

INFECTION WITH HPR-DELETED OR HPRO INFECTIOUS SALMON ANAEMIA VIRUS

1. Scope

Infection with infectious salmon anaemia virus (ISAV) means infection with the pathogenic agent highly polymorphic region (HPR)-deleted ISAV, or the non-pathogenic HPRO (non-deleted HPR) ISAV of the Genus *Isavirus* and Family *Orthomyxoviridae*.

HPR-deleted ISAV may cause disease in Atlantic salmon (*Salmo salar*), which may progress to a generalised and lethal condition characterised by severe anaemia, and variable haemorrhages and necrosis in several organs.

Detection of HPRO ISAV has not been associated with clinical signs of disease in Atlantic salmon (Christiansen *et al.*, 2011). A link between non-pathogenic HPRO ISAV and pathogenic HPR-deleted ISAV has been suggested, with some disease outbreaks potentially occurring as a result of the emergence of HPR-deleted ISAV from HPRO ISAV (Cardenas *et al.*, 2014; Christiansen *et al.*, 2017; Cunningham *et al.*, 2002; Gagne & Leblanc, 2017; Mjaaland, *et al.*, 2002).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The morphological, physicochemical and genetic properties of ISAV are consistent with those of the *Orthomyxoviridae*, and ISAV has been classified as the type species of the genus *Isavirus* (Kawaoka *et al.*, 2005) within this virus family.

ISAV is an enveloped virus, demonstrating a pleomorphic icosahedral shape, 100–130 nm in diameter, with mushroom shaped surface projections approximately 10 nm long (Falk *et al.*, 1997). However, there are studies that indicate greater morphological heterogeneity in cells of epithelial origin (Ramirez & Marshall, 2018). The virus genome consists of eight single-stranded RNA segments with negative polarity (Mjaaland *et al.*, 1997). The virus has haemagglutinating, receptor-destroying and fusion activity (Falk *et al.*, 1997; Mjaaland *et al.*, 1997).

The nucleotide sequences of all eight genome segments, encoding at least ten proteins, have been described (Clouthier *et al.*, 2002; Rimstad *et al.*, 2011), including the 3' and 5' non-coding sequences (Kulshreshtha *et al.*, 2010; Sandvik *et al.*, 2000). Four major structural proteins have been identified, including a 68 kDa nucleoprotein, a 22 kDa matrix protein, a 42 kDa haemagglutinin-esterase (HE) protein responsible for receptor-binding and receptor-destroying activity, and a 50 kDa surface glycoprotein with putative fusion (F) activity, encoded by genome segments 3, 8, 6 and 5, respectively. Segment 1, 2, and 4 encode the viral polymerases PB2, PB1 and PA. The two smallest genomic segments, segments 7 and 8, each contain two open reading frames (ORF). The ORF1 of segment 7 encodes a protein with type I interferon antagonistic properties, while ORF2 has been suggested to encode a nuclear export protein (NEP: Ramly *et al.*, 2013). The smaller ORF1 of segment 8 encodes the matrix protein, while the larger ORF2 encodes an RNA-binding structural protein also with type I interferon antagonistic properties, and also interact with the host RNAi system (Garcia-Rosado *et al.*, 2008; Thukral *et al.*, 2018).

In the HE gene, a small HPR near the transmembrane domain has been identified. This region is characterised by the presence of gaps rather than single-nucleotide substitutions (Cunningham *et al.*, 2002; Mjaaland *et al.*, 2002). A full-length gene (HPRO) has been suggested to represent a precursor from which all ISAV HPR-deleted (pathogenic) variants of ISAV originate. The presence of non-pathogenic HPRO ISAV genome has been reported in both apparently healthy wild and farmed Atlantic

salmon. Fish with clinical disease and pathological signs consistent with ISA are infected with HPR-deleted ISAV (Christiansen *et al.*, 2011; Cunningham *et al.*, 2002; Markussen *et al.*, 2008). A mixed infection with HPR-deleted and HPR0 ISAV variants has been reported in the same fish (Cardenas *et al.*, 2014; Kibenge *et al.*, 2009). Recent studies show that HPR0 ISAV variants occur frequently in sea-reared Atlantic salmon (Christiansen *et al.*, 2017). HPR0 ISAV is seasonal and transient in nature and displays a tissue tropism with high prevalence in gills (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011). To date there has been no direct evidence linking the presence of HPR0 ISAV to a clinical disease outbreak. The risk of emergence of pathogenic HPR-deleted ISAV variants from a reservoir of HPR0 ISAV is considered to be low but not negligible (Cardenas *et al.*, 2014; Christiansen *et al.*, 2011; 2017; EFSA, 2012).

