



**Opinion of the Panel on Animal Health and Welfare of the Norwegian
Scientific Committee for Food Safety
26.01.07**

**Which risk factors relating to spread of Infectious Salmon Anaemia (ISA)
require development of management strategies?**

SUMMARY

Infectious salmon anaemia (ISA) is a viral disease that was first recorded in 1984 in fish from an Atlantic salmon (*Salmo salar*) hatchery. The original disease spread in a pattern consistent with a contagious disease and the contagious nature of the disease was also confirmed experimentally.

The ISA epidemic in Norway peaked in 1990 when ISA was detected in 80 fish farms. ISA was placed on the list B of *notifiable* diseases in Norway in 1988. During 1988-1991, various biosecurity actions were implemented. The essence of these regulatory actions was to interrupt the transmission of infection and to reduce infection pressure. The result of these actions, together with significant improvements in husbandry practices, was a remarkable and a rapid reduction in the number of ISA outbreaks to only 2 outbreaks in both 1994 and 1995. The present accumulated number of ISA disease outbreaks registered in Norway is 437, of which 3 (0.7 %) have occurred in the fresh water phase. Today, the apparent annual prevalence of ISA is about 2 %.

To ensure that the Norwegian Food Safety Authority bases its management on internationally accepted knowledge, the Norwegian Scientific Committee for Food Safety was asked to consider a number of specific questions.

To prepare scientific background documents necessary to answer the questions, the Norwegian Scientific Committee for Food Safety, Panel on Animal Health and Welfare, established an *ad hoc* group consisting of 9 national and international experts. The international experts came from Scotland, Canada and the USA. The group was chaired by Professor Espen Rimstad from the Scientific Committee.

The Panel on Animal Health and Welfare discussed a preliminary version of the report in a meeting on the 12th of December and the full report in a meeting on the 15th of January, and gave its support to the conclusions drawn by the *ad hoc* group. Concerning Question 6, the Panel agrees with the opinion of the majority of the *ad hoc* group.

Conclusions of the risk assessment from the *ad hoc* group

1A) Can ISA virus transmit vertically?

The *ad hoc* group is of the opinion that vertical transmission of ISA virus cannot be excluded. However, available data are inconsistent, and there was disagreement within the *ad hoc* group on the interpretation of the available data.

1B) How high is the probability of spread of the agent and/or development of disease, as a result of vertical transmission?

The probability of spread of the ISA virus as a result of vertical transmission may depend on individual characteristics, such as clinical status and virus titre in the parent fish, intracellular or extracellular transmission, which is regarded as the dominant route; and/or strain characteristics of the virus.

The probability of further spread via eggs, fry or smolt as a result of vertical transmission will depend on the efficacy of intervening management procedures, such as disinfection and prophylactic treatment post-stripping.

Present relevant knowledge is scarce on these variables. It is not possible from the available information to estimate the probability of spreading of the agent through vertical transmission.

Spread of the *disease* as a result of vertical transmission may be regarded as a consequence of ISA virus being vertically transmitted. The low number of outbreaks in the fresh water stage (0.7 %) and lack of ISA disease in some countries that over the years have imported substantial numbers of eggs from Norway, suggest that the probability of disease emergence following vertical transmission of virus is low.

2) Do present levels of knowledge relating to the virus and disease provide a good enough basis for tracing the source of infection and routes of transmission of ISA?

The *ad hoc* group is of the opinion that epidemiological information together with phylogenetic information are both necessary to resolve the source of infection and routes of transmission. Present levels of knowledge relating to the virus and disease do not provide a good enough basis for tracing of the source of infection and routes of transmission. Each case has to be handled based on its own merits.

3A) Of what importance are the different production stages, including transport, slaughter and other related activities for spread of infectious materials?

Concerning the importance of different production stages and other related activities for spread of virus and disease, the *ad hoc* group is of the opinion that the risk of viral transmission between *freshwater production sites* is minimal.

The risk of spread of ISA disease between *marine production sites* is in general not possible to estimate. Epidemiological studies and general field observations point to clustering of outbreaks in space and time indicating horizontal transmission of the virus in seawater. The strength of the associations estimated in some early epidemiological studies may have

changed following the implementation of biosecurity measures. However, phylogenetic analyses of virus from some of the claimed clusters have not always supported that horizontal transmission has occurred between the sites. These diverging conclusions need to be followed up by more detailed studies.

Concerning *transportation*, the *ad hoc* group is of the opinion that well boat transportation is an important risk factor for the spread of ISA virus. Well boats operate in a national and international market and interchangeably carry smolt and fish for slaughter.

The number of *slaughterhouses* has been reduced, thus giving longer transportation routes. An increased number of fish is delivered for slaughter to each slaughterhouse, and these slaughterhouses are becoming centres in a large network, that may increase the risk of spreading infectious agents including ISA virus. The common use of waiting pens in the slaughterhouses instead of on-shore waiting tanks from which the effluent water is disinfected, is a possible risk factor.

3B) How long does ISA-virus remain infective under different conditions?

Concerning the persistence of infectivity of ISA virus under different conditions, the *ad hoc* group emphasizes that the ISA virus is relatively sensitive to disinfectants. However, the virus is able to survive for weeks in offal and carcasses. Although the information regarding survival of ISA virus infectivity varies, there are several findings that indicate long-term survival in water. Assessment of survival rates of viruses belonging to the same virus family indicates that ISA virus is likely to remain infective in water under field conditions for weeks.

4) Does infection with different variants of the virus pose greater or lesser risk of disease outbreak?

The outcome of any virus infection is dependent upon properties of the virus strain, infective dose, environmental factors including management practises, and the host. These factors have to be considered when assessing the virulence of the virus in any ISA outbreak.

Properties such as receptor-binding and release, fusion and interferon antagonism, which all are important factors of virulence in influenza viruses, have been identified for the ISA virus. However, it is still not known to what extent variations in these properties influence the outcome of an ISA virus infection.

The highly polymorphic region (HPR) of the haemagglutinin-esterase (HE) most likely represents an important virulence marker, and it is so far the only genetic marker that has been associated with virulence in ISA virus. The HPR is characterised as having differential deletions compared to a theoretical full-length precursor gene named HPR0.

There are no reports of detection of HPR0 from ISA-diseased fish with classical clinical and pathological changes consistent with ISA. The majority of HPR0 detections have been from healthy fish. However, based on the lack of adequate experimental infection models with HPR0 virus, caution should be exercised in stating that HPR0 is avirulent. A gene sequence corresponding to HPR0 was identified in gill samples in a case of suspicion of ISA in a marine fish farm in Scotland, and it was also found in a group of smolts with proliferative gill disease in Norway.

The circumstantial evidence as of today indicates that infection with HPR0 virus in itself poses a lesser risk of disease outbreak than infection with ISA virus with other HPRs.

5A) What are the reservoirs for ISA virus?

The *ad hoc* group is of the opinion that the most likely reservoirs for ISA virus are farmed salmon themselves and wild salmonids, of which the most important are brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*).

5B) What is the importance of various reservoirs for outbreak of disease?

Considering the importance of various reservoirs for outbreaks of the disease, the *ad hoc* group assumes that the reservoir for ISA virus causing approximately 30 % of ISA outbreaks in Norway is other farms in the proximity of and within the network of locations with recorded ISA disease. The model from which the *ad hoc* group based its opinion could not explain the remaining 70 %. Alternative reservoirs for the remaining outbreaks of disease could be:

- a. Potential disease-causing ISA virus is circulating in the industry, but is not regularly detected. This reservoir is documented; however, the relative importance is unknown. Wellboats commonly used for both transport of smolts and fish to slaughter may be an important risk factor for such circulation.
- b. Putative avirulent ISA virus is transmitted from wild fish to farmed salmon, followed by a conversion from an avirulent to a virulent form, which occurs with an unknown frequency. This reservoir is present; however, the relative importance is unknown.
- c. ISA virus is prevalent in farmed salmon and is transmitted vertically. The virus then passes through the different life stages and may cause disease in the marine stage. The virus has been detected in several life stages, but the relative importance for disease outbreaks is unknown.

6) Is screening for ISA virus in apparently healthy fish appropriate for limiting ISA distribution?

When evaluating the ability of targets for screening for ISA virus one should consider not only the target itself but also the ability to intervene after a positive finding. The basis for giving advice on screening could be clearer if the prevalence of ISA virus in different life stages in Norwegian aquaculture was known to some extent.

The majority of the *ad hoc* group recommends that screening of broodfish in the “breeding nucleus” should be encouraged. However, this recommendation should be combined with strict biosecurity procedures for handling of broodfish and sexual products. After spawning, the eggs from the breeding nucleus must be kept in separate cylinders, one for each family. This means that progeny from individual parents that have tested positive can be discarded. The group majority is also of the opinion that testing of fish ready for slaughter, prior to transport should be encouraged. A cost-benefit analysis to evaluate the testing should be performed after a sufficient time period has elapsed. Because the possible effect of the proposed screening on ISA outbreaks is currently not possible to assess, screening should be voluntary.

In the answer to question 6 of the assessment, the *ad hoc* group did not achieve consensus. One member of the *ad hoc* group disagreed with the majority. To the answer whether screening is appropriate, the conclusion of the dissent is that screening should not be applied unless it is known to be cost-efficient with regard to disease control or eradication.

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GLOSSARY

Brown trout: *Salmo trutta* (includes sea running trout)

Clade: A group of organisms consisting of a single common ancestor and all the descendants of that ancestor.

Cut-off-value: The cut-off value (k) is a pre-defined value that determines the test outcome for a diagnostic test with a continuous output, e.g. the test is positive if the result of the test is on one side of the k-value and negative otherwise.

Ct-value: The number of cycles run in a real-time RT-PCR when the fluorescence in the sample crosses a threshold value and enters a log-linear phase, i.e. when a sample is considered positive (cycle threshold).

Egg incubation: The time after fertilization before the eyed egg stage. At this time, the eggs are extremely fragile and handling is not possible.

Eyed eggs: The stage after the eyes of the embryo has appeared as two dark spots on the egg. The eggs are then robust and can be transported.

Farm site: The geographical location of a production unit.

Gold standard: A sample or an animal with known true infection status. The term has also been used to describe a diagnostic test that gives the true status of an animal.

Grow-out farm: Farms at sea that receive smolts and grow the fish to market size.

Hatcheries/smolt producers: Locations that receive eyed eggs from egg producers in order to hatch them and produce juveniles. Most hatcheries are also smolt producers, but there are some independent smolt locations.

HE: Haemagglutinin-esterase

HPR: Highly polymorphic region, that is located near the stem of the HE molecule, adjacent to the transmembrane region.

HPR0: The full-length HPR. HPR0 is, theoretically, considered to be a precursor of other HPRs.

Juvenile stage: The time between hatching and smoltification, which includes the yolk sac, start feeding and parr stages.

Molecular clock: A technique in genetics, which is used to date when two species (in this report virus) diverged.

Molecular phylogeny: The use of the structure of molecules to gain information on a virus' evolutionary relationships to other viruses.

Pathogenesis: The mechanism by which a certain aetiological factor (in this report virus) causes disease. The term is also used to describe pathological changes during disease development.

Population: A collection of animals living within geographical boundaries, - a given area or space.

Predictive value: The probability of a positive or a negative test result being a true positive or negative, respectively. Estimation of this value is based on diagnostic sensitivity, diagnostic specificity and prevalence of the pathogen in the tested population. This predictive value is of interest especially when prevalence is low.

Prevalence: The fraction of events (e.g. number of ISA outbreaks) registered in a population at a particular point in time. For example, if n animals are randomly sampled at a given time, and y animals are classified as positive for a particular disease, the prevalence (p) of that disease at that point in time is estimated to be y/n . If the entire population has been sampled, then the prevalence, p , is exactly y/n . Apparent prevalence (AP) is the fraction most often estimated since only a subset of the population is normally tested. AP may be different from p due to improper sampling strategies, registration routines or test characteristics.

Quasispecies: A viral population composed of a spectrum of mutants in which the most fit genotype dominates.

Real-time RT-PCR: A laboratory technique used to quantify and amplify simultaneously a specific part of a given DNA molecule. The DNA molecule arises from reverse transcription of RNA molecules. Diagnostically real-time RT-PCR is applied to detect rapidly the presence of genes involved in infectious diseases.

RT-PCR: PCR preceded by reverse transcription of RNA into DNA.

Screening: Systematic examination of animals or groups of animals in a population to ascertain the apparent prevalence of a specific infection or disease. The results will depend on test characteristics and sampling procedures.

Sensitivity: Diagnostic or epidemiological sensitivity is the probability that a test returns a positive result, given that the true status of the animal tested, is positive for the disease. The probability of the test to return a positive result in a positive herd (herd level sensitivity) is in general higher than the probability at individual level. Laboratory or analytic sensitivity is the test's ability to detect an agent in a sample in the laboratory (detection limit).

Smolt stage: Fish that has gone through the significant physiological changes that enable it to be transferred to sea. Smolts are silvery in appearance, as opposed to the brownish parr.

Specificity: Diagnostic or epidemiologic specificity is the probability that a test returns a negative result, given that the true status of the animal tested is negative for the disease. The probability of the test to return a negative result in a negative herd (herd level specificity) is in general lower than the probability at individual level. Laboratory or analytic specificity is the test's ability to identify a specific agent correctly.

TCID: Tissues culture infected dose. This term is used for quantification of virus. Usually the quantity of virus is expressed as the number when 50 % of the cultures are infective, $TCID_{50}$.

True negative: A negative test result for an animal that is truly negative for a particular infection or disease.

True positive: A positive test result for an animal that is truly positive for a particular infection or disease.

Validation: The evaluation of a test to determine its fitness for a particular use. This includes optimisation and demonstration of its performance characteristics.

Virulence: The relative ability of a virus to damage the host.

BACKGROUND

Infectious salmon anaemia (ISA) is a viral disease that was first recorded in 1984 in fish from an Atlantic salmon hatchery (Thorud and Djupvik, 1988). Affected fish were severely anaemic, hence the name of the disease, and the fish showed typical macroscopic lesions including ascites, petechiae on internal organs and haemorrhagic liver necrosis (Thorud and Djupvik, 1988). The original disease spread in a pattern consistent with a contagious disease and the contagious nature of the disease was also confirmed experimentally. Even though some farms that raise both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon have had ISA, disease outbreaks have only been found in Atlantic salmon, whereas the rainbow trout have remained unaffected.

The epidemic of ISA increased steadily in Norwegian salmon farming and peaked in 1990 when ISA was detected in 80 fish farms. During 1988-1991, regulatory actions were implemented to control the disease. The essence of these regulatory actions was to interrupt the transmission of infection and to reduce infection pressure. The result of these actions together with significant improvements in husbandry practices was a remarkable and rapid reduction of ISA outbreaks to only 2 outbreaks in both 1994 and 1995. The present accumulated number of ISA disease outbreaks registered in Norway is 437, of which 3 (0.7 %) have occurred in the fresh water phase.

Outside Norway, ISA was reported in the period from 1997 to 2000 in salmon farms on the Atlantic coast of Canada, and USA, in Scotland and the Faeroes. Isolation or detection of the ISA virus has also been reported from Pacific Coho salmon (*O. kisutch*) in Chile and from rainbow trout in Ireland.

PREVENTION AND CONTROL USED FOR ISA IN NORWAY

The ISA epidemic in Norway peaked in 1990 when outbreaks were diagnosed in 80 fish farms. ISA was placed on list B of *notifiable* diseases in Norway in 1988. During 1988-1991, biosecurity actions were implemented. The essence of these regulatory actions was to interrupt the transmission of infection and to reduce infection pressure. Generally, the measures were based on experience from decades of successful combating of terrestrial animal diseases, accumulating aquaculture field experience, and epidemiological information and knowledge together with ISA-specific preliminary risk analysis results generated during the epidemic (Vågsholm et al., 1994).

From the beginning of the ISA history, the disease tended not to disappear from affected sites until each site had been emptied. This experience was implemented early in the combat strategy as part of a stamping out policy. The strategy was later expanded to include fallowing of whole areas for a designated period of time, usually 6 months (Thorud and Håstein, 2002). An outbreak was defined to be present when the daily cage mortality exceeded 0.05 %.

Slaughterhouses receiving fish from an ISA site needed special approval from the Food Safety Authorities. Such approval was also needed for the chosen transportation route between the actual site and the slaughterhouse. Due to the assumed risk of horizontal transmission of the agent, an area around the affected farm was declared a combat zone and was closely monitored. All sites within this zone were controlled by restrictions on production, fallowing procedures and transportation in and out of the zone. Outside this zone, an observation zone was established with intensified surveillance and reporting duties, but

usually no restrictions were imposed. Strict segregation of generations was encouraged, although this measure was not formally incorporated in the legal acts until 2005. General hygienic measures such as frequent (daily) removal and sanitary handling of dead fish became common practice. All these biosecurity measures were quickly implemented in agreement between the authorities and the industry through a common campaign called “Stop ISA” that was initiated in 1990.

In 1996, existing experience and scientific knowledge were summed up in the first official contingency plan for combating ISA, and these guidelines became the basis of an ISA policy within the EU. The principle of a stamping out procedure was maintained in the present contingency plan released in 2004, which required all fish to be removed from infected sites within 80 days. This specific time limit was later modified “to be decided” in each case (www.mattilsynet.no).

Based on the Norwegian experience, a general approach to the control of ISA is summarised in the Diagnostic Manual for Aquatic Animal Disease published by the Office International des Epizooties (OIE): *“The incidence of ISA may be greatly reduced by implementation of general legislative measures regarding the movement of fish, mandatory health control, and slaughterhouse and transport regulations, as well as specific measures including restrictions on affected, suspected and neighbouring farms, epizootiological studies, enforced sanitary slaughtering, generation segregation (‘all in/all out’), and disinfection of offal and wastewater, etc., from slaughterhouses and fish processing plants.”*

The ISA specific legislation in Norway is described in detail in “Contingency plan for control of infectious salmon anaemia in Norway” by the Norwegian Food Safety Authority (www.mattilsynet.no). Norway has a general prohibition on vaccination against serious infectious diseases. However, the Norwegian Food Safety Authority-Head Office may give permission to vaccinate under specific conditions. *“Vaccination may, however, under certain circumstances be a useful measure to avoid multiplication of the infectious agent and spread of disease following an outbreak. This is especially applicable in areas with several disease outbreaks over relatively short periods of time and where there is extensive aquaculture activity.”*

Table 1. Chronological list of measures implemented to control ISA in Norway

1988	ISA was placed on list B of <i>notifiable</i> diseases
1989	Obligatory health certificate <ul style="list-style-type: none"> - health control in hatcheries - 12 regulatory health assessments a year - Disinfection of eggs Ban on use of sea water in hatcheries Ban on moving fish already put to sea
1990	Regulation on transport <ul style="list-style-type: none"> - disinfection of well boats Segregation of generations (‘all in/all out’) encouraged on a voluntarily basis

	Stop ISA campaign
1991	Regulation of disinfection of waste water from slaughter houses and processing plants* Regulation for disinfecting sea water for use in hatcheries Control and containment of dead fish in farms
1992	Introduction of zones to combat outbreaks
1996	Official guidelines for dealing with outbreaks
2001	Contingency plan
2004	Contingency plan revised
2005	Segregation of generations ('all in/all out') made mandatory

*The slaughterhouse regulations also regulated sanitation of dead fish and organic waste in fish farms (i.e. acid ensilage)

OCCURRENCE OF ISA OUTBREAKS

The number of ISA outbreaks in Norway and the production of Atlantic salmon during the last twenty years are presented in Fig. 1. From 1988 to 1991 some of the most important measures for controlling the disease were implemented. Due to the combined effort of industry and authorities, these measures were immediately put into action.

In addition to ISA, furunculosis, cold-water vibriosis and vibriosis were frequently diagnosed in the late 1980's and early 1990's. New bacterial vaccines proved very efficient, and almost the entire Atlantic salmon smolt population was vaccinated against the three bacterial diseases from 1991/1992 onwards. A marked decline in the number of ISA outbreaks was seen simultaneously with the introduction of efficient bacterial vaccines (Fig. 2). This favourable situation may have been due to a pure vaccination effect; however, sanitary measures and the focus on biosecurity were almost simultaneously brought into effect. The combined sets of actions may therefore have created a synergistically favourable situation as a decrease in infectious load and risk of transmission may have underpinned the effect of the vaccines and vice versa.

Obviously, the improved health status regarding bacterial diseases and the stimulation of the innate immune response through vaccines might also have had beneficial effects on the clinical occurrence of ISA.

