

## SCIENTIFIC OPINION

### Scientific Opinion on infectious salmon anaemia (ISA)<sup>1</sup>

#### EFSA Panel on Animal Health and Welfare (AHAW)<sup>2,3</sup>

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#### ABSTRACT

Atlantic salmon is the only species in which the disease infectious salmon anaemia (ISA) has been observed naturally. Initial reports of findings of infectious salmon anaemia virus (ISAV) before 2002, did not distinguish between non virulent HPR0 and virulent HPRΔ viruses, thus making interpretation of older findings difficult in the light of current knowledge. Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the relationship between HPR0 and HPRΔ, the risk of HPRΔ ISAV emerging from HPR0 ISAV, and possible risk factors for such an emergence. HPR0 ISAV does not cause clinical disease in Atlantic salmon; however, it causes a transient subclinical infection and replicates mainly in gills. There is no evidence for HPR0 ISAV leading to natural infection and replication in fish species other than Atlantic salmon. Virulent ISAV have deletions in the HPR region of the HE gene and they have either an insertion or the Q266L mutation in the F gene. The most plausible hypothesis is that virulent ISAV (HPRΔ) is derived from HPR0 ISAV. This is further supported by the close association between the genetic relatedness and spatio-temporal distances of virus strains in solitary outbreaks. Epidemiological and historical data from solitary disease outbreaks indicates that the risk of HPRΔ ISAV emerging from HPR0 is low, but not negligible. The risk factors for HPRΔ emergence from HPR0 are unknown. Nevertheless, any factor that affects virus replication or host susceptibility could possibly influence the risk of emergence. More research is needed on the drivers for transition from HPR0 to HPRΔ and factors affecting host susceptibility and thereby emergence of clinical disease. A quantitative assessment of the different evolutionary forces for ISA would be useful, as well as the prevalence of ISAV HPR0 in farmed and wild Atlantic salmon.

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#### KEY WORDS

infectious salmon anaemia virus, isavirus, virulence, highly polymorphic region, HPR0

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## SUMMARY

Following a request from the European Commission, the EFSA Panel on Animal Health and Welfare was asked to deliver a scientific opinion on infectious salmon anaemia (ISA). ISA is a fish disease caused by an orthomyxovirus (infectious salmon anaemia virus, ISAV) affecting Atlantic salmon, which has been responsible for high mortalities in salmon producing countries since its first detection in Norway in the mid-1980s.

This opinion reviews the available scientific evidence on the relationship between HPR0 and HPR $\Delta$ , and addresses the risk of HPR $\Delta$  ISAV emerging from HPR0 ISAV and, if relevant, indicates the risk factors for such an emergence.

Pathogenic HPR $\Delta$  ISAV variants cause a systemic infection, infecting endothelial cells of the blood circulatory system whereas HPR0 ISAV does not cause clinical disease in Atlantic salmon but causes a transient subclinical infection and replicates mainly in gills.

ISAV can be genetically differentiated on the basis of the sequence of a highly polymorphic region (HPR) of genomic segment 6 which encodes the Haemagglutinin-Esterase (HE) protein. A deletion within the HPR region (HPR $\Delta$  ISAV) is necessary for pathogenicity. ISAV without any deletions in the HPR region (Hereinafter: HPR0 ISAV) has been reported only in apparently healthy fish and has never been associated with clinical ISA disease.

HPR0 has been detected in farmed Atlantic salmon from several countries and it has also been detected in wild Atlantic salmon in the Faroe Islands and Norway. There is currently no evidence indicating that HPR0 ISAV naturally infects and replicates in species other than Atlantic salmon. The finding of HPR0 ISAV in wild Atlantic salmon indicates that a reservoir outside the farmed salmon population may exist.

ISAV isolates vary in virulence, as observed by differences in disease development and clinical signs in field outbreaks as well as in experimental trials. All ISAV isolates from ISA disease outbreaks have deletions in the HPR region with respect to the HPR0 variant. In addition, all virulent strains of ISAV have either an amino acid substitution or a short amino acid insertion immediately upstream or downstream of the putative arginine cleavage site in the fusion (F) protein.

The hypothesis that virulent HPR $\Delta$  ISAV is derived from HPR0 ISAV by deletions in the HPR of the HE molecule provides the best fit with current knowledge and epidemiological evidence. Epidemiological and historical data from solitary disease outbreaks indicate that the risk of emergence of virulent ISAV is low but not negligible.

Generic biosecurity measures such as segregation of generations, caution regarding contact points (water, equipment), sanitary handling of dead fish, cleaning and disinfection, and synchronous fallowing appear to have a good effect in terms of prevention and control of ISA.

Prior adaptive immunity provides some protection against subsequent infection with virulent ISAV. Little is known about antigenic variation in the haemagglutinin-esterase gene of ISAV and it is not possible to conclude whether this may impact population immunity. Likewise, it is not known if or to what extent prior infection with HPR0 ISAV may induce some degree of protective immunity.

The evolutionary relationship between virulent and low-virulent ISAV forms, where HPR0 mutates into a virulent form of the ISAV, appears plausible. However, no predisposing risk factors have been demonstrated or suggested to drive or increase such an evolutionary process.

Based on general virological knowledge, the risk of emergence of HPR $\Delta$  and subsequent development of disease can be expected to be related to the overall replication of HPR0 ISAV and the presence of susceptible hosts. Any factor that affects replication or host susceptibility would, therefore, also influence the risk of emergence of HPR $\Delta$  ISAV.

## TABLE OF CONTENTS

Abstract .....	1
Summary .....	2
Table of contents .....	3
Background as provided by the European Commission.....	4
Terms of reference as provided by the European Commission.....	4
Assessment .....	5
1. Introduction .....	5
1.1. The disease.....	5
1.2. History of ISAV diagnosis.....	5
1.3. Species range .....	5
1.4. ISAV infection.....	6
2. The capability of HPR0 ISAV to cause clinical disease (ToR1).....	6
2.1. Clinical signs.....	7
2.2. Pathogenesis.....	7
2.3. Immune responses.....	7
2.4. Geographical distribution of HPR0 ISAV .....	8
3. What is the risk of HPRΔ ISAV emerging from HPR0 ISAV (TOR 2a).....	8
3.1. Genome of ISAV .....	8
3.2. Virulence of ISAV .....	9
3.3. Phylogenetics and evolutionary dynamics of ISAV .....	9
4. Relevant risk factors for emergence of HPRΔ (ToR 2b).....	11
4.1. ISAV transmission and spread.....	11
4.2. HPRdelta emergence from endemic HPR0 infection.....	12
Conclusions and recommendations .....	12
References .....	13
Appendix .....	19
A. Diagnosis of ISA .....	19
Propagation of ISAV in cell lines .....	20
Direct methods .....	20
Detection of ISAV (HPRΔ) by RT-PCR (interpretation for surveillance and reservoirs).....	20
Glossary and abbreviations .....	22

## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

ISA is a fish disease listed in Part II of Annex IV to Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals.