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas. According to sequence differences in a partial sequence of segment 6, two groups have been defined: one designated as a European clade and one designated as a North American clade (Cardenas *et al.*, 2019; Gagne & LeBlanc, 2017).

In addition to the variations seen in the HPR of the HE gene, other gene segments are of importance for development of clinical disease. A putative virulence marker has been identified in the fusion (F) protein. Here, a single amino acid substitution, or different sequence insertion, near the protein's putative cleavage site has been found to be a prerequisite for virulence (Kibenge *et al.*, 2007; Markussen *et al.*, 2008). However, deleted ISAV variants have been found without this virulence marker on segment 5 (Cardenas *et al.*, 2019). Aside from insertion/recombination, ISAV also uses gene segment reassortment in its evolution, with potential links to virulence (Cardenas *et al.*, 2014; Devold *et al.*, 2006; Gagne & Leblanc, 2017; Markussen *et al.*, 2008; Mjaaland *et al.*, 2005).

2.1.2. Survival and stability in processed or stored samples

A scientific study concluded that ISAV retains infectivity for at least 6 months at –80°C in tissue homogenates (Smail & Grant, 2012). Isolation in cell culture has been successful even from fish kept frozen whole at –20°C for several years. The experience of diagnostic laboratories has indicated the suitability of general procedures for sample handling (see Chapter 2.3.0) for ISAV.

2.1.3. Survival and stability outside the host

ISAV RNA has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in seawater from net-pens with ISAV-positive Atlantic salmon but not from a sample collected 80–100 metres downstream of the farm (Lovdal & Enger, 2002). It is difficult to estimate exactly how long the virus may remain infectious in the natural environment because of a number of factors, such as the presence of particles or substances that may bind or inactivate the virus. A study using natural seawater at 10°C, either exposed to UVA and UVB or not, demonstrated that the starting titre of ISA diminished substantially over a period of 72 hours with some indication that infectivity in an IP challenge model was lost between 3 and 6 hours (Vike *et al.*, 2014).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with ISAV according to Chapter 1.5 of *Aquatic Animal Health Code (Aquatic Code)* are: Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with ISAV according to Chapter 1.5 of the *Aquatic Code* are: Atlantic herring (*Clupea harengus*) and amago trout (*Oncorhynchus masou*).

In addition, pathogen-specific positive RT-PCR results have been reported in the following species, but an active infection has not been demonstrated *in vivo*: Coho salmon (*Oncorhynchus kisutch*).

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

In Atlantic salmon, life stages from yolk sac fry to adults are known to be susceptible. Disease outbreaks are mainly reported in seawater cages, and a few cases have been reported in the freshwater stage, including one case in yolk sac fry (Rimstad *et al.*, 2011). Infection with HPR-deleted ISAV has been experimentally induced in both Atlantic salmon fry and parr kept in freshwater.

2.2.4. Distribution of the pathogen in the host

There is evidence of the presence of the virus in practically all organs of the fish, as well as in ovarian fluids and ova (Marshall *et al.*, 2014), however, the HPRO variant has a predilection for gills.

HPR-deleted ISAV: Endothelial cells lining blood vessels seem to be the primary target cells for ISAV replication as demonstrated by electron microscopy, immunohistochemistry and *in-situ* hybridisation. Virus replication has also been demonstrated in leukocytes, and sinusoidal macrophages in kidney tissue stain positive for ISAV using immunohistochemistry (IHC). Furthermore, red blood cells may have virus aggregates on the outer cell membrane as indicated by indirect fluorescent antibody test (IFAT) with a monoclonal antibody (MAb) against the HE protein. As endothelial cells support replication and virus may be carried on red blood cells, virus may occur in any organ. Repeated sampling over the course of a chronic infection point to kidney and heart as the organs most likely to become test-positive. Clinical disease and macroscopic organ lesions appear foremost in severely anaemic Atlantic salmon (Aamelfot *et al.*, 2012; McBeath *et al.*, 2015; Rimstad *et al.*, 2011).