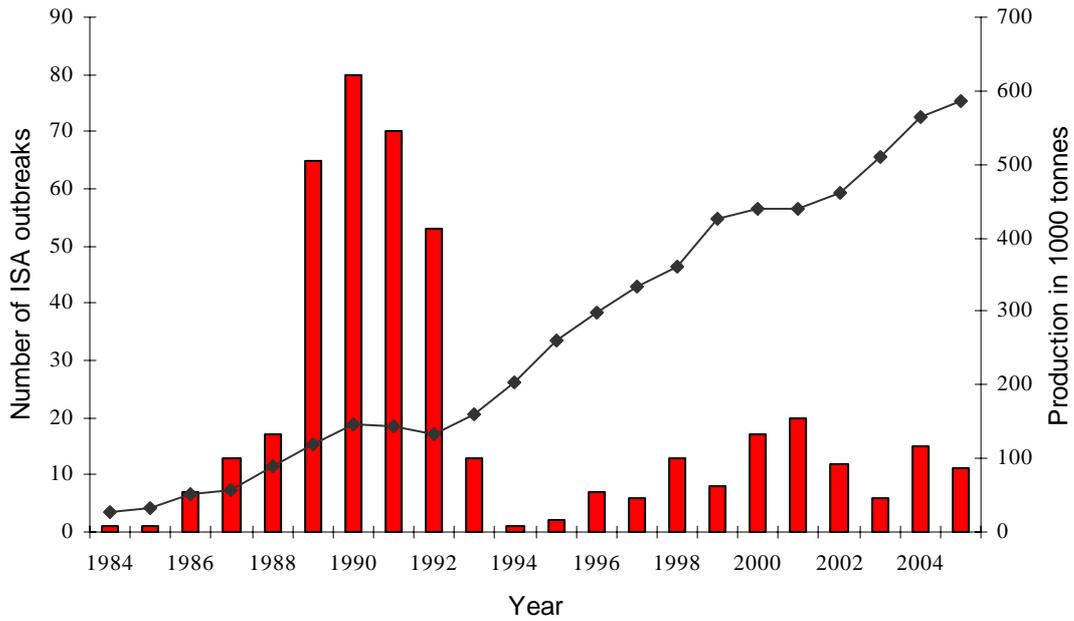


Figure 1. Number of confirmed ISA outbreaks (red columns) and salmon production in 1000 tonnes (black line) in Norway from 1984 until 2005.

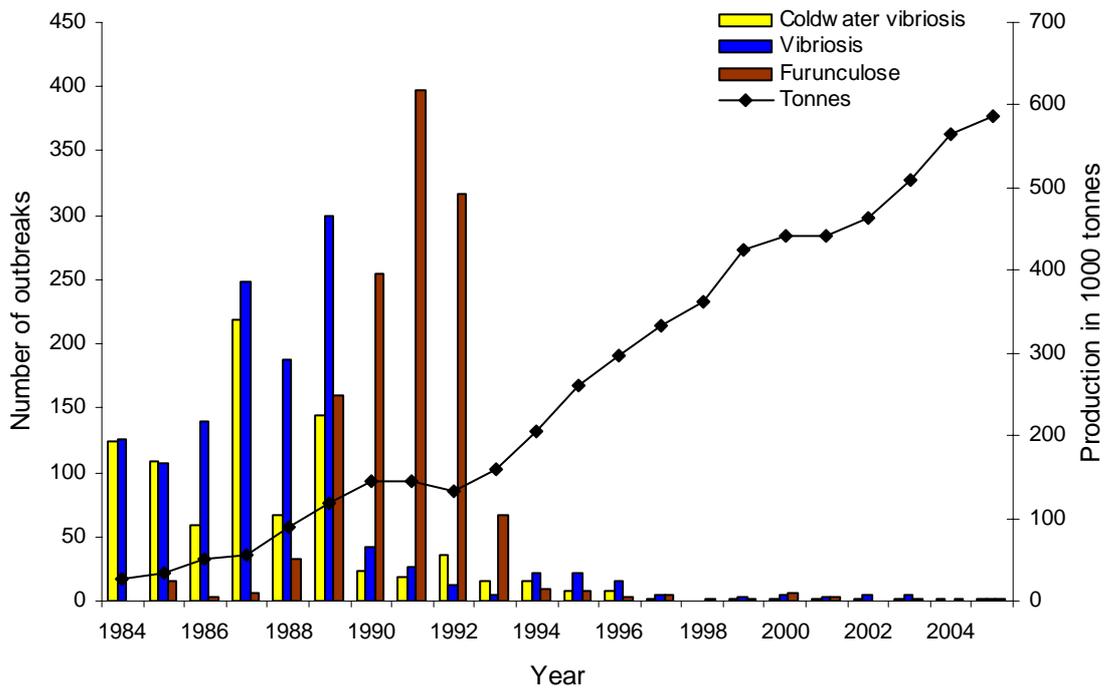


Figure 2 Number of confirmed outbreaks with cold water vibriosis, vibriosis or furunculosis together with salmon production (black line) in Norway from 1984 until 2005.

CHARACTERISTICS OF THE ISA DISEASE

ISA outbreaks are mainly seen in seawater farms, though a few outbreaks have occurred in hatcheries. Experimental infections have shown that ISA can also occur in fresh-water. The disease usually starts in one net pen, and it may take many months before disease develops in neighbouring net pens. Daily mortality generally stays low, but often increases in early summer and winter to more significant levels (0.5–1 %). Without intervention the cumulative mortality may become very high. Acute episodes of extremely high mortality may occur.

ISA is a systemic disease affecting the circulatory system, and the major target cells for the infection are endothelial cells lining blood vessels of all organs including sinusoids, endocardium and endothelial macrophages. Final stages of the disease are characterised by hemorrhage, a circulatory collapse and an extreme anaemia (haematocrit <10). Though virus infected endothelial cells are observed in all organs by immunohistochemistry (IHC), a striking observation is the lack of an inflammatory cellular response as observed by histopathological examinations.

On necropsy, the key finding is a variable set of circulatory disturbances due to endothelial injury in peripheral blood vessels. Changes indicating a systemic infection such as oedema and small bleedings of eyes, skin and serosal surfaces are present along with the changes more characteristic of ISA. Characteristic pathological changes that may be seen to a variable degree include dark livers due to haemorrhagic liver necrosis, moderately swollen kidneys with interstitial haemorrhaging and tubular necrosis, dark red guts due to bleeding within the intestinal wall, and blood accumulation in the gill filaments. It is noteworthy that hemorrhagic organ lesion can be absent or very rare in the initial stages of an ISA outbreak, leaving only the anaemia and the more subtle circulatory disturbances as a clue to the aetiology.

CHARACTERISTICS OF ISA VIRUS

Isolation in cell culture

There are no available cell lines that support propagation of all ISA virus strains. The virus may be propagated in the Atlantic salmon derived cell lines SHK, ASK and TO, but also in the Pacific salmon derived cell line CHSE-214. The CHSE-214 cell line does not or does only poorly support growth of the European variants of ISA virus.

ISA virus – physical and chemical properties

ISA virus is a pleiomorphic, enveloped virus, with surface projections associated with haemagglutination (receptor-binding), receptor-destroying and fusion activity. The genome consists of eight single-stranded RNA segments with negative polarity ranging in length from 2.4 to 1.0 kb and with a total size of approximately 14.3 kb. The properties of ISA virus are consistent with those of the *Orthomyxoviridae*, and ISA virus is classified as the type species of the genus *Isavirus* within this virus family (Fauquet et al., 2005). The organization of ISA virus genes and gene products are however unique.

The nucleotide sequences of all eight genome segments have been described (see Appendix, Table 10). The viral genome encodes at least ten proteins, where nine are known to be present in the mature virus particle. Four major structural proteins have been classified, including the nucleoprotein (NP), the fusion (F) glycoprotein responsible for virus entry, the haemagglutin-

esterase glycoprotein (HE) responsible for receptor binding and release, and the matrix (M) protein.

Survival of ISA virus infectivity outside the host

The infectivity of ISA virus may be retained for a long time outside the host (see Appendix, Tables 11-13). The virus is stable in the pH range 5-9 (Falk et al., 1997). At pH 4, the virus is completely inactivated after 30 min, and after 30 min at pH 11 the infectivity is reduced by 90 %. Five cycles of freezing (-80 °C) and thawing (20 °C) do not reduce infectivity (Falk et al., 1997).

Since ISA virus is a water-borne virus, attempts have been made to estimate the viral survival time in an aquatic environment. However, several factors present in a natural environment such as temperature fluctuations, UV-radiation, heavy metals, binding to non-living particles causing sedimentation and ocean currents may affect viral infectivity and are factors difficult to reproduce in the laboratory. Consequently, the limited information available on ISA virus survival in aquatic environments comes from laboratory experiments incubating ISA virus either in sterile seawater (MacLeod et al., 2003, Rimstad and Mjaaland, 2002), in natural seawater (MacLeod et al., 2003, Nylund, 2004), and in sterile freshwater (MacLeod et al., 2003). The samples were kept at different temperatures (4-6 °C and 15 °C), and infectivity was measured over time (Table 11).

For comparison, some data regarding survival of influenza A virus could be considered. This virus is prevalent in water birds and is transmitted partly through water. An interactive effect between salinity and pH on persistence of avian influenza A viruses in water has been demonstrated, and differences in the response to these variables are apparent, even between different types of avian influenza viruses. Duration of infectivity decreases with increasing temperature, salinity and pH; 1×10^6 TCID₅₀ was maintained for 100 d at 17 °C/0 ppt/pH 8.2, and for 9 d at 28 °C/20 ppt/pH 8.2. Persistence of infectivity of avian influenza A virus should be regarded as good outside the host (Stallknecht et al., 1990 a, b).

Available data suggest that ISA virus may remain infective for extended periods of time outside its host.

VIRULENCE OF ISA VIRUS

Virulence is not a quality of the virion alone, but a result of interactions between virions, hosts and environment. ISA disease is complex, as observed by differences in disease development.

For comparative reasons, and to illustrate the complexity of viral virulence, a few points important for the virulence of influenza virus are presented. Virulence of influenza viruses is usually the result of optimal gene constellations, although genetic changes located to a single gene or between two gene products may confer changes in virulence. Some of the genes are, however, regarded as being more central than others i.e. the surface glycoproteins responsible for receptor binding, viral entry, including fusion, and viral release from the cell. The haemagglutinin (HA) gene of influenza A virus, which is responsible for receptor binding and virus entry into the cell, plays a pivotal role in determining the severity of infection, at least in avian strains. The development of highly pathogenic viruses is associated with alteration (insertions of basic amino acids) near the cleavage sites of subtypes H5 and H7. On the other hand, the pandemic influenza virus of 1918 did not have any obvious alteration in its cleavage

site. Recently, a central role in the virulence of a human influenza A virus isolate was demonstrated for neuraminidase (NA), which is responsible for viral release. Moreover, domains on the NP, PB2, NS1 and M2 proteins may also be associated with virulence.

Functional properties associated with ISA virus virulence

ISA virus has been demonstrated to possess the major functional characteristics of the orthomyxovirus family associated with virulence and pathogenesis. These characteristics include both haemagglutinating (i.e. receptor binding) and receptor destroying (RDE) and fusion activities. In orthomyxoviruses, these functions are essential for pathogenesis, virulence and host tropism. Variation in these properties between different isolates and types of ISA virus and the relative importance of the factors for virulence are unknown.

In orthomyxoviruses, reassortment of gene segments occurs frequently and is a major contributor to the evolution of these viruses and the emergence of new virulent strains. An extensive molecular and phylogenetic sequence analysis of Norwegian ISA virus isolates provides strong evidence for the occurrence of genetic reassortment involving several ISAV gene segments (S. Mjaaland, personal communication).

The receptor binding of orthomyxoviruses are two sialic acids, which are acidic monosaccharides typically found at the outermost ends of the sugar chains of glycoproteins or glycolipids. This binding is very specific, and ISA virus has been found to bind to 4-*O*-acetylated sialic acids. Occurrence of this type of sialic acid on the cell surface is thus a prerequisite for a cell to be infected. Receptor recognition properties are of general importance for tissue and species recognition.

The function of the RDE is to cleave the receptor binding, and its major function is associated with the release of new virus particles from infected cells and to prevent agglutination of these virus particles. ISA virus haemagglutinates erythrocytes from several fish species including Atlantic salmon. RDE activity dissolved the haemagglutination for all erythrocytes that were agglutinated by ISA virus, except for Atlantic salmon erythrocytes. The inability of RDE to dissolve the hemagglutination of salmon erythrocytes may be of importance for ISA virulence and pathogenesis since virus isolates that show RDE activity with salmon erythrocytes seem to be less virulent, as demonstrated by experimental infections (K. Falk, personal communication).

The fusion activity is associated with uptake of virus into the cell during infection, and is thus essential for virulence. Analysis of the gene encoding the fusion protein has revealed a 30 nucleotide long insertion in several virus isolates, immediately upstream of the protein's putative cleavage site. The same recombination was found in two unrelated ISAV isolates which suggests the presence of a recombinational hot spot. It could be speculated that this could lead to alterations in the cleavage specificity of the fusion protein (S. Mjaaland, personal communication). However, there has so far not been revealed motifs that may be markers of variation in virulence similar to those markers seen in highly virulent avian influenza viruses.

ISA virus, as with influenza virus, encodes for a non-structural protein with an interferon antagonistic effect. This interferon antagonism may be of importance in reducing the innate response of the host, and thus is a potential virulence factor.

Genetic markers of virulence

In contrast to influenza A HA, where most of the variability is located in distal parts of the molecule, most of the variability within the ISA virus HE is concentrated to a small highly polymorphic region (HPR) near the transmembrane domain. This region is characterized by the presence of gaps, rather than single-nucleotide substitutions. The polymorphism in this region has been suggested to result from deletions, or possibly recombination, of a full-length precursor gene (HPR0). The presence of a long HPR0 gene has been confirmed in healthy wild (Cunningham et al., 2002) and farmed Atlantic salmon (Cook-Versloot et al., 2004). The number of different HPR groups is, so far, 26 and 4 among European and North American genotypes of ISA virus, respectively. The pattern of variation is constrained to the 35 amino acids defined as the HPR (Mjaaland et al., 2002, Devold et al., 2001, Nylund et al., 2003, 2007, F. Kibenge personal communication).

Recently, an alternative theory for the occurrence of HPR was presented, suggesting that insertion, rather than deletions, takes place. This suggestion is based on *in silico* estimates of the time of origin of the different viral isolates (F. Kibenge et al., 2006b poster presentation).

Virus isolated from ISA diseased fish contains gaps in the HPR region, as compared to the HPR0. However, viruses with HPR0 have been found in fish with gill disease that has not been characterised as ISA (Anonymous, 2005, Nylund et al., 2007). A gene sequence corresponding to HPR0 was identified in gill samples in a case of suspicion of ISA in a marine fish farm in Scotland in 2004 (Anonymous, 2005). There were significant mortalities, but the clinical signs and post mortem findings (mainly gill inflammation and proliferation) were not consistent with classical ISA. Similarly, Nylund et al. (2007) found HPR0 in a group of smolts with proliferative gill disease, where 6/6 smolt were real-time RT-PCR positive. No further study of a correlation between the virus and the gill inflammation was pursued.

In summary, the HE-HPR therefore most likely represents an important virulence marker. Whether there is a connection between the size or type of the gap and virulence and how deletions in the HPR influence viral virulence remains to be determined. The virulence of ISA virus, however, cannot be attributed to the HE-HPR region alone, as isolates with identical HPR vary in virulence in experimental infections using standardized experimental fish (Mjaaland et al., 2005).

Phylogenetic analysis of ISA virus

For several viruses important to fish farming, a molecular phylogenetic analysis has proven efficient in resolving epidemiological problems (Kurath et al., 2003, Einer-Jensen et al., 2004, Snow et al., 2004, Thiery et al., 2004). Such sequence analysis may provide important information on the spread of pathogens, and hence, can be used as a tool in fish disease management.

The basis for phylogenetic analysis is information on the rate of nucleotide substitutions in the genome of viruses. Substitution rates for the ISA virus are essential to the understanding of the evolution of the ISA virus in farmed populations of Atlantic salmon. The degree of changes constitutes the basis for genotyping.

Several publications demonstrate the highly conserved nature of ISA virus (Devold et al., 2006, Nylund et al., 2007, Jenkins et al., 2002). The two genomic segments with the highest variability are the two surface glycoproteins – the HE and the F. These segments may therefore be phylogenetically informative in contrast to several of the other genomic segments where very little or no phylogenetic information can be extracted.

Most of the phylogenetic analysis of ISA viruses is based on the information obtained from the 5'-end of the HE gene. The HPR region is excluded because this region varies through deletion and/or recombination between related isolates, and is therefore not useful as an indicator of relatedness. Based on the differences in 5'-end of the HE gene, ISA virus has been divided into two major clusters; the North American and the European (Devold, 2001). Analysis of the genomic segment 5 has supported this division (Devold, 2006). ISA virus isolates within the same outbreak cluster together in phylogenetic analysis, although they demonstrate some sequence variation. This finding indicates a common origin for isolates from the same outbreak (Lyngstad et al., 2005).

The European cluster has further been sub-divided into three groups (EU-G1-G3) (Nylund et al., 2007). Some ISA virus isolates from North-America are European-like and are often referred to as "European-in-North America". There are conflicting interpretations as to whether they represent as a separate group or can be placed within existing European groups (EU-G2) (Nylund, 2007, F. Kibenge, personal communications).

Molecular clock

Molecular clock is a technique in genetics, which is used to date when two viruses diverged.

For some viruses or viral genes, a molecular clock can be calculated based on the rate of nucleotide substitutions in the viral genomes.

Due to their error prone RNA-dependent RNA polymerase, RNA viruses mutate quickly and hence may evolve quickly. If viral RNA is not evolving neutrally due to undetermined effects such as codon usage, RNA secondary structures, fluctuations in adaptive environments and population size, then the observed substitution rate and its constancy over time will be affected (Jenkins et al., 2002). Hanada et al. (2004) found that the main source of the rate variation was due to differences in the replication frequency because the rates of replication error were roughly constant for different RNA viruses.

Molecular evolution will only follow a molecular clock if mutation and replication rates are constant and most substitutions neutral. It should not be expected that the molecular evolution of the ISA virus HE gene would follow a molecular clock. Recent studies have confirmed that the substitution rates of segments 5 (F) and 6 (HE) are not constant over time (Devold et al., 2006, Nylund et al., 2007). Hence, it can be concluded that mutation rates, replication rates, or undefined selective constraints vary to some extent. Moreover, identical HE sequences found in ISA virus isolates separated by at least 9 years further support the conclusion that a molecular clock cannot be assumed to be constant for ISA virus (Devold, 2006, Nylund, 2007).

Despite the lack of a molecular clock in ISA virus, there is a relationship between genetic divergence and time.

Important constraints on the evolution of ISA virus are caused by different measures implemented to combat the disease, including segregation of generations and slaughtering of infected fish. The ISA virus infecting salmon is lost, unless there is a significant horizontal transmission of the ISA virus between farms and from farms to wild fish in the marine phase.

A possible vertical transmission of ISA virus isolates could constitute a bottleneck for transmission of genetic variation accumulated in ISA virus populations.

HOST FACTORS

The outcome of a virus infection will not only depend upon the properties of the virus, but also on different host factors. These factors might be of genetic origin, and it has been shown that susceptibility towards ISA virus is linked to certain MHC class I and class II types in Atlantic salmon. Family testing performed in association with Norwegian breeding programmes indicates significant variability in ISA susceptibility between family groups (Gjøen et al., 1997).

Many researchers have experienced seasonal variability when performing controlled challenge experiments with ISA virus on Atlantic salmon. During the autumn, a chronic progression of the disease, often with low cumulative mortality, is observed. However, during the spring, an acute progression is normal. This topic is discussed by Rolland and Winton (2003). In their study, Atlantic salmon challenged during the spring experienced significantly higher mortality compared with fish challenged during the autumn. In a recent paper, Glover and co-workers (2006) show that the susceptibility of Atlantic salmon towards ISA virus infection increases markedly when the fish is in the process of smoltification. These investigators also argue that the association with smoltification might explain the observations of Rolland and Winton. Thus, it could be the physiological status of the fish, and not the season of the year, that is linked to disease progression. In the same study, Glover et al. (2006) could not find any differences between farmed, wild or hybrid fish regarding susceptibility to ISA virus infection. This finding is in contrast to a study by Nylund and co-workers (1995b), which demonstrated that farmed fish are more susceptible than wild fish.

HOST RANGE

The host range of ISA virus can be divided into species in which disease occurs naturally, which is restricted to Atlantic salmon; and secondly, into species in which virus can replicate but no disease is found. The latter may be of importance as carriers of virus and as reservoirs.

In experimental infections injecting intraperitoneally large dose of virus and also several different virus isolates, disease/histopathological changes have been described in rainbow trout inoculated with some of the isolates (Kibenge et al., 2006a).

Replication of ISA virus occurs in experimentally infected Atlantic salmon, brown trout, rainbow trout, Arctic char (*Salvelinus alpinus*), chum salmon (*O. keta*), coho salmon, herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*). No replication has been found in bivalves.

In a survey of about 50,000 non-salmonid marine fish that were tested in Maine by cell culture and RT-PCR over a period of three years, only one positive sample was found and that was in the species alewife (*Alosa pseudoharengus*) (Rolland, 2004). Other species included in that study were American eel (*Anguilla rostrata*), herring, Atlantic mackerel (*Scomber scombrus*), Atlantic cod, haddock (*Melanogrammus aeglefinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Pollock (*Pollachius virens*), American shad (*Alosa sapidissima*) and winter flounder (*Pseudopleuronectes americanus*).

However, the fact that surveys have not yet revealed a non-salmonid reservoir does not prove that a reservoir does not exist. For example, there are molecular indications from the west coast of North America that IHN virus is maintained in some marine species, but extensive surveys have not yet revealed the species (J. Winton, personal communication).