ISA mainly affects Atlantic salmon and since its first detection in Norway in mid-1980s has been causing great mortalities in salmon producing countries (Canada, Chile, USA, Norway, Faroe Islands). Within the EU, there have been only two outbreaks, in Scotland, UK (1998 – eradicated, 2009 – under eradication<sup>4</sup>).

Variants of ISAV have been genetically differentiated on the basis of the sequence of a highly polymorphic region (HPR of genomic segment 6 which encodes the Haemagglutinin-Esterase (HE) protein). A deletion within the HPR region (hereinafter: HPR $\Delta$  ISAV) in certain ISAV variants appears to be a consistent indicator of pathogenicity. ISAV without any deletions in the HPR region (Hereinafter: HPR0 ISAV) has been reported only in apparently healthy fish and to our knowledge has never been associated with clinical ISA disease.

The current prescribed diagnostic methods for confirmation of ISA in the OIE Manual of Diagnostic Tests for Aquatic Animals (2009) and Commission Decision 2003/466/EC establishing criteria for zoning and official surveillance following suspicion or confirmation of the presence of infectious salmon anaemia (ISA) do not allow for the detection of HPR0 ISA.

However, existing reverse-transcription polymerase chain reaction (RT-PCR) diagnostic methodologies do allow the detection and differentiation of both HPR0 and HPR $\Delta$  ISAV variants. Furthermore, amplification of the segment 6 and subsequent sequencing is an accurate method for discrimination between HPR0 and HPR $\Delta$  ISAV variants.

HPR0 ISA appears to be widely spread throughout the world, both in areas which are infected with and free from HPR $\Delta$  ISA.

The Commission is evaluating whether it is appropriate, proportionate and necessary to apply risk management measures to HPR0 ISA. To facilitate that evaluation it is appropriate and necessary to assess the risks posed by HPR0 ISA for the health of aquatic animals, in particular Atlantic salmon.

## TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In view of the above, and in accordance with Article 29 of Regulation (EC) 178/2002, the Commission requests EFSA for a scientific opinion on the HPR0 variant of Infectious salmon anaemia (HPR0 ISA), and in particular to assess;

1. The capability of HPR0 ISA to cause clinical disease.
2. The risk of HPR-deleted ISA emerging from HPR0 ISA and, if relevant, indicating factors for such an emergence.

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<sup>4</sup> Declaration of freedom submitted to SCoFCAH on March 9, 2012.

## ASSESSMENT

### 1. Introduction

#### 1.1. The disease

Infectious salmon anaemia (ISA) is a fish disease caused by infectious salmon anaemia virus (ISAV), an orthomyxovirus, affecting Atlantic salmon (*Salmo salar*) by inducing a systemic and lethal condition characterised by severe anaemia and variable haemorrhages and necrosis in several organs (Rimstad et al., 2011a). Current diagnostic procedures are described in Annex A.

So far the disease ISA has been described only in farmed salmon, and the Atlantic salmon is the only species in which the disease has been observed naturally. Since its first detection in Norway in the mid-1980s, ISA has been responsible for high mortalities in salmon-producing countries with high economic losses. The outbreak in Scotland in 1998 was eradicated at a cost of over £20 million (Hastings et al., 1999); the epidemic in Chile cost considerably more (Asche et al., 2009) and was sufficient to lead to shortage in supply to the market, which affected global prices of salmon. ISA is now a global disease, and outbreaks have been recorded in most countries with a significant Atlantic salmon aquaculture industry (Table 1).

**Table 1: Major outbreaks of infectious salmon anaemia**

Year	Country	Reference
1984	Norway	Thorud and Djupvik, 1988
1996	Canada	Mullins et al., 1998
1998	Scotland, UK	Rodger et al., 1998
2000	Faroe Islands	Christiansen et al., 2011
2001	USA	Bouchard et al., 2001
2007	Chile	Godoy et al., 2008 Mardones et al., 2009
2009	Scotland, UK	Murray et al., 2010

#### 1.2. History of ISAV diagnosis

The initial reports of findings of ISAV did not distinguish between HPR0 and HPRΔ, making interpretation of older studies more difficult in the light of current knowledge. The distinction between HPR0 and HPRΔ ISAV was established in 2002 (Mjaaland et al., 2002a; Nylund et al., 2003). Based on current evidence and subsequent sequencing of associated ISAV isolates, most of these older studies probably reflect infections with HPRΔ ISAV.

#### 1.3. Species range

A susceptible species is defined by Council Directive 2006/88/EC as any species in which infection by a disease agent has been demonstrated in natural cases or by experimental infection that mimics the natural pathways. This definition was used for the EFSA opinion “Aquatic species susceptible to diseases listed in Directive 2006/88/EC” (EFSA, 2008) and also informed the current opinion.