For interaction with cells the haemagglutinin-esterase (HE) molecule of ISAV, like the haemagglutinin (HA) of other orthomyxoviruses (influenza A, B and C viruses), is essential for binding of the virus to sialic acid residues on the cell surface. In the case of ISAV, the viral particle binds to glycoprotein receptors containing 4-O-acetylated sialic acid residues, which also functions as a substrate for the receptor-destroying enzyme. Further uptake and replication seem to follow the pathway described for influenza A viruses, indicated by demonstration of low pH-dependent fusion, inhibition of replication by actinomycin D and α -amanitin, early accumulation of nucleoprotein followed by matrix protein in the nucleus and budding of progeny virions from the cell surface (Cottet *et al.*, 2011; Rimstad *et al.*, 2011).

HPRO ISAV: Observed tissue tropism was foremost in the gills when PCR testing was carried out on various organs of Atlantic salmon (Christiansen *et al.*, 2011). *In-situ* immunostaining of HPRO ISAV PCR-positive gills show staining limited to the epithelium indicating replication and shedding to water, rather than invasive infection. Immunostaining was unable to demonstrate HPRO ISAV infection of internal organs.

2.2.5. Aquatic animal reservoirs of infection

Persistent infection in lifelong carriers has not been documented in Atlantic salmon, but at the farm level, infection may persist in the population by continuous infection of new individuals that do not develop clinical signs of disease. This may include infection with the HPRO ISAV variants, which seems to be only transient in nature (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011). Experimental infection of rainbow trout and brown trout with HPR-deleted ISAV indicate that persistent infection in these species could be possible (Rimstad *et al.*, 2011).

2.2.6. Vectors

Transmission of ISAV by salmon lice and sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*; Oelckers *et al.*, 2014) has been demonstrated under experimental conditions.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The disease pattern with HPR-deleted ISAV depends on many factors, including the strain of the virus. During outbreaks of infection with HPR-deleted ISAV, morbidity and mortality may vary greatly between net pens in a seawater fish farm, and between farms (Hammell & Dohoo, 2005). Morbidity and mortality within a net pen may start at very low levels, with typical daily mortality between 0.5 and 1% in affected cages. Without intervention, mortality increases and often peaks in early summer and winter. The range of cumulative mortality during an outbreak is generally insignificant to moderate, but in severe cases,

lasting several months, cumulative mortality may exceed 90%. Initially, a clinical disease outbreak may be limited to one or two net pens. In such cases, if affected fish are slaughtered immediately, further development of clinical infection with HPR-deleted ISAV at the site may be prevented.

HPR0 ISAV has not been associated with clinical disease in Atlantic salmon.

2.3.2. Clinical signs, including behavioural changes

The most prominent external signs of infection with HPR-deleted ISAV are pale gills (except in the case of blood stasis in the gills), exophthalmia, distended abdomen, blood in the anterior eye chamber, and sometimes skin haemorrhages especially of the abdomen, as well as scale pocket oedema.

Generally, Atlantic salmon naturally infected with HPR-deleted ISAV appear lethargic and may keep close to the wall of the net pen.

Affected fish are generally in good condition, but diseased fish have no feed in the digestive tract.

2.3.3. Gross pathology

Fish infected with HPR-deleted ISAV may show a range of pathological changes, from none to severe, depending on factors such as infective dose, virus strain, temperature, age and immune status of the fish. No lesions are pathognomonic to infection with HPR-deleted ISAV, but anaemia and circulatory disturbances are always present. The following findings have been described to be consistent with infection with HPR-deleted ISAV, though all changes are seldom observed in a single fish: i) oedema of the swim bladder; ii) small haemorrhages of the visceral and parietal peritoneum; iii) focal or diffusely dark red liver (a thin fibrin layer may be present on the surface); iv) swollen, dark red spleen with rounded margins; v) dark redness of the intestinal wall mucosa in the blind sacs, mid- and hind-gut, without blood in the gut lumen of fresh specimens; vi) swollen, dark red kidney with blood and liquid effusing from cut surfaces; and vii) pinpoint haemorrhages of the skeletal muscle.

2.3.4. Modes of transmission and life cycle

The main route of infection is most likely horizontally through the gills for both HPR0 and HPR-deleted ISAV, but infection via the intestine or skin cannot be excluded. Vertical transmission cannot be excluded (Marshall *et al.*, 2014).