Based on the detection of ISA virus by RT-PCR methods in wild fish, both *S. salar* and *S. trutta* are the most likely candidates as the natural hosts. Hence, it can be worthwhile to

consider the biology of *S. salar* and *S. trutta* when making assumptions on the epidemiology of the ISA virus in wild fish.

ISA VIRUS IN WILD SALMONID POPULATION

Norwegian lakes and river systems constitute a natural environment for three sea running salmonid species (*S. salar*, *S. trutta* and *Salvelinus alpinus*). The ISA virus and the populations of brown trout and salmon may have co-evolved.

The number of host species and the density, size and distribution pattern of host populations are important factors for the maintenance and spread of viruses.

The population structure of sea running salmonids is strongly influenced by migration behaviour. The highest population densities are found in rivers and lakes. The most frequent interactions between salmonids occur during spawning. The rivers are the rearing areas for salmon fry, parr and smolts, i.e. individuals that can be expected to lack acquired immunity towards viral diseases. The population densities in open seas (salmon) and in fjords (brown trout and salmon) will in most cases be significantly lower than in the rivers. Based on these facts, it is expected that a virus that has co-evolved with salmon/ brown trout should have the best opportunities to transmit in the freshwater phase, i.e. in the rivers. The interactions between individuals are highest during spawning.

Most Atlantic salmon follow an anadromous fish migration pattern, spending a large part of the life span in the ocean, while this is more variable for the different morphs of brown trout. If exposure to ISA virus for wild Atlantic salmon and brown trout mainly occurs in rivers, such exposure will possibly occur at a lower frequency for Atlantic salmon than for brown trout. There are indications that ISA virus may be more closely adapted to brown trout than Atlantic salmon (Nylund et al., 2003, Nylund and Jakobsen, 1995, Rolland and Nylund, 1998a, Devold et al., 2000).

However, co-evolution between virus and host implies adaptation of host factors such as behaviour-related (staying away from the breeding grounds) or innate immune response or factors related to the agent (i.e. low-virulent variants, cf: HPR0 discussion).

Co-evolution may explain the presence of the ISA virus, which is assumed to be avirulent, in wild salmon populations in both fresh and sea water (Nylund et al., 2003, Plarre et al., 2005). Such viruses could represent the “wild type” ISA virus, from which virulent isolates connected to clinical outbreaks of ISA in farmed populations of salmon have originated.

DIAGNOSIS OF ISA

Generally, disease diagnosis is a process involving 3 major steps: 1) description of the disease, i.e. clinical observations and pathomorphological examinations; 2) identification of putative causes/aetiological agents i.e. analyses for detection of infectious agents; 3) establishment of a causative association between 1 and 2.

A general evaluation of some aspects about diagnostic tests is presented in the Appendix.

The diagnosis of ISA (as a disease) was initially based on clinical and pathological findings only. Following the isolation of the causative agent, direct methods for the detection of virus and confirmation of the diagnosis have been established. These methods include isolation of the virus in cell culture followed by immunological identification, antibody-based demonstration of ISA virus antigen in tissues and RT-PCR techniques.

Patho-morphological evaluation

A cornerstone of this evaluation is a histopathological evaluation of formalin-fixed paraffin-embedded tissue sections. The patho-morphological evaluations are routinely supplied by an evaluation of IHC-stained tissue sections.

Cell culture isolation of ISA virus

Diagnostic cell culture isolation of ISA virus from infected fish is usually performed using either SHK-1 and/or ASK-II cell lines. Recent experiences indicate that ASK-II cells should be the first choice for primary isolation (Rolland et al., 2005). ISA virus in cell culture is usually identified by an immunofluorescent antibody technique (IFAT) test using anti-ISA virus monoclonal antibodies.

Demonstration of ISA virus antigens

These methods include detection of ISA virus using anti-ISA virus antibodies on tissue cryosections (IFAT), formalin-fixed paraffin-embedded tissue sections (IHC) and tissue imprints (IFAT). The methods are rapid, relatively cheap, robust and suitable for detection of ISA virus in fish with clinical ISA. Detection of ISA virus in subclinical infected fish is less reliable (or unsuitable) due to restricted sensitivity. IHC is currently the first choice for detection of ISA virus in diseased fish and has a major advantage of being able to associate virus detection with known target cells and pathological lesions.

RT-PCR

This method detects virus genetic material and is rapid, specific and sensitive. Different applications of this method have been described including applications using the improved real-time RT-PCR methods. The main advantages of these methods are the sensitivity and speed, and it is currently the method of choice for detection of ISA virus in subclinically infected fish (i.e. in ISA virus infected fish showing no disease symptoms). In disease diagnostic work, RT-PCR is used for confirmation of virus findings in diseased fish.

VALIDATION OF DIAGNOSTIC TESTS

Diagnostic tests need to be properly validated. In particular, performance and operating characteristics in field situations are important when healthy populations with expected low prevalence are tested. In this situation, false positive tests may have importance as an infection-free population can be classified as infected. Verification of such test results must be performed by another, independent test. In essence, validation determines whether a true finding in the laboratory is also true in the field. This requirement is not encountered in the initial optimization of the tests in laboratory simulations. General and special guidelines for validation are given in OIE's Manual of diagnostic tests for aquatic animals 2006 (http://www.oie.int/eng/normes/fmanual/A_summry.htm) and include both test optimization and documentation of performance characteristics. All screening tests that are used should be standardized, and quality control procedures should be implemented before they are used as a diagnostic test for prevention and control of ISA (Nerette et al. 2005, a, b). Cut-off values must be determined for tests with quantitative output and the analytical sensitivity and

specificity of the test should be known. Then good repeatability (i.e. within-laboratory consistency) and reproducibility (i.e. between-laboratory consistency) should be assured. A test that is used for testing of healthy fish to make regulatory decisions should also be able to distinguish between virulent and avirulent forms of the virus. However, today this requirement is restricted to determining whether the virus has HPR0 or not. The eventual pitfalls of using HPR as a virulence marker are discussed under Question 4.

The most important consideration for test validation is to measure the capacity of a positive or negative test result to predict accurately the infection status of the fish or fish population. This capacity is not only dependent on a highly sensitive, precise and accurate assay using well characterised and standardised reagents (see analytical validation above), but carefully derived estimates of diagnostic (also often termed epidemiological) sensitivity and specificity. These parameters are cumbersome and difficult to determine, but are the most important parameters to establish during a validation process. They determine - together with the infection prevalence in the population - the probability that a given test result reflects the true status of the fish (Dohoo et al., 2003).

NORWEGIAN EGG EXPORT

Norway pioneered Atlantic salmon farming, which also included a systematic breeding programme. This programme made Norwegian breeding material (i.e. fertilized eggs) attractive for other countries. Fertilized eggs have been exported during the last thirty years to most salmon producing countries. Norway is traditionally the largest exporter of Atlantic salmon eggs and probably is also the only country that currently does not import eggs. Unfortunately, official records of Norwegian egg export are not available and details concerning the volume and extent of this export have to be based on information from the exporting companies, importing countries and anecdotal information.

Official records from Chile (www.sernapesca.cl) indicate that there has been a significant yearly export from Norway to Chile since 1998. Based on information from two of the major egg producers in Norway (i.e. Aquagen and Salmo Breed), significant amounts of eggs have also been annually exported to Scotland and the Faroe Islands since 2003. In the early 1980's, there were also minor exports to Ireland and Scotland (T. Håstein personal communication). Official records from Iceland show that eggs were imported from Norway in 1984, 1986 and 1987 (G. Jónsson, personal communication). Interestingly, the 1986 and 1987 import came from the company Bolaks (location Eikelandsosen), which at that time had significant ISA problems.

EPIDEMIOLOGICAL ASSESSMENT OF VERTICAL TRANSMISSION

To assess the relative importance of vertical transmission for the spread of ISA virus and/or ISA outbreaks, detailed information on virus characteristics, company history, epidemiological information, and production procedures need to be included.

The risk of producing smolts carrying ISA virus due to vertical transmission may be quantified by a risk assessment starting at the brood stock – hatchery level. Some of the data requirements for such an assessment are listed in Table 2. For assessing the risk of outbreaks due to vertically transmitted virus, further knowledge on virus, biosecurity measures and the production system is needed, as currently most of this information is not available.

Table 2. Data required for assessing the risk of producing ISA virus infected fry resulting from vertical transmission

Probabilities	Comments
Prevalence in brood stock (male and females)	
Probability of internally- externally infected eggs	Infective stage (clinical, sub clinical), abundance of virus, strain of virus
Probability of externally contaminated milt	
Probability of eggs becoming internally infected after fertilisation	Virus load, time to water hardening
Effect of disinfection on external and internal virus	Virus load, type of disinfection, disinfection procedures
Probability of internal/external virus surviving to hatching	Disinfection procedures during incubation

The probability of ISA outbreak

In Table 3 the production volume of eggs and smolts since 1998/99 is shown. The number of production units has decreased through the years, but in 2005, 273 hatcheries were registered (Source: The Directorate of Fisheries). The annual average smolt production capacity may be estimated to 8-900 000 smolts per hatchery. Since 2000, approximately 1.4 billion eggs have been produced for the Norwegian market. There have been no reports of ISA outbreaks in hatcheries in this period. With this huge number of potentially infected eggs going through the production cycle, the probability for ISA outbreaks in hatcheries must therefore be regarded as very low.

Table 3. Egg and smolt production in Norwegian farmed salmon industry

Year	Eggs for incubation (in millions)	Year	Smolts to sea (in millions)
1998/99	192	1999	123
1999/00	183	2000	133
2000/01	224	2001	134
2001/02	248	2002	135
2002/03	260	2003	128
2003/04	215	2004	134
2004/05	225	2005	153
2005/06	245	2006	

Source: The Directorate of Fisheries

Roughly calculated, each generation occupies about 275 sea sites and a total of approximately 600 sites are actively in use at any given time every year (assuming three generations present). According to Lyngstad et al. (2005), the time from sea transfer to the diagnosis of an ISA outbreak is from 7 to 21 months. This time period indicates that the entire number of active marine sites is at risk of experiencing an ISA-outbreak. With an average of 10 official outbreaks annually in the time period 2003-2005, the apparent annual incidence of ISA outbreaks has varied from 0.8 % to 2.5 %, which may be regarded as the annual site probability of experiencing an ISA outbreak. The true prevalence may be higher assuming under-reporting of the disease.

Egg production in the Norwegian salmon industry

For the production year 2004/2005, 225 million eggs were incubated, and in the 2005 season 153 million smolts were stocked to sea (www.laksefakta.no). The corresponding numbers for rainbow trout were 35 million eggs and 19 million smolts. This production mainly originates from four commercial egg producers with 23 brood fish locations. The eggs are then distributed among 273 hatcheries/smolt producers and finally to approximately 275 grow-out locations at sea.

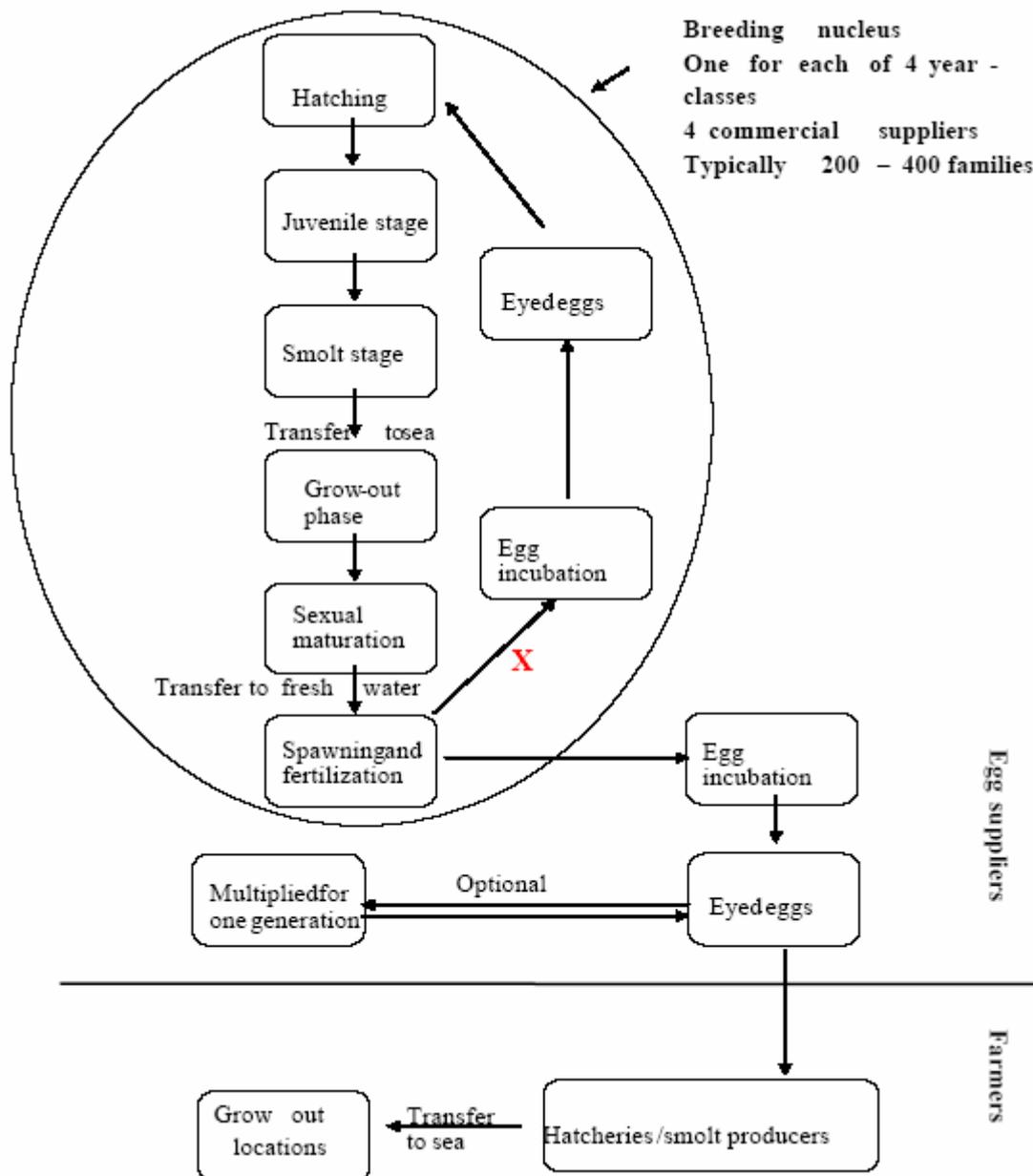
Because of the continuously changing infrastructure of the industry, detailed insight is vital when using historic information in epidemiological studies. Since the turn of the century, only a few companies have been involved in egg production of which SalmoBreed and Aqua Gen are the two main companies. These two companies act as umbrellas under which five different egg producers belong to SalmoBreed and four belong to Aqua Gen. The egg production units are located in different parts of the west-northwest coast of the country, and are clearly hydro-geographically separated. The genetic family core both for SalmoBreed and Aqua Gen dates back to about year 2000, which means that the coordinated breeding system under these umbrellas of today represents about 6-8 generations. The different sub-contractors may therefore have produced different genetic material before and just after 2000/2001. An example is shown at <http://www.sevareid.no/avl,rogn.htm> where one of the egg producers that currently belong to SalmoBreed, produced brood stock in 2001 and smolt in 2001/2002 based on eggs from Aqua Gen in 1998.

In a typical breeding system, there is 4 year-classes of salmon in production at any given time. These individuals will be the source of eggs for production, but also of eggs for the next generation of brood fish. This part of the population is termed the “breeding nucleus”. The brood stock is kept at sea, usually in separate locations for each generation, and transferred to freshwater before maturation is complete. After spawning, the eggs from the breeding nucleus are kept in separate cylinders, one for each family, whereas the eggs intended for production are usually pooled before incubation. In this setup, most of the production eggs will not be from the families that are chosen for the new breeding nucleus. It is also possible to use full siblings of the new breeding nucleus for production. In this case, it is necessary to raise these individuals to maturity for another spawning that will provide production eggs. For a schematic overview of the production, see Fig. 3.

After fertilization, the eggs are disinfected by use of iodophore (100 ppm, 10 min). This disinfection is also repeated at the eyed egg stage, before the eggs are shipped to hatcheries. In the hatcheries, normally the eggs are routinely disinfected by iodophore upon arrival. At the egg stage, it is routine to use of formalin (0.01 %, 10-30 min) to prevent fungal infections, typically every second day, depending on the water quality.

If vertical infection is a significant mode of transmission for ISA virus, the line marked with an X in Fig. 3 is a point where the cycle can be interrupted. The individuals chosen for the new breeding nucleus will be the parents of all salmon in the next generation. If each of four commercial producers has 300 families, the total number of samples to be screened would be approximately 2400 each year (300 families X 2 (female and male) X 4. Since the males normally are used for several females the male/female ratio is lower than one. The total number presented is therefore a maximum number). Also, at this stage the eggs from each family are kept in separate cylinders, making it possible to discard eggs from positive parents. All other possible control points would necessarily mean a much higher number of samples.

Figure 3. Schematic drawing of the flow of biological material within salmon breeding



ISA outbreaks versus genotyping and brood stock

A total of 46 different hatchery locations delivered smolts to the 32 sites experiencing an ISA outbreak during 2003-2005. All except one outbreak site contained smolts from two or more hatcheries. Nine hatcheries were involved with two or more ISA outbreak sites during this period. The geographical locations of these hatcheries are given in Table 4 together with year of outbreak and the virus group isolated from diseased fish. From the table, one may see that two of the hatcheries were associated with outbreaks, from which viruses belonging to EU-G2 group were found. Seven hatcheries (9 cases) were associated with two or more outbreaks within the same year. Of these, three were associated with the same group while six were associated with outbreaks of different groups. The information available is not coherent enough to conclude whether any significant association exists between hatcheries and a specific group of virus isolate.

Table 4. Hatcheries delivering smolts to two or more ISA outbreaks during 2003-2005

Hatchery (county)/group	EU-G1 ^a	EU-G2	EU-G3
1 (N-Trøndelag)	2003	2003	
2 (Sogn and Fjordane)		2004	2003
3 (Møre and Romsdal)	2004		2004
4 (N-Trøndelag)	2003	2003	
5 (N-Trøndelag)		2004, 2005, 2005	
6 (S-Trøndelag)	2003	2003	
7 (Hordaland)		2004, 2005	
8 (Hordaland)	2003		2003, 2004, 2004, 2004, 2005
9 (Finnmark)		2004, 2005, 2005	2004

^a Nomenclature according to Nylund et al., 2006.

Nylund et al. (2007) combined the information on brood stock companies (denoted A-E) with ISA virus isolates from outbreaks registered in the period 1997-2005 using information on clades (here used as the term for the phylogenetic level below the groups EU-G2; EU-G1 is treated as a clade by itself). Results are shown in Table 5. All the five clades are associated with company A, four with B, two with D, and a single clade was common to both C and E. Company B is clearly associated with EU-H2 –isolates, but any overall pattern associating clade and broodstock company, is not obvious from the data. The data are however, fragmented especially when viewed in relation to the complex infrastructure of the industry, and the low number of genotyped isolates. Also, the relative frequencies in the Table have to be considered in relation to the market shares of the different companies.

Table 5. Brood stock companies (A-E) associated with ISA virus clades isolated from ISA outbreaks during 1997-2005¹

Year/clades	EU-G1	EU-NN (G2)	EU-NW (G2)	EU-H2 (G2)	EU-MN (G3)
1997	A				
1998				B, B	

1999	A, AD			B	A
2000	A				
2001					
2002		ABD	A		
2003	AB, AB	CD, CD		B	
2004	AB, AB, AB,	A, ABE		AB, B, B, B, B, B, B, B	A, A, ABD
2005	AB, AB	AE, A, E			A

¹ Information is not available for all outbreaks in the period

Outbreaks related to contact network

In a stochastic time-space model (Scheel et al., submitted), the risk of an ISA outbreak due to proximity to an infectious site and/or contact network was estimated. Cases in this model were outbreaks from 2000-2005. The model concluded that roughly 30 % of the ISA outbreaks were related to the contact network variables in the model and distance to infectious site, assuming an ISAV infected site has an infectious period of nine months. The relative share of these two risk factors was approximately the same. The model concluded however, that the relative risk of proximity to infectious site was higher than contact network when sites were less than 5 km apart but that separation by more than five km may be of less importance if the contact network was maintained. The remaining 70 % could not be explained by the model. The model is based on the data available and the defined assumptions, which may be a subject for discussion. The small number of ISA cases in the dataset limits the precision of the results. The relatively high percentage of risk not explained by the model emphasizes the need for more data to evaluate more fully the assumed risk factors and identify others.

TERMS OF REFERENCE

Assignment to the Norwegian Scientific Committee

To ensure that the Norwegian Food Safety Authority bases its management of ISA on internationally accepted knowledge, the Norwegian Scientific Committee for Food Safety was asked in a letter of August 28th 2006 to consider the following questions:

- Can ISA virus transmit vertically? How high is the probability of spread of the agent and/or development of disease, as a result of vertical transmission?
- Do present levels of knowledge relating to the virus and disease provide a good enough basis for tracing the source of infection and routes of transmission of ISA? Is screening for ISA virus in apparently healthy fish appropriate for limiting ISA distribution?
- Of what importance are the different production stages, including transport, slaughter and other related activities for spread of infectious materials? How long does ISA-virus remain infective under different conditions?
- Does infection with different variants of the virus pose greater or lesser risk of disease outbreak?
- What are the reservoirs for ISA virus? What is the importance of various reservoirs for outbreak of disease?