Subclinically ISAV-infected feral Atlantic salmon, brown trout and sea trout (*S. trutta*) and escaped, farmed rainbow trout (*Oncorhynchus mykiss*) have been identified by RT-PCR (Raynard et al., 2001; Plarre et al., 2005). Waterborne transmission in juvenile rainbow trout, including high mortalities in genetically susceptible fish strains, have been found in some experimental set-ups (Biacchesi et al., 2007), while serial passage of a virulent strain of ISAV in larger rainbow trout did not produce clinical disease (Olsen et al., 2012). Positive RT-PCR for ISAV has also been reported in wild Atlantic cod (*Gadus morhua*), alewife (*Alosa pseudoharengus*) and pollock (*Pollachius virens*) (MacLean et al., 2003; Rolland, 2004), but contamination by virus present in the surrounding water cannot be excluded as the fish were collected close to cages with Atlantic salmon exhibiting ISA. In other studies, no transmission in cohabitant challenge of Atlantic cod has been found (Snow and Raynard, 2005; Grove

et al., 2007), and pollock cohabitating with farmed Atlantic salmon in sea cages remained PCR negative when harvested together with salmon experiencing increased mortality due to ISA (McClure et al., 2004) and was also found negative for ISAV following exposure by intraperitoneal injection of virus or by cohabitation with ISAV-infected Atlantic salmon (Snow et al., 2002). Based on these findings, both Atlantic salmon, brown trout and sea trout are candidates as natural hosts for HPR0 ISAV. There are no indications that ISAV can infect blue mussel (*Mytilus edulis*) or scallops (*Pecten maximus*) or that these shellfish play any role as reservoirs for ISAV (Skår and Mortensen, 2007).

Atlantic salmon is the only species in which the disease ISA has been repeatedly observed naturally. There is a report of isolation of ISAV from Coho salmon (*O. kisutch*) with jaundice disease in Chile (Kibenge et al., 2001a), but this has not been reported elsewhere.

#### 1.4. ISAV infection

The development of more sensitive methods for virus detection by PCR during the 1990s (Mjaaland et al., 1997) enabled studies providing evidence of ISA virus infection in apparently healthy wild fish (feral Atlantic salmon, brown trout and sea trout, and escaped, farmed rainbow trout) (Raynard et al., 2001; Plarre et al., 2005). Refinements to molecular methods enabled the description of genomic sequences of ISA virus in wild salmonids which were hypothesised to show a full-length sequence of the HPR of the haemagglutinin-esterase (HE) gene (Mjaaland et al., 2002b). Thus, the hypothesis that deletions of HPR0 were required for emergence of HPR variants (HPR $\Delta$ ) associated with virulent forms of ISAV was derived (Mjaaland, Hungnes, et al., 2002; Nylund et al., 2003).

ISAV can be genetically differentiated on the basis of the sequence of the HPR of genomic segment 6 which encodes the HE protein. Deletions within the HPR region (HPR $\Delta$  ISAV) have been identified in all virulent isolates causing clinical ISA disease and appear to be necessary for pathogenicity.

HPR0 ISAV appears to be widely distributed, both in areas infected with and areas free from HPR $\Delta$  ISAV and clinical disease. From a disease control point of view it is important to understand the dynamics and interrelationship between HPR0 and HPR $\Delta$  ISAV and, more particularly, the likelihood of and the reasons why HPR $\Delta$  arises from a background source of HPR0 ISAV.

In view of the above, the Commission is evaluating whether it is appropriate, proportionate and necessary to apply risk management measures to HPR0 ISA, in addition to those applied to HPR $\Delta$  ISAV. EFSA has been asked for a scientific opinion on the HPR0 variant of ISAV and to assess the risks posed by HPR0 ISA for the health of aquatic animals, in particular Atlantic salmon. The terms of reference (ToRs) provided by the Commission can be formulated as three questions:

1. Can HPR0 ISA cause clinical disease?
2. What is the risk of HPR-deleted ISA emerging from HPR0 ISA and, if relevant, indicating factors for such an emergence
  - a. What is the risk of HPR-deleted (HPR $\Delta$ ) ISAV emerging from HPR0 ISAV?
  - b. What are the factors relevant for such an emergence?

The above questions will be addressed on the basis of published literature, other evidence and the expertise of the scientists engaged in the opinion from the panel and Working Group.

#### 2. The capability of HPR0 ISAV to cause clinical disease (ToR1)

ISA is a systemic and lethal condition and clinical signs suggest circulatory failure. So far only HPR $\Delta$  ISAV variants have been reported to cause disease in Atlantic salmon.

## 2.1. Clinical signs

The classic external signs comprise pale gills, exophthalmia, distended abdomen, blood in the anterior eye chamber and sometimes skin haemorrhages, especially of the abdomen, as well as scale pocket oedema. Affected fish are lethargic, keeping close to the wall of the net pen. The position of the fish in water is often vertical and some diseased fish show a corkscrewing swimming behaviour. The nutritional status is usually normal, but diseased fish have no feed in the digestive tract. The development of the disease may be insidious with low mortality and few clinical signs for months; however, cumulative mortality can reach very high levels (up to 90%).

At necropsy, a variable set of haemorrhages and necroses in several organs are usually observed. Typical findings also include severe anaemia, ascites and congestion and enlargement of the liver and spleen (Evensen et al., 1991).

## 2.2. Pathogenesis

ISA can be experimentally reproduced in Atlantic salmon by intra-peritoneal injection with HPR $\Delta$  ISAV or through infected cohabitants. The incubation period in experimental infection is usually 10–20 days (Rimstad and Mjaaland, 2002). The most likely route of virus entry following cohabitation with infected fish is the gills, but other ports of entry cannot be excluded (Mikalsen et al., 2001). In infection experiments, virulent ISAV rapidly produces a systemic infection and has been detected throughout the body 5–10 days post infection with a peak in viral load at approximately 15 days post infection (Totland et al., 1996; Rimstad et al., 1999).

The major target cells for virulent ISAV *in vivo* are endothelial cells (Hovland et al., 1994; Koren and Nylund, 1997; Falk et al., 1998; Gregory, 2002). ISAV specifically binds 4-O-acetylated sialic acid at the endothelial cell surface, and the esterase part of the HE hydrolyses ISAV cell surface receptors (Hellebo et al., 2004). ISAV infects cells via the endocytic pathway and the fusion between virus and cell membrane takes place in endosomes (Eliassen et al., 2000). Virus-infected endothelial cells can be found in any organ (Aamelfot et al., 2012), leading to circulatory collapse, often accompanied by extreme anaemia, haemorrhages and necrosis of internal organs (Hovland et al., 1994).