ISAV may be shed in skin, mucous, urine, faeces (Totland *et al.*, 1996), ovarian fluid and ova (Marshall *et al.*, 2014).

Except for a single report by Ditlecadet *et al.* (2021), HPR0 ISAV has not been isolated in cell culture, which hampers *in-vivo* and *in-vitro* studies of characteristics and the life cycle of this variant.

2.3.5. Environmental factors

Generally, outbreaks of infection with HPR-deleted ISAV tend to be seasonal, occurring in early summer and winter; however, outbreaks can occur at any time of the year.

2.3.6. Geographical distribution

ISAV was initially reported in Norway in the mid-1980s (Thorud & Djupvik, 1988). It has since been reported in other countries in Europe, North America and South America. See WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Vaccination against infection with ISAV has been carried out in North America since 1999 and the Faroe Islands since 2005. In Norway, vaccination is normally carried out in regions with high prevalence of outbreaks. Chile started vaccinating against infection with ISAV in 2010. However, vaccine efficacy seems insufficient given all cases of both HPR0 and HPR-deleted ISAV that occurred in the Faroe Islands

have occurred in vaccinated fish. The same lack of efficacy has been observed in Norway after vaccination around outbreak areas.

2.4.2. Chemotherapy including blocking agents

Chemotherapy is currently not available. However, the broad-spectrum antiviral drug Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is effective in inhibiting ISAV replication both *in vitro* and *in vivo* (Rivas-Aravena *et al.*, 2011). It should also be noted that interfering peptides have recently been shown to have a non-toxic antiviral effect against ISAV (Cardenas *et al.*, 2020).

2.4.3. Immunostimulation

Not applicable.

2.4.4. Breeding resistant strains

Differences in susceptibility among different family groups of Atlantic salmon in freshwater have been observed in challenge experiments and in field tests (Gjoen *et al.*, 1997). Breeding companies are using infection trials, family selection and genomic selection to improve ISA resistance, but scientific information on the effect of this on disease incidence or prevalence of subclinical infection is lacking.

2.4.5. Inactivation methods

ISAV is sensitive to UV irradiation (UVC) and ozone. A 3-log reduction in infectivity in sterile freshwater and seawater was obtained with a UVC dose of approximately 35 Jm⁻² and 50 Jm⁻², respectively, while the corresponding value for ISAV in wastewater from a fish-processing plant was approximately 72 Jm⁻². Ozonated seawater (4 minutes with 8 mg ml⁻¹, 600–750 mV redox potential) may inactivate ISAV completely. Incubation of tissue homogenate from diseased fish at pH 4 or pH 12 for 24 hours inactivated ISAV. Incubation in the presence of chlorine (100 mg ml⁻¹) for 15 minutes also inactivated the virus (Rimstad *et al.*, 2011). Cell culture-isolated ISAV may survive for weeks at low temperatures, but virus infectivity is lost within 30 minutes of exposure at 56°C (Falk *et al.*, 1997).

2.4.6. Disinfection of eggs and larvae

Disinfection of eggs according to standard procedures is suggested as an important control measure (see chapter 4.4 of the *Aquatic Code*).

2.4.7. General husbandry

The incidence of infection with ISAV may be greatly reduced by implementation of legislative measures or husbandry practices regarding the movement of fish, mandatory health control, transport and slaughterhouse regulations. Specific measures including restrictions on affected, suspected and neighbouring farms, enforced sanitary slaughtering, generation segregation ('all in/all out') as well as disinfection of offal and wastewater from fish slaughterhouses and fish processing plants may also contribute to reducing the incidence of the disease.

Handling of fish (e.g. sorting or treatment, splitting or moving of cages) may initiate disease outbreaks on infected farms, especially if long-term undiagnosed problems have been experienced (Lyngstad *et al.*, 2008).

The experience from the Faroe Islands, where the prevalence of HPRO ISAV is high, demonstrates that the combination of good biosecurity and husbandry substantially reduces the risk of outbreaks of infection with HPR-deleted ISAV (Christiansen *et al.*, 2017).

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

For detection of HPR-deleted ISAV, clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, net-cages etc.) should be inspected and fish displaying clinical signs and gross pathology and anaemia consistent with those described in Sections 2.3.2 and 2.3.3 should be sampled.