The Norwegian Scientific Committee for Food Safety, Panel on Animal Health and Welfare, established an *ad hoc* group consisting of 9 national and international experts. The international experts came from Scotland, Canada and the USA. The group was chaired by Prof. Espen Rimstad from the Scientific Committee, Panel on Biological hazards, and has had 5 all-day meetings and one 2-day meeting.

The *ad hoc* group found it necessary to arrange the Questions from the Norwegian Food Safety Authority in a different order when answering the Terms of Reference. The questions are split up and arranged this way:

1. Can ISA virus transmit vertically? How high is the probability of spread of the agent and/or development of disease, as a result of vertical transmission?
2. Do present levels of knowledge relating to the virus and disease provide a good enough basis for tracing the source of infection and routes of transmission?
- 3.a. Of what importance are the different production stages, including transport, slaughter and other related activities for spread of disease?
- 3.b. Of what importance are the different production stages, including transport, slaughter and other related activities for spread of virus?

- 3.c. How long does ISA-virus remain infective under different conditions?
4. Does infection with different variants of the virus pose greater or lesser risk of disease outbreak?
- 5.a. What are the reservoirs of ISA virus?
- 5.b. What is the importance of various reservoirs for outbreak of disease?
6. Is screening for ISAV in apparently healthy fish appropriate for limiting ISA distribution?

The Panel on Animal Health and Welfare discussed the full report on a meeting the 15th of January 2007, and the panel supports the conclusions made by the *ad hoc* group. Concerning Question 6, the Panel supports the opinion of the majority of the group.

ASSESSMENT BY THE *AD HOC* GROUP

QUESTION 1.

CAN ISA VIRUS TRANSMIT VERTICALLY? HOW HIGH IS THE PROBABILITY OF SPREAD OF THE AGENT AND/OR DEVELOPMENT OF DISEASE, AS A RESULT OF VERTICAL TRANSMISSION?

Vertical transmission – definition

OIE's Aquatic Animal Health Code (2003) has the following definition of vertical transmission: "Vertical transmission means the transmission of a pathogen from a parent aquatic animal to its progeny via its sexual products". The *ad hoc* group uses this definition in the present context. However, the possibility of contamination of sexual products with ISA virus through other sources, such as handling or equipment, cannot be excluded, and will in a practical situation be registered as vertical transmission. It is beyond the scope of the *ad hoc* group's work to distinguish between these two possible sources of the virus. Likewise, the *ad hoc* group does not differentiate between the possibilities that the ISA virus is within or outside the egg.

Vertical transmission- literature, data, assessments

The *ad hoc* group is aware of only a few published works that have addressed vertical transmission directly (Thorud and Djupvik, 1988, Melville and Griffiths, 1999, Nylund et al., 2007, Sjøfteland et al., 2005). The latter is a report regarding ISA disease in brood fish and is available only in Norwegian.

Early experiments that tried to establish the infectious nature of ISA (Thorud and Djupvik, 1988) failed to demonstrate vertical transmission (measured by mortality). Melville and Griffiths (1999) were not able to detect vertical transmission of ISA virus by cell culture or by regular RT-PCR or by experimental injection of homogenates from eggs and progeny from ISA virus positive brood fish. A personal communication from one of these authors said that in a non-published study of progeny from a "severely infected broodfish, in very poor condition" positive results were obtained from the progeny by RT-PCR. However, the progeny never developed clinical signs of ISA. The eggs that the progeny had been derived from had been disinfected (S. Griffiths, personal communication).

In the report by Sjøfteland et al. (2005), the outcome of a natural field outbreak of ISA disease in broodfish was studied. Materials from parent fish, eggs, and juveniles were examined for ISA virus in two independent laboratories using several different methods (Sjøfteland et al., 2005). For the current purpose of evaluating the likelihood of vertical transmission, only the real time RT-PCR results are considered. Both laboratories detected positive samples. In one of the laboratories, several positive samples from eggs and juveniles were found, while in the other laboratory only three positive samples from eggs were detected. Positive results were obtained from both disinfected and non-disinfected eggs, although the number of target molecules, i.e. ISA virus RNA, was low (A. Nylund and H. Sindre, personal communications).

Nylund et al. (2007) genotyped the haemagglutinin-esterase gene (HE) obtained from outbreaks of ISA in Norway and from subclinical infections. Samples originated from fish at

sea sites, broodfish and smolt. The HE sequences obtained from smolts and most of the broodfish were not connected to outbreaks of ISA. Sequences and information on the origin of smolts, eggs, and broodfish were paired. Furthermore, screening of 24 smolt production sites by real-time RT-PCR revealed that 22 of these sites were found to be positive with this test. In the real-time RT-PCR assay, 45 cycles were conducted (Devold, 2006) and a cut-off for when the result was considered positive was not applied. The reproducibility of the assay in the range where some of the samples were obtained is unknown. The authors of this study, based on genotyping and information on brood fish, embryos, parr, smolt and post smolt, concluded that the major transmission route for ISA virus in Norwegian aquaculture is vertical transmission. In a recent study (A. Nylund, personal communication), ISA virus RNA was detected in broodfish, eggs, fry, parr and smolt by real-time RT-PCR. In broodfish that had spent three months in freshwater, 84 % tested positive in 2005, while the number had dropped to 17 % in 2006. In fry, parr and smolt (at sea for 50 days), between 2 % and 16 % were positive in real-time RT-PCR. The fry, parr and smolt did not necessarily represent the progeny of the tested broodfish population. In an independent real-time RT-PCR testing of 60 fish from each of 27 smolt producing sites, fish from 2 sites (7 %) were positive with 1-2 positive fish at each site. One additional site was regarded as possibly positive (H. Sindre and B. Dannevig, personal communications).

A report from an EU sponsored concerted action from 2005 that deals with trading of fish eggs concluded that they are not aware of any hard evidence for vertical transmission of ISA virus, and vertical transmission was regarded to be insignificant in the epidemiology of the infection (Bovo et al., 2005).

Vertical transmission – experience

In Norway, 0.7 % of all known ISA outbreaks are related to fish in the fresh water stage. In experimental infections in fresh water, it has been demonstrated that fry and parr are susceptible to ISA (Raynard, 2001b, Dannevig, 1994), although there are indications that smolts in freshwater are even more susceptible to ISA than the smaller fish (Glover et al., 2006).

A personal communication from Dr. Michael Beattie, Chief Veterinarian for Aquaculture, NBDAFA, Province of New Brunswick, Canada states “to date there has been no evidence of ISA being transmitted vertically in either NB or Maine”. He also refers to an example where the resulting fry from ISA positive brood stock fish were repeatedly tested using IFAT and RT-PCR for 5-6 months and no evidence of ISA virus was found.

A similar personal communication from Dr. Peter Østergaard, Chief Veterinarian for Aquaculture in the Faeroe Islands stated that ISA virus had not been detected in brood fish or in fish in the fresh water stage in the Faeroe Islands using RT-PCR. A comprehensive testing regime has been used after the disastrous ISA outbreaks a few years ago.

Norway has exported salmon eggs to Chile, Ireland, Scotland, Iceland, Faeroe Island, USA, Canada, and Denmark. To some of these countries, only a few hundred thousand eggs have been exported, while to others, several million eggs are exported annually. Nevertheless, ISA has never been reported in Ireland, Iceland or Denmark although ISA virus was detected in an apparently healthy rainbow trout in Ireland in 2002. Both Canada and USA have suffered outbreaks of ISA, and while the dominant virus isolated belongs to the American group, viruses from the European group are also present.

Export of fish eggs to Scotland began after 2003 and that country has had no outbreaks of ISA since 1999. A case of suspicion of ISA occurred in 2004, which was associated with gill damage, but no ISA virus was isolated. ISA virus was demonstrated using IFAT and RT-PCR. Molecular analysis of part of the ISA virus haemagglutinin gene amplified from gill tissue revealed an identical sequence (HPR0) to that found previously in a wild salmon on the east coast of Scotland (Cunningham et al., 2002) and differed significantly from the HPR sequences from the ISA virus isolated in the 1998 ISA outbreak in Scotland (Anonymous, 2005). It was not possible to establish a direct connection between the HPR0 and the gill disease.

It is assumed that eggs have been exported from Norway to Chile since approximately 1985/86. Official statistics from Chile (Sernapesca, <http://www.sernapesca.cl/>) state that eggs have been imported every year except in 2002 in the period 1998-2005. The *ad hoc* group does not have a clear overview of the ISA situation in Chile. ISA has never been reported from Chile, but it has been published that an ISA virus has been isolated from diseased, farmed Coho salmon (Kibenge et al., 2001a), and Coho salmon pooled serum samples from the affected coho farm were seropositive (by ELISA and virus neutralization) for ISA virus (Kibenge et al., 2002). The virus isolate has been grouped to the North American cluster of ISA virus (Kibenge et al., 2001b), a genogroup that has not been found in Norway, but is present in Canada. Furthermore, there are two independent publications describing experimental infection of ISA virus in Coho salmon (breeds farmed in North America) that have found this fish species to be resistant to ISA (Kibenge et al., 2006a, Rolland and Winton, 2005).

Vertical transmission - intervention procedures

One should keep in mind that the legislation introduced to minimise the spread of ISA in Norwegian aquaculture has most likely reduced the relative contribution of horizontal transmission, indicating that the relative importance of the vertical transmission route will increase. However, the legislation does not solely focus on horizontal transmission, and the mandatory disinfection of eggs is a method that will diminish vertical transmission.

The *ad hoc* group regards the use of iodophore-based disinfectants to be a good method to decontaminate egg surfaces. However, iodophore disinfection cannot be relied upon to inactivate fully any eventual virus. Iodophore disinfection (100 ppm, 10 min) is normally used after fertilization, before transportation from brood fish facilities to hatcheries, and often also after arrival at hatcheries. Although ISA virus has been shown to be sensitive to iodophore disinfection (Smail, 2004, Bovo et al., 2005), potential pitfalls include virus protection by egg products, virus localised inside the eggshell, or the potential for lapse in disinfection routines. Furthermore, timing of disinfection in relation to egg activation (hardening) can influence the outcome of disinfection, because during the activation of the egg the surface changes considerably (Bovo et al., 2005). The caution expressed by the *ad hoc* group is partly based on the experience of the ineffectiveness of iodophore disinfection to prevent vertical transmission of infectious pancreatic necrosis (IPN), infectious haematopoietic necrosis (IHN) and pancreas disease (PD) (Bovo et al., 2005).

Prophylactic anti-fungal treatment of eggs with formaldehyde (0.01 %, 10-30 min) is used routinely several times per week prior to the eyed egg stage in areas where the water quality is not optimal. Such treatment would be expected to inactivate ISA virus since 0.5 % formalin (which equals 0.02 % formaldehyde) inactivates free ISA virus (Torgersen, 1997, Bovo et al., 2005).

A: CAN ISA VIRUS TRANSMIT VERTICALLY?

Available data concerning vertical transmission are contradictory and there was disagreement within the *ad hoc* group as to the interpretation of the available data.

The *ad hoc* group focused on the results presented by Søfteland et al. (2005), which indicate that ISA virus can be transmitted vertically when the parental generation show clinical ISA disease, and on the results presented by Nylund, which show that ISA virus RNA was demonstrated in eggs and offspring from healthy brood fish (A. Nylund, personal communication).

Thus, at this time point the conclusion to the question “Can ISA virus transmit vertically?” is that vertical transmission **cannot be excluded**.

B: HOW HIGH IS THE PROBABILITY OF SPREAD OF THE AGENT AND/OR DEVELOPMENT OF DISEASE, AS A RESULT OF VERTICAL TRANSMISSION?

It is vital for the *ad hoc* group that a dual approach is used when trying to answer the question of probability of spread. One approach is to consider the spread of the agent, ISA virus, including avirulent and virulent variants and the other approach is to consider the spread of the disease, ISA. There is a general agreement that ISA virus is never, or is very seldomly, transmitted vertically in the settings of Norwegian salmon farming. This opinion is, however, based on accumulated experience-based knowledge, and not on scientifically controlled experiments. Different strains of virus may, however, have different abilities to succeed in vertical transmission, especially when considering the potential existence of non-virulent virus strains.

Importantly, some of the mentioned information given by personal communications indicates results that are in disagreement with previously available information, especially data concerning detection of ISA virus in eggs/offspring by real-time or traditional RT-PCR. Also, currently there are no field observations indicating that vertical transmission of ISA-virus plays a significant role in ISA dissemination.

Furthermore, in two of the above mentioned studies in which positive findings by RT-PCR were obtained from eggs/progeny, the brood fish had clinical ISA. Thus, to diminish vertical transmission, priority should be placed on identifying and culling of broodstock populations with clinical disease problems.

I. PROBABILITY OF SPREADING THE AGENT AS A RESULT OF VERTICAL TRANSMISSION

The probability that the ISA virus can be transmitted vertically may depend on individual characteristics such as clinical status and virus titre in the parent fish, intracellular or extracellular transmission which is the dominant route; and/or strain characteristics of the virus. At the population level, prevalence may be of importance both in wild (Plarre et al., 2005) and farmed fish (Nylund et al., 2007).

The probability for further spread via eggs, fry or smolts as a result of vertical transmission will depend on the efficacy of intervening management procedures such as disinfection and prophylactic treatment post-stripping.

A key point for reducing the probability of vertical transmission is an efficient health control scheme that is able to identify disease problems caused by ISA at an early time to prevent spread of the agent downstream in the production line.

As of today, relevant knowledge is scarce on these variables. It is not possible from the available information to estimate a probability for the spread of the agent through vertical transmission.

II. PROBABILITY OF SPREADING THE DISEASE AS A RESULT OF VERTICAL TRANSMISSION

If the spread of the disease is to be considered a consequence of the vertical transmission of ISA virus then certain central observations should be taken into account. The small number of outbreaks in fresh water stage (0.7 %) and the absence of ISA in some countries that over the years have imported substantial numbers of eggs from Norway suggest that the probability of the disease occurring as a result of vertical transmission of the agent is low.

QUESTION 2

DO PRESENT LEVELS OF KNOWLEDGE RELATING TO THE VIRUS AND DISEASE PROVIDE A GOOD ENOUGH BASIS FOR TRACING THE SOURCE OF INFECTION AND ROUTES OF TRANSMISSION?

Tracing the source of infection/disease includes assessment of several sources of information including epidemiological data at different levels, characterisation of the disease and characterisation of the virus. Thus, such evaluations should be done with adequate background information related to epidemiology, virus and disease. It is also important to emphasise that one method/analysis is not enough to base conclusions upon related to tracing the source of infections/disease.

There are many different methods that can be used to characterize a virus, including clinical appearance of the disease, properties in cell culture propagation, antigenic and genetic analysis. The *ad hoc* group regards that establishment of genetic analysis, based on nucleotide and the derived amino acid sequence analysis represents a major improvement for tracing source of infection and routes of transmission.

Due to the lack of proof-reading in RNA-dependent RNA polymerases, RNA viruses such as ISA virus are assumed to mutate and hence evolve with a high frequency. For some viruses or viral genes, this mutation rate can enable a molecular clock to be calculated, i.e. the nucleotide differences between virus isolates can be translated into an approximation of when in time these isolates diverged. There are many assumptions that have to be fulfilled before a molecular clock can be assumed to be constant. However, even for viruses for which molecular evolution indicates that a constant molecular clock is not present, there is a relationship between genetic divergence and time. This relationship is discussed in detail in the background section of this opinion. Data that have been obtained on ISA virus, including findings of identical HE sequences in isolates separated by at least 9 years, have been interpreted to indicate that a molecular clock cannot be assumed to be constant for ISA virus (Devold, 2006, Nylund, 2007). This finding implies that genotyping of single ISA virus isolates, or practically speaking, genotyping of PCR products as virus is not always isolated, cannot be used to trace events related to time.

Most of the phylogenetic analyses of ISA virus isolates (or of PCR products) that have been performed are based on the information obtained from the HE gene, excluding the HPR region that, due to its frequent variation, makes it unsuitable for this purpose. Phylogenetic analysis of ISA virus nucleotide sequences divides the virus into two major clusters; North American and European (Devold 2001, 2006; Nylund, 2007). Analysis of genomic segments other than HE has supported this division (Devold, 2006). The European cluster can further be divided into three groups (EUG1-G3) (Nylund et al., 2007). Some ISA virus isolates from North-America are European-like and are often referred to as "European-in-North America". There are differing interpretations as to whether these isolates should be regarded as a separate group or be placed within the existing European group EUG2 (Nylund et al., 2007, Kibenge et al., 2006b, poster presentation).

Based on nucleotide sequences, the European groups can be further sub-grouped into different clades, and clades may perhaps again be divided into subclades. Each subgroup (clade or subclade) is defined after phylogenetic analysis of nucleotide sequences. Each branching point in the phylogenetic tree is defined by a supporting value. A high supporting value indicates a high probability that the branching is valid. However, there is no general consensus as to the specificity of the resolution of the phylogenetic analysis, i.e. when the analysis with certainty differentiates between ISA virus isolates.

Phylogeny of nucleotide sequences is a mathematical/statistical likelihood analysis aimed at inferring a phylogeny of the input sequences from a common ancestor. Potential bias regarding how representative the input sequences are may influence the validity of the results. Some points to consider are: 1) Knowledge on genetic variation of ISA virus within disease outbreaks is scarce. Variations may be present within one fish (i.e. quasispecies or concurrent infection with different virus isolates) and between fish during an actual outbreak. RNA viruses are known to occur in quasispecies, but the relative occurrence of the different clones is unknown. In one case, samples that were collected from two different fish from the same ISA outbreak yielded sequences that belonged in different genogroups (C.M. Jonassen, personal communication). 2) Sequencing of RT-PCR products obtained directly from fish is beneficial in phylogenetic analysis compared to similar sequences obtained from virus isolated in cell cultures because cell culturing may enforce a strong selection. However, RT-PCR directly from fish will produce a consensus sequence and genetic clones (quasispecies and/or non-dominant variants) occurring in low frequency will not be detected. If one clone is dominant, this will be detected, and it is to be expected that the dominant clone causes disease. 3) Reverse transcriptase used in the RT-PCR procedure lacks proof-reading and will introduce mutations at a low frequency. These mutations will be present in the PCR products, but not in the original viral genome.

The result of phylogenetic analysis cannot stand alone as evidence for “positive” tracing of the source of infection and routes of transmission. Supplementary epidemiological information is needed to make valid conclusions. For instance, the presence of identical nucleotide sequences of PCR fragments from the HE gene of ISA virus (often orally referred to as “genetically identical ISA viruses”) at two different outbreaks does not prove that a connection exists between the sites. On the other hand, phylogenetic analysis can be used for “negative” tracing. If an analysis shows that two viruses from two different outbreaks are “sufficiently” genetically diverse to be placed in different sub-groups, this finding indicates that there is no connection between the outbreaks. However in this context, there is no consensus on the definition of “sufficiently” i.e. the limits of dissolution of phylogenetic analysis.

The *ad hoc* group is of the opinion that the use of genetic analysis of ISA today is limited to differentiation between viruses. That is, genetic analysis can be used as a basis for a negative statement that an isolate is not related to another. Genetic analysis cannot be used to relate two viruses positively. This opinion is similar to interpretations normally applied to information from the genetic analysis of human influenza viruses.

The answer to the question: “Do present levels of knowledge relating to the virus and disease provide a good enough basis for tracing the source of infection and routes of transmission?” **“is no; supplementary epidemiological information is needed as well.”**

The present level of knowledge on virus and disease is not sufficient to trace routes and sources of infection alone. In each case where it is desired to resolve the routes and/or source of infection, epidemiological information is necessary in addition to information on the viruses. Each case has to be handled based on its own merits.

QUESTION 3.

3.A. OF WHAT IMPORTANCE ARE THE DIFFERENT PRODUCTION STAGES, INCLUDING TRANSPORT, SLAUGHTER AND OTHER RELATED ACTIVITIES FOR SPREAD OF VIRUS AND DISEASE? 3.B. HOW LONG DOES ISA-VIRUS REMAIN INFECTIVE UNDER DIFFERENT CONDITIONS?

In 1988, ISA was shown to be a transmissible disease. In 1990, OIE placed ISA on the list of other significant diseases. In 1993, ISA was placed on the EU list 1. In 1995, virus specific diagnostic methods were implemented. From 1988 onwards, regulatory measures were implemented to control outbreaks. Biosecurity measures based on general principles were implemented to prevent further spread of ISA and keep infectious pressure low. Segregation of generations, i.e. no more than one generation at a sea site at a time, was introduced at approximately the same time. The segregation of generations did not become mandatory by law until 2005, but was implemented voluntarily due to the positive effect experienced from combating furunculosis. From 1991/92, the number of annual ISA outbreaks fell dramatically. Generally, the measures were based on experience from decades of successful combating of terrestrial animal diseases, on accumulated aquaculture field experience, and on epidemiological information and knowledge, together with ISA-specific preliminary risk analysis results generated during the epidemic (Vågsholm et al., 1994).