No experimental infection has been carried out so far with HPR0 ISAV. However, this variant has also been detected in naturally infected salmon, most often in the gills (McBeath et al., 2009; Christiansen et al., 2011). As opposed to the systemic and severe disease caused by HPR $\Delta$  ISAV, HPR0 ISAV replicates in (D.H. Christiansen, personal communication) and causes a localized infection of salmon gills with no signs of disease and only occasional spread to other organs (Christiansen et al., 2011). Parallel testing of kidney, heart and gill tissue for the presence of HPR0 ISAV by real-time RT-PCR disclosed a significantly higher overall detection in gill tissue compared with kidney and heart. Also, the load of HPR0 ISAV virus in positive gills was generally much higher than in kidneys and hearts (Christiansen et al., 2011; Lyngstad et al., 2011). Thus, HPR $\Delta$  ISAV and HPR0 ISAV show different infection patterns and tissue tropism, a pattern similar to that found in wild aquatic birds in which low-pathogenic avian influenza virus causes a subclinical, transient, mucosal infection whereas highly pathogenic influenza causes a systemic and lethal infection in poultry.

## 2.3. Immune responses

Both cellular and humoral immune responses against ISAV have been demonstrated in Atlantic salmon (Falk and Dannevig, 1995; Kibenge et al., 2002; Mjaaland et al., 2005; Lauscher et al., 2011). Studies by western blotting of the Atlantic salmon antibody response to ISAV revealed that ISAV antibodies bound exclusively to the viral nucleoprotein and hemagglutinin protein (Clouthier et al., 2002; Rimstad, Dale, et al., 2011). Antibody-mediated enhancement of virus infection may be involved in the pathogenesis of ISA because ISAV targets leucocytic cells, and Fc receptors for fish immunoglobulin M have been demonstrated on fish leucocytes.

Prior adaptive immunity conferred by inactivated field and experimental vaccines provides some protection against subsequent infection with virulent ISAV (Jones et al., 1999; Lauscher et al., 2011).

DNA vaccines containing the HE gene can also confer protective immunity (Mikalsen et al., 2005), indicating that this surface glycoprotein is antigenic and involved in stimulating a protective immune response. Little is known about antigenic variability in the HE protein of ISAV and it is therefore not possible to conclude whether this may impact population immunity as is seen with influenza A viruses. Likewise, it is not known if cross protection may be achieved against different variants and to what extent prior infection with HPR0 ISAV may induce some degree of protective immunity to HPRΔ variants.

#### 2.4. Geographical distribution of HPR0 ISAV

The first detection of HPR0 ISAV was done on gill tissue from a wild-caught Atlantic salmon in Scotland (Cunningham et al., 2002). In addition to Scotland, HPR0 ISAV has also been detected in farmed Atlantic salmon from the Faroe Islands, Norway, Canada, Chile and Denmark (N.J. Olesen, personal communication).

HPR0 has also been detected in wild Atlantic salmon in the Faroes and Norway. Three out of 88 confirmed wild Atlantic salmon caught by a Faroese research vessel at the feeding grounds in the North Atlantic were HPR0 positive (D.H. Christiansen, personal communication). Furthermore, 4 out of 305 Atlantic salmon caught in rivers in mid-Norway were found to be positive by PCR. Viral RNA from one of them was sequenced to HPR0, clustering phylogenetically with the Faroes cluster. The amount of RNA from the other three was too scarce for sequencing, but still empirically indicated the presence of HPR0. All salmon were caught in an area with on-going ISA outbreaks with virus subtypes associating with a cluster different from that identified as HPR0. The four positive ones were all from the same river and confirmed as “wild salmon” according to fish scale examination. (R. Grøntvedt and T. Lyngstad, personal communication).

The Faroe Islands documented findings of HPR0 in Atlantic salmon in their monitoring from 2005 to 2009. HPR0 was detected on gills 1–13 months post sea transfer (mean 7.7 months). The various cohorts (49) were sampled 5–12 times each year, and the presence of HPR0 on gills showed peaked transient infection profile with peak prevalence up to 100 % lasting for 4 months. Almost all of the cohorts were positive for HPR0. No clinical disease or histopathological consequences have been reported in association with this HPR0 infection in the Faroes (Christiansen et al., 2011).

In Chile, all ISAV strains detected in 2011 were identified as HPR0 (Kibenge et al., 2012). No outbreaks were observed and HPRΔ was not detected.

In a retrospective study in Norway (Lyngstad et al., 2012), ISAV was present in 23 % of 210 cohorts of marine farmed Atlantic salmon along the coast, with no suspicion of ISA. HPR0 ISAV was confirmed in 59 % of these ISAV-positive groups. The rest of the positive groups were not sequenced due to lack of RNA, but the low titres may indicate the presence of HPR0.

The groups were sampled once and at various points in time after sea transfer. In other screening studies, HPR0 has been detected in gill samples from juvenile salmon and in brood stock in the freshwater environment (M. Devold and D. H. Christiansen, personal communication). A low level of HPR0 has also been detected in ovarian fluid of farmed Atlantic salmon (D. H. Christiansen, personal communication).

### 3. What is the risk of HPRΔ ISAV emerging from HPR0 ISAV (TOR 2a)

In order to address this question the genomic structure of ISAV is briefly described and the evidence available regarding virulence factors and phylogenetics and evolutionary dynamics is considered.

#### 3.1. Genome of ISAV

The ISA virus belongs to the genus *Isavirus* of the family *Orthomyxoviridae*. The genome of ISAV consists of eight negative-sense single-stranded RNAs. Each segment contains one or more open reading frames (ORFs) flanked by 5' and 3' untranslated regions (Sandvik et al., 2000; Clouthier et al.,

2002; McBeath et al., 2006) and together they encode 10 or 11 proteins (Mjaaland et al., 1997; Rimstad and Mjaaland, 2002; Kibenge et al., 2007a). Whereas segments 1 to 6 encode one protein each, segment 7 encodes two or three proteins and segment 8 encodes two proteins. Functional characterisation of the two viral surface proteins, the HE protein coded by segment 6 and the F protein coded by segment 5 has disclosed that ISAV possess the major functional characteristics of the influenza virus family including receptor-binding, fusion and receptor-destroying activities (Falk et al., 1997, 2004; Krossøy et al., 2001; Rimstad et al., 2001; Aspehaug et al., 2005). The sequence of segments 1, 2 and 4 have been identified to encode the RNA polymerases PB2, PB1 and PA, respectively (Krossøy et al., 1999; Clouthier et al., 2002; Snow et al., 2003a) whereas segment 3 encodes a nucleoprotein (Ritchie et al., 2001; Aspehaug et al., 2004). The unspliced mRNA of segment 7 encodes a non-structural protein and ORF2 of segment 8 encodes an RNA-binding structural protein. Both proteins have interferon antagonistic properties (Biering et al., 2002; McBeath et al., 2006; García-Rosado et al., 2008). The spliced mRNA of segment 7 encodes a putative nuclear export protein (Kibenge et al., 2007b) and ORF1 of segment 8 encodes the matrix protein (Biering et al., 2002; Falk et al., 2004).