For detection of HPRO ISAV, gills from randomly selected individuals should be sampled at different time points throughout the production cycle.

For the purposes of disease surveillance, fish to be sampled are selected as follows:

- i) The most susceptible species should be sampled preferentially (see Section 2.2.3). Other susceptible species listed in Section 2.2.1 should be sampled proportionally.
- ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.
- iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present (e.g. during surveillance of apparently healthy populations), the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with ISAV should be collected. Ideally, fish should be collected while alive, however, recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

3.2. Selection of organs or tissues

3.2.1. Detection of HPR-deleted ISAV

Only internal organs that have not been exposed to the environment should be used for diagnostic testing.

The organs or tissue material to be sampled can include: i) for histology: mid-kidney, liver, heart, pancreas, intestine, and spleen; ii) for immunohistochemistry: mid-kidney and heart including valves and bulbus arteriosus; iii) for RT-PCR (conventional and real-time) analysis: mid-kidney and heart; and iv) for virus culture: mid-kidney, heart, liver and spleen.

3.2.2. Detection of HPRO ISAV

Gill tissue is recommended.

3.3. Samples or tissues not suitable for pathogen detection

Information on samples or tissues not suitable for pathogen detection is lacking; follow recommendations in Section 3.2 for virus detection.

3.4. Non-lethal sampling

Blood is preferred for non-lethal sampling for HPR-deleted ISAV based on a study by Giray *et al.* (2005) in which blood and mucus was compared with kidney samples derived from both infected fish with or without clinical signs and tested by RT-PCR and virus isolation in cell culture. Gill swabs are recommended for non-lethal sampling for HPRO (Aamelfort *et al.*, 2016).

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples for real-time RT-PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen. Commercial RNA preservatives are available, which have better efficacy than ethanol at room temperature. Commercial fixatives validated to be at least as effective as the fixatives described above may be used.

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.3.0 *General information* (diseases of fish).

3.5.4. Samples for electron microscopy

ISAV has been characterised by transmission electron microscopy (TEM) using general procedures (Falk *et al.*, 1997).

3.5.5. Samples for other tests

At present, other tests, for example serology tests, are not used for diagnostic purposes.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry can be pooled to provide the minimum amount of material needed for testing. If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

- +++ = Methods are most suitable with desirable performance and operational characteristics.
- ++ = Methods are suitable with acceptable performance and operational characteristics under most circumstances.
- + = Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
- Shaded boxes = Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

OIE Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to OIE Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Gross signs					+	+	+	1				
Histopathology ³					++	++	++	1				
Cell culture					++	++	++	1	++	++	++	1
Real-time RT-PCR	+++	+++	+++	1	+++	+++	+++	1	++	++	++	1
Conventional RT-PCR	+	+	+	1	++	++	++	1	++	++	+	1
Amplicon sequencing ⁴									+++	+++	+++	NA
<i>In-situ</i> hybridisation												
Immunohistochemistry					++	++	++	1	++	++	++	1
IFAT on kidney imprints or blood smears					++	++	++	1	+++	+++	+++	1
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not applicable; RT-PCR = reverse-transcription polymerase chain reaction;

LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Susceptibility of early and juvenile life stages is described in Section 2.2.3.

³Histopathology and cytopathology can be validated if the results from different operators have been statistically compared. ⁴Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Not applicable.

4.2. Histopathology and cytopathology

Histological changes in clinically diseased Atlantic salmon are variable, but can include the following:

- i) Numerous erythrocytes in the central venous sinus and lamellar capillaries where erythrocyte thrombi also form in the gills.
- ii) Multifocal to confluent haemorrhages and/or hepatocyte necrosis at some distance from larger vessels in the liver. Focal accumulations of erythrocytes in dilated hepatic sinusoids.
- iii) Accumulation of erythrocytes in blood vessels of the intestinal lamina propria and eventually haemorrhage into the lamina propria.
- iv) Spleen stroma distended by erythrocyte accumulation.
- v) Slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney.
- vi) Erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

Virus has been observed in endothelial cells and leukocytes by electron microscopy of tissue preparations, but this method has not been used for diagnostic purposes.

- Haematocrit <10 in end stages (25–30 often seen in less advanced cases). Haematocrit <10 should always be followed up by investigation for infection with HPR-deleted ISAV in seawater reared Atlantic salmon.
- Blood smears with degenerate and vacuolised erythrocytes and the presence of erythroblasts with irregular nuclear shape. Differential counts show a reduction in the proportion of leucocytes relative to erythrocytes, with the largest reduction being among lymphocytes and thrombocytes.