It has been suggested that the introduction of oil-adjuvanted vaccines may have made fish more resistant to ISA, though no data documenting this effect or the duration of such non-specific stimulation have been published.

All these changes have independently been beneficial in combating ISA. The *ad hoc* group is of the opinion that the most important factor for the large reduction of ISA disease outbreaks seen in the 1990's was the implemented measures aimed at reducing infectious pressure and the interruption of infection routes.

Two epidemiological case control studies published in retrospect found a tendency for disease outbreaks to cluster in time and space, indicating a capability of the contagious disease causing agent to transmit horizontally from infectious sites (Vågsholm et al., 1994, Jarp, 1999).

The improved biosecurity is as such anticipated to have reduced the transmission pressure of infectious agents in general and of ISA specifically. The opinion of the *ad hoc* group is that an eventual reduced control of horizontal transmission of virus either through transport, slaughtering or other related activities would pose a big threat for the local spread of ISA.

The aquaculture industry has gone through large structural changes in the last decade resulting in a few large companies controlling many smolt production sites and sea farms. These changes could potentially result in more contact between farm sites. The farms and individual nets have become larger and the number of slaughterhouses has been reduced. The slaughterhouses are becoming hubs in a large network of farms. The effects of these changes on disease transmission should be examined through a broad epidemiological study to identify risk factors and intervention measures adapted to an economically efficient industry.

QUESTION 3A. OF WHAT IMPORTANCE ARE THE DIFFERENT PRODUCTION STAGES, INCLUDING TRANSPORT, SLAUGHTER AND OTHER RELATED ACTIVITIES FOR SPREAD OF VIRUS AND DISEASE?

Transmission from the fresh water phase

The probability of vertical transmission and the risk of spreading of the virus through vertical transmission are dealt with in Question 1.

Eggs both after fertilization and during incubation are submitted to an intense disinfection regime as mentioned in Question 1. Any risk of spreading the virus through environmental contamination must therefore be regarded as very low.

It is assumed to be little exchange of equipment and personnel between different smolt production sites. Subsequently, **the risk of viral transmission between fresh water production sites is minimal.**

Transmission in seawater phase

Epidemiological studies and general field observations do indicate that there is a clustering of outbreaks in space and time indicating a certain probability for horizontal transmission of the virus in seawater (Vågsholm et al., 1994, Jarp, 1999). The strength of the associations estimated in the epidemiological studies may have decreased following the implementation of biosecurity measures. Phylogenetic analyses of virus from some of the claimed clusters have, however, not always supported horizontal transmission between the sites (A. Nylund, personal communication).

A recent mathematical model supports the contention that a certain proportion of outbreaks are associated with horizontal contact networks (distance, equipment, ownership) (Scheel et al., submitted).

As none of these techniques stands completely on its own, the diverging conclusions need to be followed up by more detailed studies.

It is not possible for the *ad hoc* group to give a quantitative assessment regarding the risk of spread of ISA disease between sea production sites in general. The risk has been reduced as a consequence of the measures implemented. However, any quantitative judgement of risk is dependent on the local conditions, contacts between farms such as transport with well boats, feed boats and different kinds of service boats, and sharing of personnel and equipment. These factors may possibly be influenced by the structural changes that have occurred in the industry.

Transportation

There are several transportation steps in the flow of live material in the industry.

In general, a lot of stress is imposed on fish during transportation as a consequence of factors such as high densities and changes of environment. This stress may make the fish more vulnerable to infections. Transport (as a form of stress) of terrestrial animals is known to reactivate subclinical infections, which is a phenomenon that should also be regarded as possible in fish. High densities of fish will facilitate the transmission of contagious diseases.

The two largest transportations of fish are the transport of smolt to sea sites and the transport of slaughter-ready fish to slaughterhouses/processing plants. The well boats used are normally out-sourced, i.e. not owned by the farming company. Considerable emphasis is placed on the cleaning and disinfection of well boats by both the legislative authorities and the farming industry.

Transportation of smolt and the use of well boats

The main bulk of the transport of smolt takes place in two hectic periods during spring and autumn. Well boats mainly used for this transport are at the same time used for other transport activities including the transport of slaughter-ready fish. This busy biological and economic schedule for the boats and their very complex tube system for water and air circulation, make proper cleaning and disinfection between operations difficult. It should be mentioned that well boats are used to assist other operations on fish farms including sorting of fish and treatment for lice. Well boats have been implicated in the spread of ISA virus from an initial outbreak in Scotland (Stagg et al., 2001). As long as this system is operating, the *ad hoc* group is of the opinion that **well boat transportation is an important risk factor for transmission of ISA virus.**

Slaughtering infrastructure

All fish in Norway are live-hauled to the slaughterhouses. The fish are delivered to the slaughterhouses/processing plants, either live in waiting pens, or live in on-shore cooling tanks. The number of slaughterhouses has been reduced, but the individual slaughterhouses are becoming larger and this trend is set to continue. This change implies longer transportation routes. Larger slaughterhouses need an increasing number of fish deliveries for slaughter. This change in turn means that these slaughterhouses receive numerous well boat visits with live fish from many different places. The fewer, but larger slaughterhouses are becoming a centre (hub) in a large network for both close and more remote geographical areas.

It is the opinion of the *ad hoc* group that such a hub will, by its nature, increase the risk of spreading infectious agents including ISA virus.

Transport of fish for slaughter

ISA is a disease often developing over months before diagnosis is verified (Lyngstad et al., 2005). This change increases the probability of unknowingly transporting infectious fish to slaughter. In such a situation, routine sanitary measures for slaughtering ISA infected fish will not be implemented, either for the chosen transportation route, at the slaughtering house, or for the cleaning and disinfecting of the well boat. As well boats operate in a national market and interchangeably carry smolts and fish for slaughter, the unknown infectious status of the transported fish may increase the probability of transmitting the virus.

Slaughtering process

An epidemiological study published in 1994 using information gathered before many of the current regulatory measures were implemented stated that proximity to slaughterhouses was a significant risk factor for ISA outbreak (Vågsholm et al., 1994). Today, sanitary slaughter is implemented and the production sites are not located as close to slaughterhouses as before.

However, slaughtering is a technical process and failures in this process might occur. The common use of waiting pens instead of on-shore waiting tanks from which the effluent water is disinfected is a possible weakness.

There is no literature from recent years stating that the slaughter process represents a risk factor for virus transmission.

Other contacts between farms

There are several other possible contacts between farms such as different kinds of service boats and net-washing companies. Although some of these contacts may be of importance for virus transmission, it is beyond the scope of this report to assess all of these possible sources of contact.

QUESTION 3B. HOW LONG DOES ISA-VIRUS REMAIN INFECTIVE UNDER DIFFERENT CONDITIONS?

Infective virus means that the virus is able to replicate. Testing for infectivity of viruses implies that tests have to be performed in a live system. This requirement means that the samples should be tested in cell culture or in experimental fish. Quantitatively, the amount of infective virus present in a sample is described as the number of infective doses in cell cultures or of experimental animals. This unit is termed titre of virus. An assessment of infectivity should therefore preferably state the reduction of virus titre over time and not only whether infective virus is present or not.

Disinfections

A list showing the susceptibility of ISA virus to physical and chemical disinfectants is given in the background section / appendix. In general, ISA virus is not particularly resistant to disinfectants and is for instance susceptible to the iodophore disinfection used for eggs.

Offal, carcasses

Torgersen (1997) found that ISA virus infected material from fish that was stored for 6 days caused 30-40 % mortality when injected in experimental fish. There were no data for storage for more than 6 days and the endpoint, i.e. the time when the material was no longer found to be infectious, was not determined. Measuring mortality in experimental fish injected with material from organs is indeed a very crude method and may have several pitfalls. The outcome of an infection, induction of disease and possible death are not solely dependent on exposure to the virus but also on the infectious dose and environmental temperature, among other things. Fish that did not die in the experiment were not tested for the presence of ISA virus. Assuming that the dead fish died of ISA, the virus dose administered to the fish that died was considerable.

In experimental infections of juvenile fish with virus propagated in cell culture, virus amounts of 10^{2-3} TCID₅₀ normally produce more than 70 % mortality within 2-3 weeks (Dannevig et al., 1994, Rimstad et al., 1999, Mikalsen et al., 2001). Others have administered much higher doses of virus, 10^{6-7} TCID₅₀ (Kibenge et al., 2006a). The mortality in such experiments may

vary a lot, dependent on the physical condition, genetics and other factors relating to the fish used in the experiment and the primary route of infection/exposure.

Assuming that material is stored at environmental temperature, the opinion of the *ad hoc* group is that infectious ISA virus is able to survive for **weeks** in offal and carcasses.

In water

Water in this context is not a constant entity, but varies depending on factors such as temperature, presence of bacteria, enzymatic activities, organic material or UV radiation. The effect of these factors may not necessarily be negative for virus survival, for instance UV radiation is effectively stopped in water, and organic material may be protective for virus survival. ISA is an enveloped virus with glycosylated surface proteins and accordingly is easily attached to different particulate material, which could affect virus survival as well as spread. Laboratory experiments are thus limited in their ability to reproduce natural conditions and their effect on ISA virus.

In studies using sterile sea water, MacLeod et al. (2003) found that virus was infective after 21 d at 15 °C, and Rimstad and Mjaaland (2002) found a 3-log reduction in virus titre after 105 d at 4 °C. In "natural sea water", MacLeod et al. (2003) found infective virus after 7 d, while Nylund found virus to be infective only for 48 h (A. Nylund, personal communication).

There is available information for environmental survival for other members of the orthomyxovirus family. Viruses within a family have broadly similar construction. When comparing survival rates within a virus family, one assumes that the construction of the virus particle is important for survival. There may be differences in, for instance, the robustness of outer glycoproteins between viruses that may influence survival rates. For avian influenza viruses (influenza viruses belong to the orthomyxoviruses), that are excreted from the intestine of ducks, it has been observed that viruses are infective for 207 d in distilled water (17 °C), with a 90 % reduction per 34.5 d (EFSA, 2005), and in natural water there is a 90 % reduction per 8.4 d. In wet manure in field (faeces), infectivity was present after 105 d, but for other virus strains infectivity was not present after a week (15-20 °C) (EFSA, 2005). There are variations in survival between strains and serotypes of avian influenza viruses, and this may also be the case for ISA virus.

Although the information regarding survival of ISA virus infectivity varies, there are several findings that indicate long-term survival in water or the marine environment. Following an assessment of survival rates for viruses belonging to the same virus family, the *ad hoc* group is of the opinion that the infectivity of ISA virus in water in natural conditions may remain for **weeks**.

QUESTION 4

DOES INFECTION WITH DIFFERENT VARIANTS OF THE VIRUS POSE GREATER OR LESSER RISK OF DISEASE OUTBREAK?

The outcome on any virus infection in any animal is dependent upon properties of the virus strain, infective dose, environmental factors including management practices, and genetic constitution of the host. This has to be considered when assessing the virulence of the virus in any ISA outbreak.

Host

It is not known whether the susceptibility to ISA virus varies between the different strains of salmon used in Norwegian aquaculture. In an experiment using a farmed salmon strain, wild salmon and hybrids between the two, no difference in susceptibility to ISA virus was found between the three groups (Glover, 2006). However, family testing in association with Norwegian breeding programmes indicates significant variability in ISA susceptibility between family groups (Gjøen et al., 1997). A similar significant variability in ISA susceptibility was also revealed when smolts from 7 different smolt producers were compared (K. Falk, personal communication).

Environment

Environmental and management factors that promote outbreaks of contagious diseases in general, should be considered to be important also for the development of ISA.

Virulence - general

Virulence may in principal be influenced by any viral gene. Generally, most viruses that infect animals (including fish) occur in forms that have different levels of virulence, and even avirulent forms exist. The variation in virulence for viral haemorrhagic septicaemia (VHS) virus is an example of this phenomenon.

Virulence of the comparative model influenza A virus is usually the result of optimal gene constellations, although genetic changes located to a single gene or between two gene products may be attributed to virulence. Some of the genes are however regarded as being more central than others i.e. the surface glycoproteins responsible for receptor binding, viral entry, including fusion, and viral release from the cell. The cleavability of the HA, which is a textbook favourite of viral virulence markers, is more or less the sole marker for the virulence of highly pathogenic avian influenza, but this example should probably best be regarded as an exception, and not a general rule.

A traditional way of measuring virulence is by experimental infections. However, one should be careful when comparing the outcome because of the lack of standardisation of experimental infections for ISA. Preparation of inoculums including cell type used for propagation and occurrence of defective interfering particles, injection or cohabitant inoculation, dose, fish, density and temperature are some of the variables that will influence the outcome. There is empirical experience that the same virus isolate administered in the same manner and with the same titre, has produced large variations in mortality of experimental fish (K. Falk and E. Rimstad, personal communications).

There are publications describing experimental infections of different ISA virus isolates that could be categorised in different virulence groups, according to the mortality they induced (Mjaaland et al., 2005, Kibenge et al., 2006a). Although the number of fish used in these experiments was small and the individual experiments were not repeated, the *ad hoc* group is of the opinion that, most probably ISA virus can be divided into variants that differ in virulence.

Functional properties associated with ISA virus virulence

ISA virus has been found to possess the major functional characteristics of the orthomyxovirus family associated with virulence and pathogenesis. These characteristics include haemagglutinating (i.e. receptor binding), receptor destroying (RDE) and fusion activities as well as innate immune response antagonism.

Molecular markers for virulence

Based on circumstantial evidence, the highly polymorphic region (HPR) is so far the only genetic marker that has been associated with virulence in ISA virus. The HPR is characterised by having differential deletions compared to a theoretical full-length precursor gene named HPR0.

All HPRs that have been sequenced from ISA outbreaks may have derived from such a full-length sequence. It is assumed that a deletion or possibly recombination process converting HPR0 to other HPRs is a prerequisite for induction of disease. There are no publications that have addressed the frequency of a conversion of HPR0 to other HPRs. Whether there is a connection between the size/type of deletion and virulence has not been determined. The mechanisms for how HE-HPR deletions influence viral virulence remain speculative.

The presence of a long HPR0 gene has been detected by different RT-PCR methods in healthy wild salmon in Scotland (Cunningham et al., 2002) and farmed Atlantic salmon in Scotland, Norway and USA (Anonymous, 2005, Nylund et al., 2007) and in cell culture and healthy salmon in Canada (Cook-Versloot et al., 2004).

There is no report of successful propagation of HPR0 virus in cell culture. Cook-Versloot et al. (2004) reported detection of HPR0 by PCR in cell culture, but these investigators could not demonstrate replication. As a consequence, there are no reports or publications that the *ad hoc* group is aware of, that describe experimental infections using cell-cultivated HPR0 virus.

In an experimental infection using tissue homogenate obtained from conventional and real-time RT-PCR positive wild salmon (kidney samples), no disease was induced (Plarre et al., 2005). Although there was neither information regarding HE or HPR in this publication detailing the nature of viruses detected in the wild fish, nor information on the procedures for the experimental infections, preparation of homogenate, number of fish or controls, the *ad hoc* group has been notified that the virus was characterised as HPR0 (A. Nylund, personal communication).

A gene sequence corresponding to HPR0 was identified in gill samples in a case of suspicion of ISA in a marine fish farm in Scotland in 2004 (Anonymous, 2005). There was significant mortality, but the clinical signs and post mortem findings (mainly gill inflammation and proliferation) were not consistent with classical ISA. In the fish, 28/30 gill samples and 4/30 of kidney samples generated positive results in one of the ISA virus real-time RT PCR procedures used. All ISA virus sequences examined were of the HPR0 type. No obvious

aetiology of the disease problems and mortality was identified, thus the possibility that this case could represent a new manifestation of ISA, could not be completely ruled out. No source of the ISA virus infection was identified.

Similarly Nylund et al. (2007) found HPR0 in a group of smolts with proliferative gill disease, where 6/6 smolt were real-time RT-PCR positive. No further study of a possible correlation between the virus and the gill inflammation was pursued.

Conclusions

There are no reports of detection of HPR0 from ISA-diseased fish with classical clinical and pathological changes consistent with ISA. Except for the above-mentioned two reports, there are no reports of HPR0 detection in diseased fish. There are no reports of the induction of ISA disease in experimental fish after injection of tissue homogenate from fish with assumed HPR0 virus. However, based on the Scottish experience and on the lack of adequate experimental infection models with HPR0 virus, one should be careful to state that HPR0 is avirulent. However, the circumstantial evidence indicates that the majority of HPR0 detections have been from healthy fish and that HPR0 has never been found in connection with classical ISA. This evidence indicates that infection with **HPR0 virus in itself poses a lesser risk of disease outbreak than infection with ISA virus from other HPR groups.**

An important reservation regarding this conclusion is the lack of information regarding the probability of conversion of HPR0 to other HPRs. The *ad hoc* group is not aware of studies that have dealt with this question. If this conversion is a frequent occurrence in farmed fish, the justification for distinguishing between HPR0 and other HPRs in disease surveillance makes less sense.

QUESTION 5.

5.A. WHAT ARE THE RESERVOIRS FOR ISA VIRUS? 5.B. WHAT IS THE IMPORTANCE OF VARIOUS RESERVOIRS FOR OUTBREAK OF DISEASE?

Natural hosts

Virus transmitted from wild fish originally caused the ISA epidemic in farmed salmon.

ISA disease has only been reported in farmed Atlantic salmon. In farmed fish in Europe, ISA virus has also been detected in rainbow trout in Norway and Ireland. In Chile, ISA virus has been isolated from Coho salmon (Kibenge, 2001a) (further discussion on the latter is given in Question 1).

In wild fish, ISA virus has been detected by RT-PCR methods in Atlantic salmon and brown trout in Norway and in Scotland (Raynard et al., 2001a, Cunningham et al., 2002, Nylund, 2004, Plarre et al., 2005). There have been reported positive RT-PCR from other species, including alewife (Rolland, 2004) and eel (Stagg et al., 2001). There are also detections of virus in a few other species, however, the close proximity to ISA virus infected farms in these cases makes it difficult to assess whether these detections represent significant reservoir species or result from accidental transmission from the outbreak.

In experimentally infected fish, i.e. usually this means exposure to the virus by intraperitoneal injection of a large dose of virus, the salmonids Atlantic salmon, Brown trout, Arctic char, Rainbow trout, Chum salmon and Coho salmon have been found to support replication (Raynard et al., 2001a, Nylund, 2004, Plarre et al., 2005, Snow et al., 2001, Rolland and Winton, 2003). In the case of rainbow trout, even clinical disease and mortality occurred (Kibenge et al., 2006a). Similarly, herring have been found to sustain replication of ISA virus (Nylund et al., 2002). Snow and Raynard (2005) were not able to show any replication of ISA virus in Atlantic cod and Atlantic halibut after challenge; however, others claim to have shown that the ISA virus replicates in Atlantic cod (S. Grove, personal communication).

Present knowledge indicates that **brown trout and Atlantic salmon appear to represent important natural reservoirs**. However, the fact that surveys have not yet revealed other reservoirs is not valid evidence that they do not exist. Other naturally occurring marine species may be found to maintain the virus for extended lengths of time, and Atlantic salmon is a species that could represent a non-natural and possibly somewhat more susceptible host. However, in the absence of further evidence, this remains speculative. There are molecular indications from the west coast of North America that IHN virus is maintained in some marine species, but extensive surveys have not yet revealed these species (J. Winton, personal communication).

Natural hosts versus farmed fish as reservoirs

In farmed salmon, there have been in total 30 ISA outbreaks in the time period 2003-2005, and the annual apparent incidence of ISA outbreaks has varied from 0.8 % to 2.5 %. This should be regarded as a minimum number as the presence of virus without disease outbreak cannot be neglected. The disease ISA usually starts in one net pen and daily mortality generally stays low. It may take months before disease develops in neighbouring net pens. There are also findings of ISA virus in farms without any overt ISA disease (Stagg et al.,

2001, Nylund et al., 2007). The possible vast number of fish excreting virus in a farm and the length of the period of time this excretion occurs make the farming industry an important reservoir of the virus.

In ISA outbreaks from which the virus has been examined, the virus has been of a non-HPR0 type, although some of the non-HPR0 types have been detected only once. HPR0 has been detected in fish with gill disease in Norway and Scotland, however, no direct correlation between the presence of virus and the occurrence of the disease was pursued or examined on these occasions. In virus from wild fish, the situation is almost the opposite. There is a limited number of observations of ISA virus in wild fish, and even less where the HPR has been described. Most virus isolates that have been typed have been of the HPR0 type; however, non-HPR0s have been found as well (Stagg et al., 2001).

Regardless of what kind of molecular mechanisms that are used in nature, the different non-HPR0s may all have originated from HPR0. Some of the non-HPR0s may have originated from other non-HPR0s.

Whether the conversion of HPR0 to non-HPR0 may happen in wild fish and/or farmed salmon has not been determined. ISA as a disease has only been reported in farmed Atlantic salmon, where the high population density would be beneficial for virus transmission, and thus virus replication, and subsequently the appearance of virus mutants. Furthermore, an increase in pathogenicity would not be deleterious for viral fitness in dense fish populations, as there would be a continuous supply of susceptible hosts, as opposed to the situation in wild salmon. Comparing the substitution rates of RNA viruses, Hanada et al. (2004) found that the main source of the rate variation was due to differences in the replication frequency. It is therefore possible to speculate that the conversion of HPR0 to other HPRs happens more often in farmed salmon with dense fish populations than in wild salmon. There has been an increasing rate of detection of HPR0 from different countries indicating that the HPR0 may occur commonly in farmed Atlantic salmon (Cook-Versloot et al., 2004, Cunningham et al., 2002, Anonymous, 2005, Nylund et al., 2007).