### 3.2. Virulence of ISAV

ISAV isolates vary in virulence, as observed by differences in disease development and clinical signs in field outbreaks, as well as in experimental trials. In experimental infections, induced mortality for different ISAV isolates varied from 0 % to 47 % in the injected fish and from 3 % to 75 % in the cohabitant fish (Mjaaland et al., 2005).

Only a few potential virulence markers have been identified in the ISAV genome. The functional role of these markers, if any, has not yet been determined. The HE gene includes an HPR of 35 amino acids close to the transmembrane region (Devold et al., 2001; Kibenge, Kibenge, et al., 2001; Krossøy et al., 2001; Rimstad et al., 2001; Mjaaland, Hungnes, et al., 2002; Falk et al., 2004). Approximately 30 different HPR variants have been identified in Europe, North America and Chile (Nylund et al., 2006; Kibenge et al., 2009; Christiansen et al., 2011). The HPR variants can be explained as various deletions (Mjaaland, Hungnes, et al., 2002) from a putative ancestral sequence (HPR0) first identified in a wild salmon in Scotland (Cunningham et al., 2002). Whereas all ISAV isolates from ISA disease outbreaks have deletions in the HPR region with respect to the HPR0, the HPR0 subtype has not been associated with clinical or pathological signs of ISA disease (Cunningham et al., 2002; Cook-Versloot et al., 2004; Nylund et al., 2006; McBeath et al., 2009; Vike et al., 2009; Christiansen et al., 2011). This strongly suggests that the HPR region is an important virulence marker of ISAV (Mjaaland et al., 2005).

In addition, all virulent strains of ISAV examined so far have the Q266→L266 amino acid substitution or short amino acid insertions immediately upstream or downstream of the putative arginine cleavage site R267 in the F protein (Devold et al., 2006; Markussen et al., 2008; Cottet et al., 2011).

However, pathogenic ISAV isolates with identical HPR deletions and mutations around the putative cleavage site R267 of the F gene vary in virulence (Mjaaland et al., 2005; Markussen et al., 2008) suggesting that other as yet unknown genetic changes have an influence on virulence. This is supported by findings in influenza viruses where specific mutations in the PB1 (Conenello et al., 2007) and PB2 (Shinya et al., 2004) genes have been linked to virulence.

### 3.3. Phylogenetics and evolutionary dynamics of ISAV

Based on phylogenetic analysis of segment 2 and segment 8, ISAV isolates have been divided into two major genogroups: the North American (NA) genogroup including isolates from Canada and the USA, and the European (EU) genogroup consisting of ISAV isolates from Norway and Scotland (Blake et al. 1999; Krossøy et al. 2001). Based on genetic analysis of only the extracellular region of the HE gene on segment 6, the EU isolates have been further divided into four subgroups (with HPR0 being represented in all subgroups) (Nylund et al., 2003, 2006; Devold et al., 2006; Lyngstad et al., 2008;

McBeath et al., 2009; Vike et al., 2009; Christiansen et al., 2011). It should be noted that this subgrouping may not be consistent across different segments (Plarre et al., 2012).

Phylogenetic analysis of Faroese HPR0 ISAV variants identified a lineage with close genetic similarity to HPRΔ ISAV associated with the Faroese ISA disease epidemic (Christiansen et al., 2011). Lyngstad et al (2012) showed that genetic and geographic distances between pairs of HPR0-ISAV sequences are positively correlated, suggesting that the population of HPR0 ISAV is geographically structured. They also showed that virulent ISAV HE gene sequences from isolated ISA outbreaks were significantly closer in geographical distance to the genetically closest HPR0 ISAV HE gene sequences than would be expected by chance. This association between genetic and geographical distance supports the hypothesis that HPRΔ ISAV may evolve from HPR0 ISAV (Cunningham et al., 2002; Mjaaland, Hungnes, et al., 2002; Nylund et al., 2003).

Several hypotheses have been put forward to explain the origin of HPRΔ and its relationship with HPR0. The original hypothesis was that the virulent HPRΔ ISAV is derived from non-virulent HPR0 ISAV by deletions in the HPR of the HE molecule (Cunningham et al., 2002; Mjaaland, Hungnes, et al., 2002).

A postulated “insertion hypothesis” whereby virulent HPRΔ ISAV undergoes mutations involving insertions into the HPR, resulting in HPR0 ISAV and thus attenuation (Kibenge et al., 2012), is not consistent with these findings and does not explain the presence of geographical variants of HPR0.

A third hypothesis brought forward is that HPR0 is the consensus sequence of all HPRΔ sequences during virus replication (Kibenge et al., 2012). If this hypothesis is correct, fish should be HPR0 ISAV positive while being infected by a range of virulent HPRΔ ISAV (“HPRΔ cloud”), of which the combined HPR consensus sequence would be HPR0. Selection by farming would then be on a variety of HPRΔ ISAV mutants rather than on individual HPR0 ISAV sequences.

Although quasispecies are a common feature of RNA virus infections, i.e. a mixed population of mutants rather than clonally expanded virus particles, there is no experimental support of an “HPRΔ cloud” in all ISAV infected fish. Nucleotide sequences from fish found to be HPR0 positive have not reported the presence of a variety of HPRΔ sequences (Christiansen et al., 2011, Lyngstad 2012). Lyngstad et al (2012) found a geographical relationship between HPR0 and HPRΔ in the nucleotide sequences of the HE gene (excluding the HPR region) (Lyngstad et al., 2012). More recently, the use of new generation sequencing/pyrosequencing of HPR0-positive fish and of ISA-diseased fish demonstrated the presence of a very small fraction of HPRΔ in HPR0 positive fish and an even smaller fraction of HPR0 ISAV in diseased fish having one dominant HPRΔ form, thus rejecting the third hypothesis (Markussen et al., 2012). The original deletion hypothesis has, on the other hand, been supported by the findings from sequencing of viral RNA from ISA outbreaks.

