area shows that a minimum of six very different ISA viruses were the causes for the outbreaks, and hence, horizontal transmission of ISA virus from a primary outbreak can not explain the observations (Figure 5, **Paper III**). Admittedly, based on the fact that the majority of the ISA viruses identified had nearly identical genomes (four segments sequenced), the same HPR, and the presence of escaped salmon infected with ISA virus, the hypothesis of horizontal transmission can be supported (**Paper III**). However, it should be added that the dominating ISA virus in southern Troms (2007 – 2010) was closely related to ISA viruses from earlier outbreaks in western and mid Norway, and not a new emerging ISA virus with no previous history in Norway (cf. figure 4, **Paper III**). Phylogenetic analysis of segment

6 (the HE protein gene) from ISA viruses shows that the clade including the dominating ISA viruses from Southern Troms (2007 – 2010) also contains several HPR0 ISA viruses, including one from a fresh water site (western Norway) belonging to the brood fish company from where salmon in the infected farms originated (**Paper III**).

A large amount of evidence, supporting the hypothesis of vertical/transgenerational transmission of ISA viruses, has accumulated during the last six years (Multiple authors 2005, Vike et al 2008, **Paper II & III,** A. Nylund pers. com.). Brood fish companies in Norway have experienced several outbreaks of ISA in their brood fish stock during the last decades, and screening of brood fish in normal production has on several occasions shown that close to 100 % of the fish were infected with HPR0 ISA viruses (A. Nylund pers.com., Multiple authors 2005, **Paper II**). HPR0 ISA viruses belonging to the same clades as the brood fish ISA viruses can also be found in salmon smolt in fresh water and in salmon in marine farms (**Paper II & III**), and the evidence from existing literature clearly suggests that the pathogenic ISA viruses originate from these HPR0 ISA viruses (**Paper II & III**, Christiansen et al 2011). The emergence of ISA in Chile, caused by a Norwegian ISA virus, i.e. ISA virus in a country on the southern hemisphere with no natural populations of salmonids importing large amounts of salmon embryos every year for industrial production, strongly supports the hypothesis of vertical/transgenerational transmission of ISA viruses (Vike et al 2008).

Based on the existing evidence of vertical/transgenerational transmission of ISA viruses, presence of ISA viruses in smolt in fresh water, and outbreaks of ISA in geographically very distant areas caused by nearly identical ISA viruses, strongly suggests that vertical transmission plays an important role in the maintenance and spreading of ISA virus in Norwegian salmon production (**Paper II** & **III**). This does not, of course, exclude the possibility that horizontal transmission may play a role during outbreaks of ISA in local areas, but based on the wide distribution of the few ISA virus clades present in farming of Atlantic salmon in Norway, the most important transmission route seems to be movement of HPR0 ISA viruses in connection with smolt transport (cf. **Paper II**). Since HPR0 ISA viruses are present in both brood fish and smolt of Atlantic salmon in pure fresh water, and since offspring (embryos and fry) from ISA virus positive brood fish stay positive throughout production in fresh water, it seems highly likely that the salmon industry is circulating a small number of ISA viruses in the production system (**Paper II** & **III**).

The existing knowledge about hosts susceptible to ISA viruses in the North Atlantic, results from studies of genotyping of ISA viruses from healthy and ISA suffering salmon, and presence of vertical transmission of ISA viruses strongly suggests that the main reservoir for ISA viruses in Norway is farmed Atlantic salmon. In this system the brood fish seem to play a central role in the dissemination of these viruses.

FUTURE WORK

A future aim of ISA virus research is to fully understand the molecular basis for interactions of the virus and the host. The sequencing of the complete genome of Atlantic salmon will soon be finished and the sequences of some ISA viruses have already been established. Hence it should be possible in the coming years to resolve the genetic control of the interactions of ISA viruses and the host (Atlantic salmon), which should give a new and better understanding of pathogenesis and possibly new insight into how to control the spreading of ISA viruses and the mechanisms behind outbreaks of ISA. It is difficult to imagine that the ISA virus can be eradicated from the natural populations of salmonids in the North Atlantic, but strategies to eradicate the virus from farmed populations can already be envisioned. The remarkable advances in molecular biology, including reverse genetics, during the last decade also point towards new strategies for vaccine development as another option in the control of ISA viruses and development of ISA in farmed populations of Atlantic salmon.

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