Prevalence of virus in wild fish

The prevalence of ISA virus in *Salmo trutta* was tested in a few selected rivers and was found to vary from 6-100 %, depending on year of sampling. The detected prevalence was 100 % in 2001 and fell to 6 % in 2003 (Plarre et al., 2005). The rivers tested were in regions with endemic ISA at the time of sampling, which may have biased the sampling. Most marine salmon farms close to these rivers were empty in the period summer 2002 to summer 2003 due to earlier ISA outbreaks (Plarre et al., 2005). The HPRs were not typed in this study.

A survey of brown trout and salmon from Scotland, performed around the time of the Scottish outbreaks of ISA and two years after, found a prevalence of 0.02 % and 0.004 %, respectively. The findings indicated that many of the positive brown trout were found in proximity to affected farms in 1998, but findings were also from brown trout/salmon in fresh water river systems remote from the areas where the industry is located (Stagg et al., 2001).

In summary, the studies of prevalence of ISA virus in wild fish have so far only been from limited geographical areas in Norway and Scotland (Plarre et al., 2005, Stagg et al., 2001). It is not known whether the data are representative for fresh water reservoirs in these countries. However, the prevalence in the wild population seems to some degree to be dependent on the level of infection in the farmed population.

WHAT ARE THE RESERVOIRS FOR ISA VIRUS?

Current data and knowledge suggest that the reservoirs for ISA virus are wild brown trout, wild Atlantic salmon and farmed Atlantic salmon.

WHAT IS THE IMPORTANCE OF VARIOUS RESERVOIRS FOR OUTBREAK OF DISEASE?

Is there a continuous transmission of ISA virus in and between Norwegian farmed salmon, which then causes all ISA outbreaks? In the Faeroe Islands and east coast of Canada /USA, the outbreaks have been severe. This severity could be due to the short distance between farms, and thus be a demonstration of the importance of horizontal transmission (McClure et al., 2005). In an opinion expressed by an expert panel on the strongest independent predictors of ISA infection in Maine and New Brunswick, a site's proximity to other farms with clinically infected fish, and a previous history of ISA on the site were rated first (Gustafson et al., 2005). In an epidemiological study, the risk of being infected with ISA virus due to proximity to an infectious site, i.e. a farm with an outbreak was estimated (Scheel et al., submitted). Cases in this model were outbreaks from 2000-2005 and were related to contact networks such as for instance ownership, distance and exchange of equipment. The model estimated that **the reservoir for ISA virus causing approximately 30 % of ISA outbreaks in Norway is other farms in the proximity and a network with recorded ISA disease.** The remaining 70 % could not be explained by the model.

The remaining 70 % of outbreaks could not be explained in the model and thus may be explained by other reservoirs:

- 1) It could be that assumed virulent ISA virus, i.e. for the sake of simplicity in this context assumed to be non-HPR0s, are circulating in the industry, but are not regularly detected. This assumption implies that these ISA viruses do not always cause overt disease, or cause a slowly developing disease. Disease outbreaks are dependent upon factors such as management procedures and host properties, which are common for virus infections in general. It could be argued that there is a limited number of non-HPR0s that can originate from other non-HPR0, so if non-HPR0s are prevalent they need a regular supply of HPR0. The spread of these potentially virulent viruses can be assumed to be restricted to the marine stage, since there have been no outbreaks in the hatcheries from the year 2000 and onwards. The spread in the marine stage that are not related to farms with outbreaks in the vicinity, may for instance be through well boats, as documented to be involved in transmission in the outbreak in Scotland. Two independent screenings of smolt production sites in Norway indicated that a high and a moderate number of these sites were positive for ISA virus, respectively (Nylund et al., 2007, H. Sindre and B. Dannevig personal communications). **Smolt as reservoirs are therefore documented, but the relative importance is unknown.**
- 2) ISA virus could be transmitted from wild fish to farmed salmon. The detection of ISA virus in relatively high frequency in wild brown trout, as has been cited above, is supportive of this theory. The findings of HPR0 in Scotland in wild fish, but the current absence of ISA disease in that country could be explained by a low frequency of conversion from HPR0 to other HPRs. The mechanisms generating virulent virus remain unknown. It cannot be ruled out that wild salmonids, (or possibly hitherto not detected reservoir in naturally-occurring marine species) more or less regularly furnish farmed salmon with ISA virus. As mentioned earlier, ISA virus occurring in farmed

fish must originally have been transmitted from wild fish. **This reservoir is therefore present; however, the relative importance is unknown.**

- 3) A third possibility is that avirulent ISA virus is prevalent in farmed salmon. This virus then passes through the different life stages and may cause disease, preferably in the marine stage. Furthermore, that this transmission causes an “infectious circle” that does not need input of virus from wild fish, but is sustained as consequence of the structure and flow of biological material in the industry. The probability that ISA virus is transmitted vertically, and hygienic barriers that have been introduced to minimise vertical transmission are dealt with under Question 1. The difference in presence and prevalence of ISA disease in different salmon farming countries is difficult to reconcile with an “all industry caused recycling of avirulent virus” theory. However, Nylund et al. (2007) found 22/24 smolt production sites to be positive using real-time RT-PCR, when testing between 30-60 individuals per site, and Sindre and Dannevig (personal communications) found 2/27 smolt production sites to be positive and 1/27 possibly positive following testing of 60 individual fish per site. Two of the HE genes from the study by Nylund et al. (2007) were sequenced and one was found to be HPR0. The study indicates that ISA virus is prevalent in farmed salmon, but the number of HPRs found so far is too small to draw conclusions.

The *ad hoc* group does not have enough information to rate the importance of farmed salmon as reservoir of avirulent virus, nor to distinguish the importance of a farmed salmon reservoir from the relative importance of wild fish as reservoir.

QUESTION 6

IS SCREENING FOR ISA VIRUS IN APPARENTLY HEALTHY FISH APPROPRIATE FOR LIMITING ISA DISTRIBUTION?

In the answer to question 6, the *ad hoc* group did not achieve consensus. The alternative conclusion is presented beneath the conclusion from the majority of the group.

The wording of the question implies that the purpose of the screening is to detect subclinically infected fish. The results from the screening may be used to remove or isolate positive fish or populations with the goal to decrease the infection pressure downstream in the production cycle. Evaluation of possible routine screening for ISA virus in dead or diseased fish is not considered to be a part of the question.

Any screening of healthy fish should have a long-term goal, i.e. this can be the control of the disease, a decrease in numbers of disease outbreaks, or “freedom of infection” for particular populations of fish and others. The *ad hoc* group has defined decrease in numbers of disease outbreaks as the most likely goal to achieve. Screening may be used in a national context or in a local context i.e. within a combat zone after an ISA outbreak.

Screening for ISA virus in healthy fish could be used as a supplement to other methods, or as a replacement for other methods. The suitability of screening thus has to be compared to other optional interventions. These other forms of intervention include a problem-oriented approach focusing on mortality control and disease diagnostics.

Screening should have a positive cost-benefit outcome. Presently, the cost-benefit outcome of screening healthy fish population is difficult to predict and will depend on virus prevalence in subclinically infected fish and the regulatory consequences of detection.

Method

The *ad hoc* group regards real-time RT-PCR to be the method of choice in screening for the presence of ISA virus RNA. In general, real-time RT-PCR is well suited to mass screening, since it is fast and has the capacity to detect small amounts of viral RNA even in subclinically infected individuals. However, it should be noted that a positive test does not necessarily indicate that the fish is actively shedding virus or that the virus is virulent.

The *ad hoc* group considers that if a real-time RT-PCR test uses a cut off where the tested samples have a high concurrence of similar results in repetitive testing, then positive results should be considered to indicate the presence of infectious virus.

Diagnostic tests need to be properly validated. In particular, performance and operating characteristics in field situations are important when healthy populations with expected low prevalence are tested. In this situation, false positive tests may have importance as an infection-free population can be classified as infected. Verification of such test results must be performed by another, independent test. In essence, validation determines whether a true finding in the laboratory is also true in the field. This requirement is not encountered in the initial optimization of the tests in laboratory simulations. General and special guidelines for validation are given in OIE’s Manual of diagnostic tests for aquatic animals 2006 (http://www.oie.int/eng/normes/fmanual/A_summry.htm) and includes both test optimization and documentation of performance characteristics. All screening tests that are used should be standardized, and quality control procedures should be implemented before they are used as a

diagnostic test for prevention and control of ISA (Nerette et al., 2005, a, b). Cut-off values must be determined for tests with quantitative output and the analytical sensitivity and specificity of the test should be known. A test that is used for testing of healthy fish to make regulatory decisions should also be able to distinguish between virulent and avirulent forms of the virus. However, today this requirement is restricted to determining whether the virus has HPR0 or not. The eventual pitfalls using HPR as a virulence marker are discussed under Question 4.

The most important consideration for test validation is to measure the capacity of a positive or negative test result to predict accurately the infection status of the fish or fish population. This capacity is not only dependent on a highly sensitive, precise and accurate assay, but also on carefully derived estimates of diagnostic sensitivity and specificity. These parameters are cumbersome and difficult to determine. Together with the infection prevalence in the population, they determine the probability that a given test result reflects the true status of the fish (Dohoo et al., 2003).

If a test is to be used to demonstrate the absence of ISA virus (“freedom of infection”), as opposed to the absence of positive responders, an increased level of documentation of the method as well as evaluation of the sampling programme should be required. This consideration is also discussed in a separate chapter in the OIE manual. Preferably, a reliable estimate of the prevalence of the ISA virus in a population that is tested should be available to assist in validating the method, which can be anticipated to be difficult to obtain. If screening is used to postulate freedom of infection in a fish population, this postulate must be related to the sensitivity of the test and the sample analysed in relation to the population in question (representability). Knowledge on the potential uneven distribution of infected fish between cages/tanks or groups of fish on a site are important if the population in question and the validity of the result are to be defined.

Target populations

There are many possible stages in the production cycle that are possible targets. When evaluating the ability of targets, one should consider not only the target itself, but also the ability to intervene after a positive finding.

Testing of broodfish, eggs and ovarian fluid, smolt before seawater transfer, fish in farms within ISA combat zones as part of surveillance, and others are all targets that may be considered.

Broodfish

If screening is to be used then the *ad hoc* group is of the opinion that broodfish and fish ready for slaughter represent suitable targets in the production cycle. The purpose of screening broodfish would be to remove positive fish or their sexual products to block potential vertical transmission of the virus. This is possible for the “breeding nucleus” part of the brood stock, i.e. the origin of eggs for the next generation of broodfish. This population should be kept, for each generation, at separate locations at sea. After spawning, the eggs from the breeding nucleus are kept in separate cylinders, one for each family. This means that progeny from individual parents that have tested positive can be discarded. The number of brood fish in the breeding nucleus is limited.

There should be a close disease surveillance of the broodfish. All fish suspected of possessing clinical or pathological changes caused by ISA should be discarded before stripping. These changes should be looked for in broodfish by clinical/pathological examination.

Screening of broodfish in this context thus means screening of healthy, possibly subclinically infected individuals. One should be aware of that there is limited information that such fish may transmit virus to its offspring. In the answer to Question 1, the probability of spread of the disease as a result of vertical transmission was assessed. In Canada and Faeroe Islands, vertical transmission of ISA has not been observed. In Norway since 2000, approximately 1.4 billion eggs have been produced for the Norwegian market. There have been no reports of ISA outbreaks in hatcheries in this period. A quantitative assessment of the effect of screening of broodfish on distribution of ISA disease in early life stages is therefore not possible. Whether possible vertical transmission may be of importance for ISA outbreaks in the seawater stage is discussed under Question 1.

There are other possible measures to stop vertical transmission besides testing and culling, such as swelling of fertilized eggs in disinfecting iodophores, if this is compatible with egg fertility. Vaccination of fish that will be used as broodfish may also be a possible positive intervention.

The heart and kidney are the preferred organs when testing for ISA virus in general. However, the use of ovarian fluid as a better choice of sample for such a test should be investigated. The presence of virus in ovarian fluid is also a stronger indication for the possibility of vertical transmission.

Slaughter-ready fish

Testing of fish that are ready for slaughter would make it possible to require special biosecurity measures when transporting fish with positive testing results, i.e. as is required for sanitary slaughtering after ISA outbreaks today. It would also bring attention to the disinfection process of well boats after transportation of known ISA virus infected fish. Screening of this population and the subsequent outlined intervention may thus reduce the risk of horizontal spread of ISA virus.

Biosecurity

If screening of broodfish is used for the purpose of blocking vertical transmission, it will require that the possibility for horizontal transmission during stripping and subsequent processes is strictly minimised. Any contamination of eggs with ISA virus through factors such as handling of virus positive fish or equipment would be detrimental to the purpose of the screening. This possible source of contamination implies that any screening must be accompanied by strict biosecurity measures. If the observed vertical transmission does not come from the parents, but is a consequence of accidental contamination, then the accurate testing of individual fish may end up being a waste of resources.

Conclusions

The best-suited targets for screening for the presence of ISA virus infection in apparently healthy fish is considered to be **broodfish** and **slaughter ready fish** by use of real-time RT-PCR. A quantitative assessment of the effect of screening on ISA and subsequent culling of positive broodfish or intervention in transport of slaughter ready fish is not possible to make.

However, the cost of screening and the reduced benefit if cross contamination is prevalent will stimulate the use of strict biosecurity measures with positive implications for other infectious agents that are also considered to transmit vertically.

If a positive post-screening effect on the number of ISA outbreaks is observed, it would thus be difficult to credit this to screening only.

The majority of the *ad hoc* group therefore recommends that screening of broodfish in the “breeding nucleus” combined with strict biosecurity procedures for handling of broodfish and sexual products is encouraged, and similarly that testing of slaughter-ready fish is encouraged. A cost-benefit analysis should be performed after a sufficient time period has elapsed. Since the eventual effect of the proposed screening on ISA outbreaks is currently not possible to assess, screening should be **voluntary**.

Dissent:

Knut Falk could not support the conclusion recommending screening of healthy fish in particular broodfish, by RT-PCR at present due to the following reasons:

- The strategy in Norway has been to control the occurrence of ISA disease through general and specific regulations based on detection of clinical ISA. This strategy has been successful, and a shift of strategy to detect the viral infection in apparently healthy fish has not been evaluated with respect either to benefit or to cost for the industry and the authorities. In general, focusing on laboratory tests on healthy animals is traditionally mainly used when eradication of an exotic infectious agent is the goal.
- Real time RT-PCR for detection of ISA virus is a powerful diagnostic tool. However, the method has so far, not been properly validated for screening of healthy animals. Also, current knowledge does not allow us to distinguish reactions caused by virulent virus and avirulent virus.
- Though it is concluded in Question 1 that vertical transmission of ISA virus cannot be excluded, especially when the broodfish are suffering from clinical ISA, there are no data indicating that eliminating such possibly low level vertical transmission will result in fewer actual ISA disease outbreaks.
- There is no documentation indicating that testing and removal of test-positive broodfish will result in fewer ISA disease outbreaks in the seawater phase.

In conclusion, screening should not, in the opinion of Knut Falk, be applied unless it is known to be cost-efficient with regard to disease control or eradication. Presently, both central test parameters and the beneficial effect of removing broodfish that are positive by real-time RT-PCR are unknown. A recommendation of further, controlled research into these subjects can be supported, but not the practical application of a test and removal procedure in broodfish populations as a measure against ISA.

LACK OF KNOWLEDGE

- The *ad hoc* group believes that the limited knowledge of the prevalence of ISA virus infection in fish in different life stages implies that some of our assessments might not be based on solid evidence. The group recommends that such information should be gathered and be made available to the Food Safety Authority.
- There is a lack of epidemiological knowledge on possible changes for the risk of spread of contagious infections caused by the structural changes that have occurred in the Atlantic salmon farming industry.
- There is a lack of information regarding a scientific basis for differentiation between avirulent and virulent variants of ISA virus. This may be of importance when testing for the presence of ISA virus in healthy fish. The frequency of conversion between HPR0 and other HPRs is also unknown. This parameter could be important information for a scientific approach to handle findings of HPR0.

ASSESSED BY

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Dr. Are Nylund was a member of the *ad hoc* group until 5th of January 2007. He contributed to the report during the time when the report was produced, but withdrew from the group in the closing phase.

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REFERENCE

- Anonymous. 2000.** Final Report of the Joint Government/industry. Working Group on Infectious Salmon Anaemia (ISA) in Scotland. Published by Scottish Executive.
- Anonymous. 2005.** Epizootiological Investigation into a case of suspicion of infectious salmon anaemia (ISA) in Scotland in November 2004. Report by FRS Marine Laboratory, Aberdeen AB11 9DB.
- Anonymous. 2006.** Infectious salmon anaemia, p. 186-201. In - Anonymous (ed.), OIE Manual of Diagnostic Tests for Aquatic Animals 2006. Office International des Epizooties, Paris (http://www.oie.int/eng/normes/fmanual/A_00026.htm).
- Antec International Ltd** (2003) Vicon S efficacy against specific fish pathogens. <http://www.antecint.co.uk/main/virkaqua.htm>.
- Aspehaug, V., K. Falk, B. Krossoy, J. Thevarajan, L. Sanders, L. Moore, C. Endresen and E. Biering.** 2004. Infectious salmon anemia virus (ISAV) genomic segment 3 encodes the viral nucleoprotein (NP), an RNA-binding protein with two monopartite nuclear localization signals (NLS). *Virus Res.* **106**:51-60.
- Aspehaug, V., A. B. Mikalsen, M. Snow, E. Biering and S. Villoing.** 2005. Characterization of the infectious salmon anemia virus fusion protein. *J. Virol.* **79**:12544-12553.
- Biering, E., K. Falk, E. Hoel, J. Thevarajan, M. Joerink, A. Nylund, C. Endresen and B. Krossoy.** 2002. Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV); the co-linear transcript from Segment 7 probably encodes a non-structural or minor structural protein. *Dis. Aquat. Org.* **49**:117-122.
- Bovo, G., B. Hill, A. Husby, T. Håstein, C. Michel, N. J. Olesen, A. Storset and P. J. Midtlyng.** 2005. Report. European Commission QLK2-CT-2002-01546: Fish Egg Trade
- Christiansen, D.H., and P. Østergård.** 2006. Vaccination against ISA-virus infection in Atlantic salmon on the Faroe Islands. 10th Annual Meeting of EU National Reference Laboratories for Fish Disease, Copenhagen, Denmark, May 22-24, 2006.
- Clouthier, S. C., T. Rector, N. E. Brown and E. D. Anderson.** 2002. Genomic organization of infectious salmon anaemia virus. *J. Gen. Virol.* **83**:421-428.
- Cook-Versloot, M., S. Griffiths, R. Cusack, S. McGeachy and R. Ritchie.** 2004. Identification and characterisation of infectious salmon anaemia virus (ISAV) haemagglutinin gene highly polymorphic region (HPS) type 0 in North America. *Bull. Eur. Assoc. Fish Pathol.* **24**:203-208.
- Cunningham, C.O. and M. Snow.** 2000. Genetic analysis of infectious salmon anaemia virus (ISAV) from Scotland. *Dis. Aquat. Org.* **41**:1-8.
- Cunningham, C. O., A. Gregory, J. Black, I. Simpson and R. S. Raynard.** 2002. A novel variant of the infectious salmon anemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. *Bull. Eur. Assoc. Fish Pathol.* **22**:366-374.
- Dannevig, B. H., K. Falk and E. Skjerve.** 1994. Infectivity of internal tissues of Atlantic salmon, *Salmo salar* L., Experimentally Infected with the etiologic agent of infectious salmon anemia (ISA). *J. Fish Dis.* **17**:6: 613-22.
- Dannevig, B. H., K. Falk and E. Namork.** 1995. Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *J. Gen. Virol.* **76**:1353-1359.
- Devold, M., B. Krossøy, V. Aspehaug and A. Nylund.** 2000. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. *Dis. Aquat. Org.* **40**: 9-18.
- Devold, M., K. Falk, B. Dale, B. Krossoy, E. Biering, V. Aspehaug, F. Nilsen and A. Nylund.** 2001. Strain variation, based on the hemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. *Dis. Aquat. Org.* **47**:119-128.
- Devold, M., M. Karlsen and A. Nylund.** 2006. Sequence analysis of the fusion protein gene from infectious salmon anemia virus isolates: evidence of recombination and reassortment. *J. Gen. Virol.* **87**:2031-2040.
- Dohoo, I., W. Martin and H. Stryhn.** 2003. Veterinary Epidemiologic Research. AVC Inc., Charlottetown, Canada
- Einer-Jensen, K., P. Ahrens, R. Forsber and N. Lorenzen.** 2004. Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *J. Gen. Virol.* **85**:1167-1179.