To gain further insights into the process of virulence evolution, a quantitative assessment of the different evolutionary forces for ISA would be useful, in particular the relative contribution of mutation and reassortment processes. The evolutionary rate of different ISAV segments has been estimated to be about an order of magnitude lower than that of influenza A viruses (Plarre et al., 2012), but for the HE gene, the rate estimate was also lower than that obtained for a different ISAV dataset (Castro-Nallar et al., 2011). This implies that the tempo and mode of ISAV evolution may need further investigation. Although transitional viruses having only the relevant virulence mutations in either the HE or the F gene have not been identified, reassortment may also be important in generating virulent combinations of these segments. Reassortment appears to occur relatively frequently for ISA (Plarre et al., 2012), but more formal assessments (e.g., comparisons with influenza) are required to determine how pervasive the reassortment is. Finally, the fitness aspects of the virulence mutations in the HE and F gene segments remain to be determined, such as the synergistic or compensatory nature of these mutations, or the preferred evolutionary trajectory towards virulence.

#### 4. Relevant risk factors for emergence of HPR $\Delta$ (ToR 2b)

There is an evolutionary relationship between virulent and low-virulence forms of ISAV, whereby HPR0 – possibly through several steps – mutates into a virulent form of the ISAV. However, no predisposing risk factors have been demonstrated or suggested to drive or increase such an evolutionary process. Hence, there is a lack of knowledge on specific risk factors for HPR $\Delta$  emergence from HPR0. The available evidence on transmission within populations and spread between populations and control thereof is almost entirely based on studies of HPR $\Delta$  (ISAV); however, the observations from the Faroe Islands and Norway have provided some insight into the dynamics of HPR0.

##### 4.1. ISAV transmission and spread

Horizontal transmission is the major pathway for spread of ISA and vertical transmission is not considered to play a significant role (Rimstad et al., 2011b). In one study proximity to other ISA-infected holdings and management practices that increased exposure to foreign biological material were found to be major risk factors (Vågsholm et al., 1994). The findings indicated that ISA was spread by infected live salmon or infected biological material, i.e., animal waste or discharge from normal operations and slaughter.

The knowledge gaps concerning host (Atlantic salmon) susceptibility should be acknowledged. As the ISAV (HPR $\Delta$ ) is found in other species without clinical disease being observed, at least a species difference in susceptibility among salmonid fish appears important. Furthermore, genetic differences in disease susceptibility following infection with HPR $\Delta$  ISAV have been described within Atlantic salmon (Odegard et al., 2007; Li et al., 2011).

As ISA as a disease has been found only in farmed Atlantic salmon, conditions under which the fish are farmed are assumed to facilitate this process and possibly provide a positive selection for deletion mutants. This hypothesis fits well with subsequent findings that HPR0 ISAV is widespread in healthy, farmed Atlantic salmon and that it has also been detected in wild Atlantic salmon (Christiansen et al., 2011).

Strategies for controlling ISA disease have differed between countries, but there is strong evidence that the disease can be controlled to limit the number of affected farms by the application of biosecurity measures that include movement restrictions as well as depopulation and fallowing of infected farms (Thorud and Håstein, 2003). It is also possible to eradicate ISA over different scales of geographical area in an economically advantageous way by the application and maintenance of good biosecurity practice by industry, together with Government regulation (Stagg, 2003; Murray et al., 2010; Christiansen et al., 2011).

The study from the Faroes suggests that HPR0 ISAV is introduced from an endemic source (reservoir) during the marine phase of the salmon life cycle and that HPR0 ISAV is capable of rapid horizontal spread through local populations (Christiansen et al., 2011).

Generic control measures such as segregation of generations, caution regarding contact points (water, equipment), sanitary handling of dead fish, cleaning and disinfection, and synchronous fallowing have had a good effect in terms of the prevention and control of the disease in the Faroe Islands.

A geographically structured pattern is coherent with the hypothesis of an on-going local HPR0 ISAV propagation in the local biotope (Lyngstad et al., 2012). The presence of HPR0 at various stages in the production cycle opens up possibilities for a potential transfer between generations during the stripping/fertilisation phase due to breaks in the biosecurity system and, as such, HPR0 may also be carried by smolts to marine sites (Nylund et al., 2003, 2006).

#### **4.2. HPRdelta emergence from endemic HPR0 infection**

Fish susceptibility to virulent viruses may be modulated by acquired immunity. HE is a major antigen of ISAV and contributes to stimulating a protective immune response (see Section 2.4). Previous infections with ISAV, like vaccination with attenuated viruses, may induce a level of protection against subsequent infections with the same or other ISAV variants. The possibility that a subclinical infection with HPR0 might prevent or mitigate subsequent infections with virulent variants has not been investigated.

Industrialised farming of salmon is exposed to a range of environmental challenges, such as high density, low physical activity, water with periodically suboptimal oxygen levels and changing temperatures, intensive feeding and growth rate, and handling. Such exposures may be regarded as chronic or temporary stress inducers, harming their defence mechanisms and aggravating disease outbreak dynamics, as well as potentially influencing the evolution and replication ability of the infectious agents themselves. Moreover, high-density farming increases the number of possible infectious contacts whereby an infection and disease in a single salmon can become a communicable disease in an industrialised high-density setting.

### **CONCLUSIONS AND RECOMMENDATIONS**

#### **GENERAL CONCLUSIONS**

Atlantic salmon is the only species in which the disease ISA has been observed naturally.

ISAV can be genetically differentiated on the basis of the sequence of an HPR of genomic segment 6 which encodes the HE protein.

Initial reports of findings of ISAV before 2002 did not distinguish between HPR0 and HPRΔ, making interpretation of older findings more difficult in the light of current knowledge.

A single observation of HPR0 ISAV in wild Atlantic salmon indicates that the virus may exist outside the farmed salmon population.

#### **TOR 1 THE CAPABILITY OF HPR0 ISAV TO CAUSE CLINICAL DISEASE.**

HPR0 ISAV does not cause clinical disease in Atlantic salmon.

It is known that HPR0 ISAV causes a transient subclinical infection and replicates mainly in gills.