- Falk, K., E. Namork, E. Rimstad, S. Mjaaland and B.H. Dannevig.** 1997. Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *J. Virol.* **71**:9016-9023.
- Falk, K., V. Aspehaug, R. Vlasak and C. Endresen.** 2004. Identification and characterization of viral structural proteins of infectious salmon anemia virus. *J. Virol.* **78**:3063-3071.
- Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger and L. A. Ball.** 2005. *Virus Taxonomy*, 8th Report of the ICTV, p. 681-693. Amsterdam, Elsevier Academic Press.
- Garcia-Rosado, E., T. Markussen, S. Mjaaland, E. Bækkevold and E. Rimstad.** Functional characterization of infectious salmon anemia virus genomic segment 7 un-spliced mRNA and the segment 8 ORF2 proteins: two putative interferon antagonists (submitted).
- Gjøen, H. M., T. Refstie, O. Ulla and B. Gjerde.** 1997. Genetic correlations between survival of Atlantic salmon in challenge and field tests. *Aquac.* **158**: 277-288.
- Glover, K. A., C. Skår, K. E. Christie, J. Glette, H. Rudra and Ø. Skaala.** 2006. Size-dependent susceptibility to infectious salmon anemia virus (ISAV) in Atlantic salmon (*Salmo salar* L.) of farmed, hybrid and wild parentage. *Aquaculture*; **254**:82-91.
- Gustafson, L.L., S.K. Ellis and C. A. Bartlett.** 2005. Using expert opinion to identify risk factors important to infectious salmon-anemia (ISA) outbreaks on salmon farms in Maine, USA and New Brunswick, Canada. *Prev. Vet. med.* **70**:17-28.
- Hanada, K., Y. Suzuki and T. Gojobori.** 2004. A large variation in the rates of synonymous substitution for RNA viruses and its relationship to diversity of viral infection and transmission modes. *Mol. Biol. Evol.* **21**:1074–1080.
- Hellberg, H., E. M., C. Hoel, M. Skår, B. Myrmel, B. Dannevig and S. Mortensen.** 2005. Bivalves as possible vectors for fish pathogens: mussels (*Mytilus edulis* L.) and infectious salmon anaemia virus (ISAV). 12th International Conference of the EAAP- Diseases of Fish and Shellfish, September 11-16, Copenhagen. (Poster)
- Jarp, J.** 1999. Epidemiological aspects of viral diseases in the Norwegian farmed Atlantic salmon (*Salmo salar* L.) *Bull Eur. Ass. Fish Pathol* **19**: 240-244.
- Jenkins, G. M., A. Rambaut, O. G. Pybus and E. C. Holmes.** 2002. Rates of molecular evolution in RNA viruses: A quantitative Phylogenetic analysis. *J. Mol. Evol.* **54**:156–165.
- Joseph, T., A. Cepica, L. Brown, B.O. Ikede and F.S.B Kibenge.** 2004. Mechanism of cell death during infectious salmon anemia virus infection is cell type-specific. *J. Gen. Virol.* **85**:3027-3036.
- Kibenge, F. S. B., O. N. Garate, G. Johnson, R. Arriagada, M. J. T. Kibenge and D. Wadowska.** 2001a. Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. *Dis. Aquat. Org.* **45**:9–18.
- Kibenge, F. S., M. J. Kibenge, P. K. McKenna, P. Stothard, R. Marshall, R. R. Cusack and S. McGeachy.** 2001b. Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene. *J. Gen. Virol.* **82**:2869-2879.
- Kibenge, M.T., B. Opazo, A. H. Rojas and F. S. B Kibenge.** 2002. Serological evidence of infectious salmon anemia virus (ISAV) infection in farmed fish, using an indirect enzyme-linked immunosorbent assay (ELISA). *Dis. Aquat. Organ.* **51**: 1-11.
- Kibenge, F. S. B., M. J. T. Kibenge, D. Groman and S. McGeachy.** 2006a. In vivo correlates of infectious salmon anemia virus pathogenesis in fish. *J. Gen. Virol.* **87**:2645-2652.
- Kibenge, F. S. B., M. J. T Kibenge, Y. Wang, B. Qian, S. Hariharan and S. McGeachy.** 2006b. Correlates of virulence of infectious salmon anaemia virus. Poster on OIE Global Conference on Aquatic Animal Health in Bergen, Norway, 9-12 October 2006.
- Krogsrud, J., K. Falk, B. H. Dannevig and O. B. Reite.** 1991. Inaktivering av ILA-agens. Forskningsprogrammet Frisk Fisk, årsmøteseminar, Gol.
- Krossoy, B., I. Hordvik, F. Nilsen, A. Nylund and C. Endresen.** 1999. The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. *J. Virol.* **73**:2136-2142.

- Krossøy, B., F. Nilsen, K. Falk, C. Endresen and A. Nylund.** 2001a. Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. *Dis Aquat. Org.* **44**:1–6.
- Krossoy, B., M. Devold, L. Sanders, P. M. Knappskog, V. Aspehaug, K. Falk, A. Nylund, S. Koumans, C. Endresen and E. Biering.** 2001b. Cloning and identification of the infectious salmon anaemia virus haemagglutinin. *J. Gen. Virol.* **82**:1757-1765.
- Kurath, G., K. A. Garver, R. M. Troyer, E. J. Emmenegger, K. Einer-Jensen and E. D. Anderson.** 2003. Phylogeography of infectious haematopoietic necrosis virus in North America. *J. Gen. Virol.* **84**:803–814.
- Lyngstad, T., E. Brun, P.A. Jansen and B.T. Heier.** 2005. Summary of epidemiological reports regarding outbreaks of ISA in Norway 2004. Report to Food Safety Authority from National Veterinary Institute, Oslo, Norway.
- MacLeod, L. A., R. S. Raynard, A. G. Murray and A. Gregory.** 2003. Survival of infectious salmon anaemia virus (ISAV) in aquatic environments. Abstract from 11th EAAP-meeting, Malta, 2003.
- McBeath, A. J., B. Collet, R. Paley, S. Duraffour, V. Aspehaug, E. Biering, C. J. Secombes and M. Snow.** 2006. Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. *Virus Res.* **115**:176-184.
- McClure CA, K.L. Hammell, I.R. Dohoo and Gagne N.** 2004. Lack of evidence of infectious salmon anemia virus in pollock *Pollachius virens* cohabitating with infected farmed Atlantic salmon *Salmo salar*. *Dis Aquat Organ.* **61**:149-52.
- McClure, C. A., K. L. Hammel and I. R. Dohoo.** 2005. Risk factors for outbreaks of infectious salmon anemia in farmed Atlantic salmon, *Salmo salar*. *Prev. Vet. Med.* **72**: 263-280.
- Melville, K. J. and S. G. Griffiths.** 1999. Absence of vertical transmission of infectious salmon anemia virus (ISAV) from individually infected Atlantic salmon *Salmo salar*. *Dis.Aquat.Org.* **38**: 231-234.
- Mikalsen A. B., A. Teig, A. L. Helleman, S. Mjaaland, and E. Rimstad.** 2001. Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon *Salmo salar*. *Dis. Aquat. Organ.* **47**:175-181.
- Mjaaland, S., E. Rimstad, K. Falk and B. H. Dannevig.** 1997. Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. *J. Virol.* **71**:7681-7686.
- Mjaaland, S., O. Hungnes, A. Teig, B. H. Dannevig, K. Thorud and E. Rimstad.** 2002. Polymorphism in the infectious salmon anemia virus hemagglutinin gene: importance and possible implications for evolution and ecology of infectious salmon anemia disease. *Virol.* **304**:379-391.
- Mjaaland, S., T. Markussen, H. Sindre, S. Kjøglum, B. H. Dannevig, S. Larsen and U. Grimholt.** 2005. Susceptibility and immune responses following experimental infection of MHC compatible Atlantic salmon (*Salmo salar* L.) with different infectious salmon anaemia virus isolates. *Arch. Virol.* **150**:2195-2216.
- Nerette, P., I. Dohoo, L. Hammel, N. Gagne, P. Barbash, S. MacLean and C. Yason.** 2005a. Estimation of the repeatability and reproducibility of three diagnostic tests for infectious salmon anaemia virus. *J. Fish Dis.* **28**:101-110.
- Nerette, P., I. Dohoo and L. Hammel.** 2005b. Estimation of specificity and sensitivity of three diagnostic tests for infectious salmon anaemia virus in the absence of a gold standard. *J. Fish Dis.* **28**:89-99.
- Nylund, A., C. Wallace and T. Hovland.** 1993. The possible role of *Lepeophtheirus salmonis* (Krøyer) in the transmission of infectious salmon anaemia virus (ISAV) in Atlantic salmon, *Salmo salar*. In: Pathogens of wild and farmed fish: sea lice. London: Ellis Horwood Ltd.: 367-373.
- Nylund, A., and P. Jacobsen.** 1995. Sea trout as a carrier of infectious salmon anemia virus. *J. Fish Biol.* **47**:174-176.
- Nylund, A., T. Hovland, K. Watanabe and C. Endresen.** 1995a. Presence of infectious salmon anaemia virus (ISAV) in different organs of *Salmo salar* L. collected from three fish farms. *J. Fish Dis.* **18**:135-145.
- Nylund, A., A. M. Kvenseth and B. Krossøy.** 1995b. Susceptibility of wild salmon (*Salmo salar* L.) to infectious salmon anaemia (ISA). *Bull. Eur. Assoc. Fish Pathol.* **15**:152-156.
- Nylund, A., M. Devold, J. Mullins and H. Plarre.** 2002. Herring (*Clupea harengus*): A host for infectious salmon anaemia virus (ISAV). *Bull. Eur. Assoc. Fish Pathol.* **22**:311-318.

- Nylund, A., M. Devold, H. Plarre, E. Isdal and M. Arseth.** 2003. Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: A new hypothesis. *Dis. Aquat. Org.* **56**:11–24.
- Nylund, A.** 2004. National ISA-seminar. For interested parties. Bergen, Norway. Oral presentation published on VI's web-sites. October 2004.
- Nylund, A., H. Plarre, M. Karlsen, F. Fridell, K. F. Ottem, A. Bratland and P.A. Sæther.** 2007. Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). *Arch. Virol.* **152**:151-179.
- OIE Manual of Diagnostic Tests for Aquatic Animals.** 2003. Fourth Edition Office. International des Epizooties, Paris, France.
- Oye, A. K, and E. Rimstad.** 2001. Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC irradiation. *Dis. Aquat. Organ.* **48**:1-5.
- Plarre, H., M. Devold, M. Snow and A. Nylund.** 2005. Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Dis. Aquat. Org.* **66**:71-79.
- Raynard, R. S., A. G. Murray and A. Gregory.** 2001a. Infectious salmon anaemia virus in wild fish from Scotland. *Dis. Aquat. Org.* **46**:93-100.
- Raynard, R. S., M. Snow and D. W. Bruno.** 2001b. Experimental infection models and susceptibility of Atlantic salmon *Salmo salar* to a Scottish isolate of infectious salmon anaemia virus. *Dis. Aquat. Org.* **47**:169-74.
- Rimstad E., K. Falk, A.B. Mikalsen, A. Teig.** 1999. Time course tissue distribution of infectious salmon anaemia virus in experimentally infected Atlantic salmon *Salmo salar*. *Dis Aquat Organ.* **36**:107-12.
- Rimstad, E., S. Mjaaland, M. Snow, A. B. Mikalsen, and C. O. Cunningham.** 2001. Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin. *J. Virol.* **75**:5352-5356.
- Rimstad, E. and S. Mjaaland.** 2002: Infectious salmon anaemia. An orthomyxovirus causing an emerging infection in Atlantic salmon. *Acta Pathologica, Microbiologica et Immunologica Scandinavica (APMIS)* **110**: 273-282. Invited review
- Rodger, H. D., T. Turnbull, F. Muir, S. Millar and R. H. Richards.** 1998. Infectious salmon anaemia (ISA) in the United Kingdom. *Bull. Eur. Ass. Fish Pathol.* **18**: 115 - 116.
- Rolland, J. B. and A. Nylund.** 1998a. Sea running brown trout: carrier and transmitter of the infectious salmon anemia virus (ISAV). *Bulletin of the European Association of Fish Pathologists.* **18**:173-180.
- Rolland, J. B. and A. Nylund.** 1998b. Infectiousness of organic materials originating in ISA-infected fish and transmission of the disease via salmon lice (*Lepeophtheirus salmonis*). *Bull. Eor. Assoc. Fish Pathol.* **18**:173-180.
- Rolland, J. B., and J. R. Winton.** 2003. Relative resistance of Pacific salmon to infectious salmon anaemia virus. *J. Fish Dis.* **26**:511-520.
- Rolland, J. B.** 2004. Studies of factors affecting the epizootiology of infectious salmon anemia (ISA). Thesis. Dr. Scient. University of Bergen.
- Rolland, J. B., D. Bouchard, J. Coll and J. R. Winton.** 2005. Combined use of the ASK and SHK-1 cell lines to enhance the detection of infectious salmon anemia virus. *J. Vet. Diagn. Invest.* **17**:151–157.
- Royal Society of Edinburgh.** 2002. The scientific issues surrounding the control of infectious salmon anaemia (ISA) in Scotland. A report of the Royal Society of Edinburgh Working Party on Infectious Salmon Anaemia. Edinburgh: The Royal Society of Edinburgh. 13p.
- Scheel, I., M. Aldrin, A. Frigessi and P. Jansen.** Factors determining the spread of infectious salmon anemia (ISA) in Atlantic salmon farming. A stochastic space time model (manuscript).
- Smail, D. A., R. Grant, D. Simpson, N. Bain and T. S. Hastings.** 2004. Disinfectants against cultured Infectious Salmon Anaemia (ISA) virus: the virucidal effect of three iodophors, chloramine T, chlorine dioxide and peracetic acid/hydrogen peroxide/acetic acid mixture. *Aquaculture* **240.1-4**: 29-38.
- Snow, M., R. S. Raynard and D. W. Bruno.** 2001. Comparative susceptibility of Arctic char (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to the Scottish isolate of infectious salmon anaemia virus. *Aquacult.* **196**:47-54.

- Snow, M., and C. O. Cunningham.** 2001. Characterisation of the putative nucleoprotein gene of infectious salmon anaemia virus (ISAV). *Virus Res.* **74**:111-118.
- Snow M., R. Raynard, D.W Bruno, A. P. Van Nieuwstadt, N. J. Olesen, T. Lovold and C. Wallace.** 2002. Investigation into the susceptibility of saithe *Pollachius virens* to infectious salmon anaemia virus (ISAV) and their potential role as a vector for viral transmission. *Dis. Aquat. Org.* **50**, 13–18.
- Snow, M., R. Ritchie, O. Arnaud, S. Villoing, V. Aspehaug and C. O. Cunningham.** 2003. Isolation and characterisation of segment 1 of the infectious salmon anaemia virus genome. *Virus Res.* **92**:99-105.
- Snow, M., N. Bain, J. Black, V. Taupin, C. O. Cunningham, J. A. King, H. F. Skall and R. S. Raynard.** 2004. Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Dis. Aquat. Org.* **61**:11–21.
- Snow, M., and R. S. Raynard.** 2005. An investigation into the susceptibility of Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*) to infectious salmon anaemia virus (ISAV). *Bull. Eur. Assoc. Fish Pathol.* **25**:189:195.
- Stagg, R. M., D. W. Bruno, C.O. Cunningham, R. S. Raynard, P. D. Munro, A. G. Murray, C.E.T. Allan, D.A. Smail, A. H. McVicar and T. S. Hastings.** 2001. Epizootiological investigations into an outbreak of infectious salmon anaemia (ISA) in Scotland. Report No. 13/01, FRS Marine Laboratory, Aberdeen.
- Stagg, R. M.** 2003. The eradication of clinical infectious salmon anaemia from Scotland. In: Miller O, Cipriano RC (eds) *International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication: Proceedings of a Symposium 3-4 September 2002.* Department of Agriculture, Animal and Plant Health Inspection Service, New Orleans, LA.
- Stallknecht D.E., M. T. Kearney, S. M. Shane and P. J. Zwank.** 1990a. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. *Avian Dis.* **34**:412-8.
- Stallknecht D.E., S. M. Shane, M.T. Kearney and P. J. Zwank.** 1990b. Persistence of avian influenza viruses in water. *Avian Dis.* **34**:406-11.
- Søfteland and editors:** "Feltforsøk med ILA-infisert stamfisk av laks- er det mulig å overføre ILA-virus via rogn og melke?". Report October 2005. Mattilsynet, Universitetet i Bergen, Akvaforsk, Fomas.
- Thierry R., J. Cozien, C. de Boisseson, S. Kerbart-Boscher and L. Nevarez.** 2004. Genomic classification of new betanodavirus isolates by phylogenetic analysis of the coat protein gene suggests a low host-fish species specificity. *J. Gen. Virol.* **85** (Pt 10):3079-3087.
- Thorud, K. E. and H. O. Djupvik.** 1988. Infectious anaemia in Atlantic salmon (*Salmo salar* L.). *Bull. Eur. Assoc. Fish Pathol.* **8**:109-111.
- Thorud, K. and Y. Torgersen.** 1994. Havforsknytt, nr 22-1993.
- Thorud, K., and T. Håstein.** 2002. Experience with Regulatory Responses to Unfectious Salmon Anemia in Norway. In *International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication.* Proceedeening of a symposium 3.-4. September 2002; New Orleans. Eds: O Miller, R C Cipriano.
- Torgersen, Y.** 1997. Physical and chemical inactivation of the infectious salmon anaemia (ISA) virus. Workshop on ISA, St. Andrews, New Brunswick, 26. November 1997.
- Vågsholm, I., H.O. Djupvik, F. V. Willumsen, A. M. Tveit and K. Tangen.** 1994. Infectious salmon anaemia (ISA) epidemiology in Norway. *Prev. Vet. Med.* **19**: 277-290.
- www.laksefakta.no
- www.mattilsynet.no
- www.oie/int/eng/normes/fmanual/A_summry.htm
- www.sernapesca.cl
- www.sevareid.no/avl,rogn.htm
- www.thechronicleherald.ca/NovaScotia/550550.html
- www.ukas.com

APPENDIX I.

REGIMES FOR ISA DISEASE CONTROL IN SCOTLAND, CANADA AND FAEROE ISLAND

Combating and Control of ISA in Scotland

ISA is a List I disease in the European Union and is managed under EU directives, which require that management of ISA in Scotland be based on three elements:

- Passive surveillance to identify the first occurrence of the disease
- Application of biosecurity measures to prevent spread
- Elimination of the source of infection.

Surveillance

Since 1991, ISA has been notifiable to the Official Service in the UK, which requires action following suspicion or confirmation of ISA. In Scotland, farms were confirmed as having ISA if fish showed clinical disease characteristic of ISA and there was evidence of infection with ISA virus by culture, RT-PCR or IFAT. The first case of ISA was reported in Scotland in Loch Nevis in May 1998 (Rodger et al., 1998) with a further 10 cases being subsequently confirmed (Stagg et al., 2001). The incidence of new cases peaked in April 1998 with the last case being confirmed in May 1999. During the investigation, 25 farms held fish suspected of being infected with ISA virus but the disease did not progress sufficiently to allow confirmation.

Application of biosecurity measures to prevent spread

From the outset, the objective of the control regime was to contain and eradicate the disease swiftly, since initial evidence revealed that the epidemic arose from a single primary site located in Loch Nevis (for reviews see Stagg et al., 2001). In addition to complete and immediate depopulation of fish from confirmed farms, the following measures supported by legislation designed to implement the EC directive for control of fish disease were implemented:

- Prevention of movements of live fish
- Placing conditions on the movements of dead fish, personnel and material liable to transmit disease
- Fallowing and disinfecting farms to break the cycle of infection.

In support of these actions, a rational basis for defining the area of the coastal zone affected by the disease in addition to the fallowing period necessary to eradicate disease was scientifically determined.

Elimination of the source of infection

The average time for depopulation of infected farms during the Scottish epidemic was 21 days. Farmers were allowed to harvest fish not showing signs of clinical disease. Suspect cases were investigated until November 1999, after which an independent scientific panel of experts concluded that clinical cases of ISA had been eradicated from Scottish salmon farms

(Royal Society of Edinburgh, 2002). The rapid rate of depopulation is likely to have been an important factor in the successful eradication of ISA from Scottish farms compared to the situation in other countries where cages not suffering mortality or clinical disease have remained until harvest and epidemics have persisted (Stagg, 2003).

The source of ISA virus in Loch Nevis that was responsible for causing disease is unclear. Evidence suggests the disease was either imported from Norway or emerged from a possible subclinical infection among wild fish (Stagg et al., 2001). Indeed, evidence for infection without signs of disease has been identified in wild fish- often in areas remote from infected farms (Raynard et al., 2001a). This observation has included detection of a putative ancestral form of ISA virus (HPR0) in Scotland (Cunningham et al., 2002), which has also been detected in Norway, Canada and USA (Nylund et al., 2007) without association with clinical ISA. More recently in 2004, this variant was detected in the gills and salmon suffering unexplained mortality on a smolt farm in Scotland. Clinical signs of ISA were not apparent and the cause of disease was not attributable to the presence of virus (Anonymous, 2005). Fish were voluntarily culled and the site fallowed within 16 days of declaration of suspicion of ISA by the Official Service. ISA suspicion was lifted after an epizootiological investigation found no evidence for ISA in other freshwater or marine farm sites that had direct or indirect contact with the infected farm.

Measures used to reduce ISA infection in New Brunswick, Canada.

ISA virus is one of the viral pathogens having a severe economic impact on the aquaculture industry in the Northern Hemisphere, and is probably the most important viral pathogen to the Atlantic Canada and Maine salmon farming industry. Moreover, there is no treatment for ISA virus infection. Presently in both New Brunswick and Maine, the only way to minimize losses due to ISA has been by prophylactic surveillance in which the virus is detected in a cage well before the clinical disease occurs and infected cages are removed before the virus spreads to other cages at the same site or to adjacent sites. This is a very expensive control method since a cage can hold up to 25,000 fish at a time. Presently, there is only one commercially available licensed ISA vaccine used in USA and Canada. Although this vaccine preparation, which consists of inactivated virus, has shown some protection in freshwater laboratory trials, it has not produced the kind of protection desired in seawater challenges, with relative percent survival (RPS) ranging from 39-57 % in short- and long-term laboratory trials, respectively. Despite the use of vaccination since 2002 on all salmon placed in net pens at marine aquaculture sites, high levels of losses have continued, indicating that the vaccine is not fully protective against the virus. Moreover, there is no test to identify fish that have been immunized against the virus infection. Consequently, vaccination is incompatible with the currently used depopulation control methods.

The specific measures used by the Atlantic salmon industry in New Brunswick include:

1. ISA Surveillance Program (consists of monthly Veterinary visits; early detection and depopulation* – cage by cage basis; control zones).
2. *Threshold for depopulation (at infection level) is 2 separate tests (RT-PCR, IFAT, or virus isolation), 2 fish, 2 visits.

3. Increased biosecurity.
4. Control and treatment of processing waste.
5. Biosecurity audits of sites and harvest vessels.
6. Year class separation/single year class sites and bays.
7. Controls on wharf usage.
8. Controls on harvest vessel traffic/New Brunswick standards and certification.
9. Implementation of control and containment plans on sites.
10. Cleaning and disinfection.

At a recent ISA Research Strategy Workshop (in Saint John, New Brunswick, December 6-7, 2006) organized by the salmon industry and government departments, the Canadian Food Inspection Agency (CFIA) agreed to fund a study to determine whether ISA virus in New Brunswick can be eradicated or managed. CFIA is considering making ISA a reportable disease, which would mean that veterinarians, fish farmers and laboratories must inform the CFIA of any suspected outbreak, and if destruction of stock is ordered, then the fish farmers would receive compensation (<http://thechronicleherald.ca/NovaScotia/550550.html>).

Control of ISA in Faeroes

In the Faeroes, the ISA epidemic was considered to be present in more or less all marine farms. After a fallowing period, the farmers were permitted to restock the sea farms only if the fish had been vaccinated against ISA.

The Faeroes was the first European country where the vaccine was one of the measures used in the fight against the disease. The first vaccinated fish were reintroduced to the marine farms in March 2005 and ISA has not been observed in the Faeroes since then. The fish farms have been kept under surveillance for ISA-virus (ISAV?) by monthly sampling, and until spring 2006, two fish out of more than 4600 tested were found positive for ISAV by RT-PCR. Sequence analysis of segment 6 revealed a full-length HPR-region (HPR0) of both virus samples (Christiansen and Østergård, 2006).

ANALYTICAL VALIDATION AND USE OF REAL-TIME PCR ASSAYS FOR DETECTION OF FISH PATHOGENS; SCOTTISH GUIDELINES

Molecular diagnostics within Fisheries Research Services (FRS), Scotland is conducted under the auspices of the United Kingdom Accreditation Service (www.ukas.com) within the framework of ISO/IEC 17025. FRS implements and maintains a management system capable of controlling a flexible scope of accreditation for the molecular diagnosis of fish pathogens using real-time PCR. In practice, this means that FRS can claim accreditation of any new test, which is developed, validated and implemented according to the accredited generic set of procedures. Analytical validation procedures were based on those recommended by the OIE and detailed in chapter 1.1.3 (Validation and quality control of PCR methods used for the diagnosis of infectious diseases) of the Manual of Diagnostic Tests for Aquatic animals (OIE, 2003). Internal controls are required (e.g. elongation factor, ELF) for all accredited assays, and the ELF ct-value obtained in Real-time RT-PCR must fall within a pre-defined range for the assay to be considered valid. Positive controls are not conducted routinely, though new batches of reagent are tested at regular intervals. Work is in progress to develop positive

controls, which can be distinguished from true positives and thus provide a more robust system. The principal steps of the assay validation system are fully reviewed and documented at each stage and may be summarized in Table 6.

Table 6. Analytical validation of Real-Time PCR assays

Steps	Actions
Sequence review	All available and relevant sequences are retrieved, aligned and reviewed prior to design of a new assay.
Primers and probe design	
Primer and probe specificity	BLAST searches based on primer/probe sequences
Determination of assay parameters (standard reaction conditions used)	
Initial feasibility study	Assay of at least 10 positive, 10 negative samples in varying concentrations and representing all strains assay designed to detect.
Evaluation of reaction efficiencies	Serial dilution experiment. Reaction efficiency must be in range -3.2-3.6
Repeatability	At least 10 reactions on same run (within ct range 15-35). Repeat three times and ensure variation less than 3 cycles for each sample.
Analytical sensitivity/ Assay range	Limit of detection determined by end point assay. The ct-value at which the assay can no longer detect the target in question in three replicates is determined. The average ct-value from the highest dilution generating a positive result in all replicates is defined as the assay limit of detection. In practice "positive" results above this threshold are reported as "suspect".
Analytical specificity	Test of assay using material from other members of the same pathogen group to ensure specificity to intended target

SOME ASPECTS ABOUT DIAGNOSTIC TESTS

Diagnostic tests are in general imperfect, i.e., the outcome is not always correct. It should therefore be realized that the classification into positives and negatives is imperfect. The term validity expresses to what degree the test measures what it is supposed to measure, and the terms sensitivity (Se) and specificity (Sp) are characteristics of the test and are tools used to judge this validity. Se is defined as the number of diseased animals classified by the test as positive/total number of positive animals, while Sp is the number of not-positive animals classified by the test as negative/total number of negative animals (Table 7).

Table 7. Table for evaluation of sensitivity (se) and specificity (sp)

	True positive	True negative	
Test positive	a	B	a+b
Test negative	c	D	c+d
	a+c	b+d	N

$$Se = a / a+c, Sp = d / b+d$$

Relation between diagnostic sensitivity, specificity, prevalence and predictive value

The measured prevalence (apparent prevalence (Ap)) in a population will depend on Se, Sp and the true prevalence (p) in the population.

The proportion of animals testing positive when they are truly positive is termed positive predictive value (PV+), $a/a+b$ from Table 7. The predictive value is a characteristic of the population as it depends on the prevalence in the population.

The relationship between prevalence and PV+ is shown in Table 8.

Table 8. Relationship between true prevalence and positive predictive value (PV+) using a test of Se=99 % and Sp=99.9 % in a population of 100 000

Prevalence (%)	True positives	False positives	Total positives	PV+ (%)
10	9900	90	9990	99
1	990	99	1089	91
0.1	99	100	199	50
0.01	10	100	110	9
0.001	1	100	101	1
0	0	100	100	0

PV+ will fall as the prevalence decreases when using the same test due to the lower number of positives (as p decreases) while false positives stay relatively constant. Further, when the prevalence is high, the number of false positives is relatively low compared with total positives, but accounts for most of the positives as the prevalence approaches zero. By using the same test in a control program, the number of true positives will gradually fall while false positives will stay constant and dominate the number of test positives in a low-prevalent sample. When low prevalence is expected, a test with high specificity should be used to exclude false positives– or a regime of re-testing of positives should be applied.

Testing at herd level

If individuals are tested to determine health status at an aggregated level (herd), herd sensitivity (HSE) and herd specificity (HSP) should be used. HSP decreases with increasing

numbers of tested animals within a herd whereas HSE increases. HSE also increases with increasing true prevalence while HSP is not influenced by prevalence (Table 9).

Table 9. Herd level specificity (HSP) and sensitivity (HSE) by true prevalence p given an individual animal test sensitivity of 95 % and a specificity of 99 %

Number tested in the herd	HSP ($p=10\%$, 30%)	HSE ($p=10\%$)	HSE ($p=30\%$)
1	99	10	28
2	98	20	48
5	95	42	81
10	90	66	96
30	74	96	100
60	55	100	100
100	37	100	100

Epidemiological sensitivity

The terms sensitivity and specificity are often used in connection with a specific laboratory test, and the probability for truly detecting an agent in a sample is a result of these characteristics. However, the probability that the sample tested actually contains the agent in question- given its presence in the fish is not included in the test characteristics. Therefore, the probability of detecting an agent in a fish may therefore be dependent on the probability of the agent's presence in test sample given its presence in the fish, and the test sensitivity. This may be described as the epidemiological sensitivity of the test system. Knowledge of prudent sampling for a specific agent will contain information on necessary sample size, the distributing pattern of positive fish in the population as such, distribution between cages/tanks, distribution between target organs and distribution within organs. This last aspect may be more critical the lesser the amount of the agents (RNA-fragments) a test may detect. This effect is well illustrated when using PCR- techniques to detect an agent in a possible carrier fish with no clinical disease. If the virus load in for instance the kidney from one fish is very low (as it may be in carriers), PCR may detect small amounts of RNA in one test but not necessarily in a parallel sample from the same kidney due to uneven distribution in the organ. This challenge is specifically noticeable in screening of non-diseased fish and underlines the need for validation of applied testing procedures.

Awareness of epidemiological sensitivity is of the up-most importance if conclusions are to be drawn on test results and the infectious status of a population.

Aetiological fractions (attributable proportion)

For control and management purposes, it is important to evaluate the magnitude of the different fractions of the total probability of obtaining a disease. Vertical transmission may be regarded as one part of the aetiological fractions contributing to the total probability of an ISA -outbreak.

CHEMICAL AND PHYSICAL CHARACTERISTICS OF ISA VIRUS

Table 10. Genomic segments and encoded proteins of ISA virus

Segment [kb]	Encoded protein	Protein [kDa]
1 [2,3]	Polymerase, PB2	80.5 ^a
2 [2,3]	Polymerase, PB1	79,5 ^a
3 [2,2]	Nucleoprotein, NP	66-74 ^b
4 [2,0]	Polymerase, PA	65,3
5 [1,7]	Fusion, F	53
6 [1,5]	Haemagglutinin-esterase, HE	38-46 ^b
7 [1,3]	Three open reading frames: <ul style="list-style-type: none"> • ORF1 = non-structural (NS) • ORF2 = spliced mRNA • ORF3 = spliced mRNA 	34 17 11
8 [1,0]	Two open reading frames <ul style="list-style-type: none"> • Matrix, M • RNA-binding protein 	22-24 ^b 26

- a) Estimations based on amino acid sequence
- b) The estimated molecular masses of some of the proteins differ slightly in the literature, probably due to differences in experimental conditions. For HE, this could also be due to differences in glycosylation as well as in the highly polymorphic region (HPR); see text.
- c) References: Segments 1 (PB2; Krossøy et al., 1999), segment 2 (PB1; Snow et al., 2003), segment 3 (NP; Snow and Cunningham, 2001, Aspehaug et al., 2004, Falk et al., 2004), segment 4 (PA; Clouthier et al., 2002), segment 5 (F; Aspehaug et al., 2005), segment 6 (HE; Rimstad et al., 2001, Krossøy et al., 2001b), segment 7 (Biering et al., 2002; McBeath et al., 2006; Garcia-Rosado et al., submitted, Kibenge et al., 2004), segment 8 (Mjaaland et al., 1997, Falk et al., 2004, Garcia-Rosado et al., submitted)

Table 11. ISA virus survival in aquatic environments^a

	Virus in supernatant ^b		Tissue preparation ^{b,c}			Sterile seawater ^{d,e}		Natural seawater ^{d,e}		Sterile freshwater ^{d,e}	
	4°C	15°C	0°C	10°C	15°C	4°C	15°C	4-6°C	15°C	4°C	15°C
Survival Time	14 d	10 d	48 h	24 h	12 h	105 d ^g	21 d	7 d ^e /48 h ^f	7 d ^e	7 d	7 d

^a The table does not describe end-points of virus survival, but states that the virus was infective at the indicated time. ^bFalk et al., 1997 ^cTorgersen, 1997 ^dRimstad and Mjaaland, 2002, ^eMacLeod et al., 2003 ^f, A. Nylund, personal communication. ^ga 2-log₁₀-reduction in titre after 2 weeks (MacLeod et al., 2003); a 3-log₁₀ reduction in titre after 4 months (Rimstad and Mjaaland, 2002)

Table 12. Inactivation of ISA virus

Method	Dose	Comment	Contact time	Outcome / titre reduction	Reference
Heat	45 °C		5 min	+	Torgersen, 1997
	50 °C		1 min	+	Torgersen, 1997
	50 °C		2 min	-	Torgersen, 1997
	55 °C		1 min	-	Torgersen, 1997
	55 °C		10 min	-	Krogstad et al., 1991
UVC (J/m²)	5	Diluted ISA-infective tissue homogenate		+	Torgersen, 1997
	40-100			-	Torgersen, 1997
	200			-	Torgersen, 1997
	33±3.5	Freshwater		3-log red.	Øye and Rimstad, 2001
	51±13	Saltwater		3-log red.	Øye and Rimstad, 2001
	72±16.31	Wastewater		3-log red.	Øye and Rimstad, 2001
	1200	Processing plant effluents		-	Anonymous, 2000
Formic acid	pH 3.5	0 °C, H ₂ CO ₂	8 hours	-	Torgersen, 1997
	pH <3.9		24 hours	-	Anonymous, 2000
	pH 4.0	0 °C, H ₂ CO ₂	24 hours	-	Torgersen, 1997
NaOH	pH 11.5	0 °C	48 hours	-	Torgersen, 1997
	pH 12	0 °C	24 hours	-	Torgersen, 1997
	pH 12		7 hours	-	Krogstad et al., 1991
Chlorine	50 mg/ml		30 min<	+	Torgersen, 1997
	100		15 min	-	
Formaline	0.5 %			-	Krogstad et al., 1991
	0.5 %		16 hours	-	Anonymous, 2000
Ozone	8 mg/ml	600-750 mV redox potential	4 min	-	Anonymous, 2000
Virkon S (peroxygen compound)	1:100	Tested on ISA virus in SHK cells	10 and 20 min at 20°C	Virucid activity confirmed	Antec, 2003
	1:200				
	(in hard water)				

Table 13. Influence of storage on the infectivity of muscle, offal from the head and internal organs

Days of storage	Per cent mortality after storage and subsequent injection in fish		
	muscle	head	internal organs
0	5	20	13
3	60	77.5	64.1
6	32.5	39.5	32.5

Torgersen, 1997.

Table 14. Detection of ISA virus in wild fish and replication of ISA virus in different fish species after experimental infection

Species	Systematic name	Wild fish	Experimental infection	Days after challenge	Detection method	References
Salmon	<i>Salmo salar</i>	pos	pos	>200	several	Raynard et al., 2001a, Cunningham et al., 2002, Nylund 2004, Plarre et al., 2005
Brown trout	<i>S. trutta</i>	pos	pos	>200	several	Raynard et al., 2001a, Nylund, 2004, Plarre et al., 2005
Artic char	<i>Salvelinus alpinus</i>		pos	>40	RT	Snow et al., 2001
Rainbow trout	<i>Oncorhynchus mykiss</i>		pos	>200	several	Several authors
Chum	<i>O. keta</i>		pos	>15	Cc	Rolland and Winton, 2003
Chinook	<i>O. tshawytscha</i>		neg	15	Cc	Rolland and Winton, 2003
Coho salmon	<i>O. kitsutch</i>	pos ^a	pos	>15	RT, Cc	Kibenge et al., 2001a, Rolland and Winton, 2003
Halibut	<i>Hippoglossus hippoglossus</i>	neg	neg		RT	Rolland, 2004, Nylund pers. comm.
Turbot	<i>Scophthalmus maximus</i>		neg		RT, Cs	Nylund pers. comm., Thorud and Torgersen, 1994
Herring	<i>Clupenga harengus</i>	neg	pos	>42	RT, Cs	Nylund et al., 2002
Eel	<i>Anguilla anguilla</i>	pos	neg	7	RT	Stagg et al., 2001, Nylund pers. comm.
American eel	<i>Anguilla rostrata</i>	neg				Rolland, 2004
Goldsinny	<i>Ctenolabrus rupestris</i>		neg	7	RT,Cs	Nylund pers. comm., Thorud and Torgersen, 1994
Ballan wrasse	<i>Labrus bergylta</i>		neg		RT	Nylund pers. com.
Cod	<i>Gadus morhua</i>	neg	neg/pos ^b		RT, ReTi	Rolland 2004, Snow and Raynard 2005, Grove pers. comm.
Haddock	<i>Melanogrammus aeglefinus</i>	neg			RT	Rolland, 2004
Pollock	<i>Pollachius virens</i>	neg			RT	McClure et al., 2004
Winter Flounder	<i>Pseudopleuronectes americanus</i>	neg			RT	Rolland, 2004
Mackerel	<i>Scomber scombrus</i>	neg			RT	Rolland, 2004
Alewife	<i>Alosa pseudoharengus</i>	pos			RT	Rolland, 2004
Saithe	<i>Pollachius virens</i>		neg	<7	RT	Snow et al., 2002
Sea Bass	<i>Dicentraxus labrax</i>		neg		Cs	Thorud and Torgersen, 1994
American shad	<i>Alosa sapidissima</i>					
Blue mussel	<i>Mytilus edulis</i>	neg ^c			RT, Cc	Hellberg et al., 2005

Great scallop	<i>Pecten maximus</i>	neg		RT, Cc	Mortensen pers. comm.
Salmon louse	<i>Lepeophtheirus salmonis</i>	neg (pos)	<24 hours	Cs	Nylund et al., 1993, Rolland and Nylund, 1998b

Cs = ISA virus detected by challenging salmon, Cc = isolation of ISA virus in cell culture, RT = ISA virus detected by RT-PCR, ReTi = ISA virus detected by real-time RT-PCR.

^aFarmed fish. ^bAfter injection of virus. ^cCollected from a salmon farm positive for ISA.

Table 15. Highly polymorphic region (HPR) variants of infectious salmon anaemia virus (ISAV)

HPR	N-term	HPR aa no							TMR
		1 - 5	6 - 10	11 - 15	16 - 20	21 - 25	26 - 30	31 - 35	
Europe									
HPR0a	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA
^a HPR0b	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAN	SVLSN	IFISM	GVA
^b	TDV	KIRVD	AIPPQ	LNQTF	NTN			FISM	GVA
HPR1	TDV	K				PAT	SVLSN	IFISM	GVA
HPR2	TDV	KIRVD	AIPPQ	LNQT				M	GVA
HPR3	TDV	KIRVD	AIPPQ	LNQT				FISM	GVA
HPR4a	TDV	KIRVD	AIPPQ	L			SN	IFISM	GVA
HPR4b	TDV	KIRVD	AIPPQ	L			SN	TFISM	GVA
HPR5	TDV	KIRVD	AIPPQ	L				ISM	GVA
HPR6	TDV	KIRVD	AI		QV	EQPAT	SVLSN	IFISM	GVA
HPR7a	TDV	K				T	SVLSN	TFISM	GVA
HPR7b	TDV	K				T	SVLSN	IFISM	GVA
HPR8	TDV	KIRVD	AIPPQ	L					GVA
HPR9	TDV	KIRVD	AIPPQ	LNQTF	NT			M	GVA
HPR10	TDV	KIK				QPAT	SVLSN	IFISM	GVA
HPR11a	TDV	KIRVD	AIPP				RN	IFISM	GVA
HPR11b	TDV	KIRVD	AIPP				RN	IFVSM	GVA
HPR12	TDV	GIGVD	AIPPQ	L			N	IFISM	GVA
^a HPR13	TDV	K				EQPAN	SVLSN	IFISM	GVA
HPR14	TDV	KIRVD	A		NQV	EQPAT	SVLSN	IFISM	GVA
HPR15	TDV					ET	SVLSN	IFISM	GVA
HPR16	TDV	KIRVD	AIPPQ	LNQTF				M	GVA
HPR17	TDV	KIR			LEV	EQPAT	SVLSN	IFISM	GVA
HPR18	TDV	KIRV				PAT	SVLSN	IFISM	GVA
HPR19	TDV	KIRVD	AIPPQ	LNQTL					GVA
HPR30	TDV	KIRVD	AIPPQ	LNQ				ISM	GVA
America									
HPR00	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPST	TVLSN	IFISM	GVA
^c HPR00	TDV	KIRVD	AIPPQ	LNQTF	NTNQV	EQPAT	SVLSN	IFISM	GVA
HPR20	TDV	NNRVD	AI	L	GVNQV	EQPST	SVPSN	IFISM	GVA
HPR21	TDV	NNRVD	AIPPQ	L			SN	IFISM	GVA

N-term = N terminal region with respect to the HPR, TMR = transmembrane region.

^aThese two HPR groups have only been found in Scotland. ^bGenBank Acc. # AY963263, "European-in-North America", found in New Brunswick (Kibenge pers. comm.) ^cThis HPR0 have been found in Nova Scotia, Canada.