There is currently no evidence indicating that HPR0 ISAV leads to natural infection and replication in fish species other than Atlantic salmon.

#### **TOR 2 THE RISK OF HPR-DELETED ISA EMERGING FROM HPR0 ISA AND, IF RELEVANT, INDICATING FACTORS FOR SUCH AN EMERGENCE.**

##### **2a Risk of HPRΔ emerging from HPR0**

All virulent ISAV have deletions in the HPR region of the HE gene and they have either an insertion or the Q266L mutation in the F gene. Transitional viruses having only one of the HE or F mutations have not been found.

The most plausible hypothesis is that virulent ISAV (HPRΔ) is derived from HPR0 ISAV.

The close association between genetic relatedness and the spatio-temporal distance of virus strains in solitary outbreaks further supports the hypothesis that virulent ISAV evolve from HPR0-ISAV.

Epidemiological and historical data from solitary disease outbreaks indicate that the risk of emergence of virulent ISAV is low but not negligible.

## 2b Risk factors

There is a lack of knowledge on risk factors for HPR $\Delta$  emergence from HPR0.

The risk of emergence of HPR $\Delta$  ISAV and subsequent development of disease can be expected to be related to the overall replication rate of HPR0 ISAV and the presence of susceptible hosts. Any factor that affects replication or host susceptibility would therefore also influence the risk of emergence of HPR $\Delta$  ISAV.

### RECOMMENDATION

Baseline monitoring is needed to estimate the prevalence of ISAV HPR0 in farmed Atlantic salmon and wild fish in proximity to farming facilities.

### RECOMMENDATIONS FOR RESEARCH

Research should be done to address the drivers for transition from HPR0 to HPR $\Delta$  and factors affecting host susceptibility and thereby emergence of clinical disease.

To gain further insights into the process of virulence evolution, a quantitative assessment of the different evolutionary forces for ISA would be useful, in particular the relative contribution of mutation and reassortment processes.

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## APPENDIX

### A. DIAGNOSIS OF ISA

As ISA was described and surveyed for approximately eight years before the causative agent was identified and described, the diagnosis of the disease was traditionally based on clinical and pathological signs, combined with clinical chemistry, as haematocrit values below 10 in Atlantic salmon should lead to suspicion of ISA. A number of methods for detection of the virus have since been developed and have contributed to increasing the sensitivity and specificity of ISA diagnosis. However, the diagnosis of confirmed ISA is still based on a combination of clinical signs and specific detection of the causative agent ISAV.

The HPR0 variant is not associated with clinical ISA or any ISA-associated pathological changes in any tissue. HPR0 has been detected from fish with gill lesions, but so far no causal association has been shown or suggested. Findings of HPR0 in apparently healthy fish groups, and often those with low virus loads (high cycle threshold (Ct) values), support the hypothesis that HPR0 is of low virulence (Cunningham et al., 2002; Mjaaland et al., 2002; Markussen et al., 2008; Kibenge et al., 2009, Lyngstad et al 2011).

The ISA-typical gross macroscopic changes are present late in disease development. At necropsy, haemorrhage may be found in the liver, kidney, gut and gills. The spleen is often swollen and dark (Mjaaland et al., 1997; Devold et al., 2000; Mikalsen et al., 2001; Snow et al., 2003b). The haemorrhagic lesions can be absent or rare in the initial stages of ISA. In the more slowly developing, chronic forms of ISA, clinical signs and pathological changes may be more subtle.

When opening the fish for gross pathological examination the changes can vary from almost none to severe. No signs are pathognomonic but anaemia is always present, with haematocrit values of < 10 % at the end stage (normal values for non-infected fish > 30 %). The following signs are, however, rather consistent with ISA:

- oedema and yellowish fluid in the abdominal cavity
- petechial haemorrhages in the viscera and peritoneum
- focal or diffusely dark liver (liver colour is given a number from 1 to 5 according to liver colour card scale used at necropsy in the field)
- swollen, dark-red spleen with rounded margins
- swollen, dark-red kidney with blood and liquid effusing from cut surfaces
- pinpoint haemorrhages of especially the dorsal skeletal muscle.

ISA virus can be detected by a range of direct and indirect immunochemical and molecular methods.

According to the World Organisation for Animal Health (OIE), the following criteria should be met for confirmation of ISA: mortality, clinical signs and pathological changes consistent with ISA and detection of ISAV in tissue preparations by means of specific antibodies against ISAV, in addition to either isolation and identification of ISAV in cell culture or detection of ISAV by RT-PCR.

According to OIE the definition of confirmed ISAV HPRΔ infection requires two criteria to be met:

- isolation and identification of ISAV in cell culture from at least two independent samples (targeted or routine) from any fish on the farm tested

- isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm with corroborating evidence of ISAV in tissue preparations using either RT-PCR or immunofluorescent antibody test (IFAT)/immunohistochemistry (IHC).

Similarly, the confirmation of infection with HPR0 ISAV requires:

- an absence of clinical signs consistent with ISA disease or mortality (= apparently healthy fish)
- detection of ISAV by RT-PCR followed by independent amplification and sequencing of the HPR region of segment 6 to confirm the presence of HPR0 only.

### **Propagation of ISAV in cell lines**

The HPR $\Delta$  ISAV variants can be propagated in various cell cultures. The first cell line supporting replication of ISAV was SHK-1, established from a culture of Atlantic salmon head kidney cells. Cytopathic effect (CPE) was observed 12–14 days after inoculation (Dannevig et al., 1995). More recently Devold et al. (2000) and Wergeland and Jakobsen (2001) reported the isolation of an Atlantic salmon head kidney cell line (ASK) and TO cell line, respectively, that supported replication of HPR $\Delta$  ISAV showing CPE in only seven to eight days. Other cell lines have also been used to propagate HPR $\Delta$  ISAV; however, they all display low yields of virus and poorly defined CPE compared with SHK-1, TO and ASK.

Only ISAV HPR $\Delta$  isolates have hitherto been able to multiply in cell cultures, and even among these isolates CPE is not always obtained. When cultivating, cell cultures with no CPE should always be tested in an haemadsorption test using salmonid erythrocytes, by IFAT or by RT-PCR, as virus replication regularly occurs without development of apparent CPE. ISAV in cell cultures is identified by antibody-based antigen detection methods such as IFAT or by molecular techniques such as RT-PCR or real time RT-PCR.

Although several laboratories have tried to propagate and isolate HPR0 ISAV in conventional ISAV permissive cell lines they have so far been non-culturable with no cytopathic effect (Nylund et al., 2006). Despite the unsuccessful attempts to cultivate ISA HPR0, it was recently demonstrated by Christiansen et al. (2011) that ISA HPR0 could in fact multiply in ASK cells as they demonstrated that both ORF1 and the cellular splicing dependent ORF1/2 and possibly the ORF1/3 of segment 7 could be detected in HPR0-infected ASK cells as a means of proving cellular translation. Whether the multiplication resulted in fully developed virions or not remains to be shown.

### **Direct detection methods**

ISAV can be detected directly in fish tissue material by IFAT on smears of kidney imprints using monoclonal antibodies, or by IHC, in which the strongest staining usually is observed in endothelial cells of heart and kidney, or, as is most widely used, by RT-PCR on tissue material.

ISAV HPR0 infection is found on the gills, and this organ has to be collected if a survey targeting ISAV HPR0 is conducted. ISA HPR0 is, however, occasionally detected also in internal organs. As no specific immunochemical or PCR-based methods have been developed yet, ISAV HPR0 can so far be confirmed only through sequencing the ISAV-positive PCR-products of segment 6.

### **Detection of ISAV (HPR $\Delta$ ) by RT-PCR (interpretation for surveillance and reservoirs)**

ISAV HPR $\Delta$  is identified by molecular methods and often also genotyped and characterised by subsequent sequencing. The primers described in the OIE manual for RT-PCR and real-time RT-PCR will detect both HPR0 and HPR $\Delta$  HPR $\Delta$  ISAV, and the methods may be used for detection of ISAV in tissue samples or in samples from cell cultures. Since Mjaaland et al. (1997) reported the first RT-PCR for ISAV, which targeted genomic segment 8, several methods have been reported.

The primer sets derived from genomic segment 8 (ILA1/ILA2) have been used by several laboratories and have been found suitable for detection of ISAV during disease outbreaks and in carrier fish (ILA2 reverse primer does not, however, match isolates from North America). The segment 6 primers may be useful for verification of PCR results based on segment 8 primers as an alternative to sequencing the PCR product. With the widespread occurrence of HPR0 variants in mind, for any positive PCR results based on the segment 8 primers, it is essential to follow up by sequencing the HPR of segment 6 in order to determine the ISAV HPR variant present (HPR $\Delta$  or HPR0 or both). For this purpose the primers described by Kibenge et al. (2009) are adequate.

**Table 1:** Primers targeting segment 6 and 8, respectively, in conventional RT-PCR

RT-PCR: primer sequences	Named	Genomic segment	Product size	Reference
5'-GGC-TAT-CTA-CCA-TGA-ACG-AAT-C-3' 5'-GCC-AAG-TGT-AAG-TAG-CAC-TCC-3'	ILA1 ILA2	8	155	Mjaaland <i>et al.</i> , 2002b
5'-GGA-ATC-TAC-AAG-GTC-TGC-ATT-G-3' 5'-CTT-CAA-AGG-TGT-CTG-ACA-CGT-A-3'	Seg6U Seg6L	6	130	Designed by OIE Ref. Lab.
5'-GCC-CAG-ACA-TTG-ACT-GGA-GTA-G-3' 5'-AGA-CAG-GTT-CGA-TGG-TGG-AA-3'	ISAV HPR Fwd ISAV HPR Rev	6	319–391	Kibenge <i>et al.</i> , 2009

The use of real-time RT-PCR has several advantages, when compared with conventional RT-PCR, in terms of its higher analytical specificity and often also sensitivity, especially when including a sequence-specific probe. This method is also more rapid, the risk of contamination is reduced and at the same time it is possible to estimate the relative amount of viral RNA in the sample, but none of the hitherto published methods are yet able to discriminate between ISAV HPR0 and HPR $\Delta$

Snow et al. (2006) developed a real-time RT-PCR with primer and probe sequences targeting segment 7 and 8, respectively, that have been used for screening for ISAV. Both primers and probes target conserved regions and ensure detection of all documented ISAV strains, and a positive real-time RT-PCR will thus demand a subsequent sequencing of segment 6 after a conventional RT-PCR.

**Table 2:** Primers targeting segment 7 and 8, respectively, in real-time RT-PCR

Real-time RT-PCR: primer and probe sequences	Named	Genomic segment	Reference
5'-CAGGGTTGTATCCATGGTTGAAATG-3' 5'-GTCCAGCCCTAAGCTCAACTC-3' 5'-6FAM-CTCTCTCATTGTGATCCC-MGBNFQ-3'	Forward primer Reverse primer Taqman <sup>®</sup> probe	7	Snow <i>et al.</i> , 2006
5'-CTACACAGCAGGATGCAGATGT-3' 5'-CAGGATGCCGGAAGTCGAT-3' 5'-6FAM-CATCGTCGCTGCAGTTC-MGBNFQ-3'	Forward primer Reverse primer Taqman <sup>®</sup> probe	8	Snow <i>et al.</i> , 2006

## GLOSSARY AND ABBREVIATIONS

F	Fusion protein
HE	Hemagglutinin-Esterase
HPR	Highly Polymorphic Region
HPR0	Low-virulence ISAV having a full-length HPR
HPR $\Delta$	Virulent ISAV having a deletion in HPR
ISA	Infectious Salmon Anaemia
ISAV	Infectious Salmon Anaemia Virus

## COMMON AND LATIN NAMES OF SPECIES MENTIONED IN THE TEXT.

Latin name	Common name
<i>Alosa pseudoharengus</i>	Alewife
<i>Oncorhynchus kisutch</i>	Coho salmon
<i>Gadus morhua</i>	Atlantic cod
<i>Mytilus edulis</i>	Blue mussel
<i>Oncorhynchus mykiss</i>	Rainbow trout
<i>Pecten maximus</i>	Scallop
<i>Pollachius pollachius</i>	Pollock
<i>Pollachius virens</i>	Saithe
<i>Salmo salar</i>	Atlantic salmon
<i>Salmo trutta</i>	Brown trout; Sea trout