Liver pathology will lead to increased levels of liver enzymes in the blood.

4.3. Cell culture for isolation

ASK cells (Devold *et al.*, 2000) are recommended for primary HPR-deleted ISAV isolation, but other susceptible cell lines, such as SHK-1 (Dannevig *et al.*, 1995), may be used. However, strain variability and the ability to replicate in different cell lines should be taken into consideration. The ASK cells seem to support isolation and growth of the hitherto known virus isolates. A more distinct cytopathic effect (CPE) may appear in ASK cells. Both the SHK-1 and ASK cell lines appear to lose susceptibility to HPR-deleted ISAV with increasing passage.

The SHK-1 and ASK cells are grown at 20°C in Leibovitz's L-15 cell culture medium supplemented with fetal bovine serum (5% or 10%), L-glutamine (4 mM), gentamicin (50 µg ml⁻¹) and 2-mercapto-ethanol (40 µM) (this latter supplement may be omitted).

For virus isolation, cells grown in 25 cm² tissue culture flasks or multi-well cell culture plates, which may be sealed with parafilm or a plate sealer to stabilise the pH of the medium, may be used. Cells grown in 24-well plates may not grow very well into monolayers, but this trait may vary between laboratories and according to the type of cell culture plates used. Serially diluted HPR-deleted ISAV-positive controls should be inoculated in parallel with the tissue samples as a test for cell susceptibility to HPR-deleted ISAV (this should be performed in a separate location from that of the test samples). See Chapter 2.3.0 for the methods used for inoculation of cell monolayers, monitoring the cultures and sub-cultivation.

Inoculated cell cultures are incubated for at least 14 days and examined as described in Chapter 2.3.0. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification by immunofluorescence (IFAT) (see Section 4.9), real-time PCR or conventional PCR (see Sections 4.4.1 and 4.4.2) as virus replication may occur without apparent CPE.

The procedure has been successful for isolation of HPR-deleted ISAV from fish with clinical signs or from suspect cases.

Until 2022, for HPRO ISAV, there had been no reports of cultivation of HPRO ISAV and no reports of virulence markers in the F segment. However in 2022, a HPRO-like ISAV variant within the North American clade was described and cultured on ASK and SHK-1 cells (Ditlecadet *et al.*, 2022). This variant was found to have F virulence markers, but experimental studies in fish for this variant have not yet been published.

Cell lines should be monitored to ensure that their susceptibility to targeted pathogens has not changed.

4.4. Nucleic acid amplification

4.4.1. Real-time RT-PCR

The primers and probes shown in Table 4.4.1.1 for real-time RT-PCR will detect both European and North-American HPR-deleted ISAV and HPRO ISAV. Real-time RT-PCR may be used for detection of ISAV from total RNA (or total nucleic acid) extracted from recommended organs/tissues (see Section 3.2) and is recommended over RT-PCR (see Section 4.4.2.) as it has increased specificity and, probably, also sensitivity. The primer sets derived from genomic segment 8 and segment 7 have been used by several laboratories and have been found suitable for detection of ISAV during disease outbreaks and in apparently healthy carrier fish.

With the widespread occurrence of HPRO ISAV variants, it is essential to follow up any positive RT-PCR results based on segment 7 or 8 primer sets by sequence analysis of the HPR in segment 6 in order to determine if the virus is either HPR-deleted or HPRO ISAV or both. Primers, designed and validated by the OIE Reference Laboratory, are given in Table 4.4.2.1. Validation of the HPR primer set for the North American HPRO isolates is restricted by the limited sequence data available in the Genbank for the 3' end of ISAV segment 6 (Marshall *et al.*, 2014).

The primers for segment 7 and 8 as well as sequencing primers for segment 6 HPR, are listed below and may also be used for conventional RT-PCR if necessary.

Table 4.4.1.1. Primer and probes sequences and cycling conditions for ISAV real-time RT-PCR

Primer and probe sequences (5'→3') (concentration)	Cycling conditions	Genomic segment	Amplicon size (bp)	Reference
For: CAG-GGT-TGT-ATC-CAT-GGT-TGA-AAT-G (900nM) Rev: GTC-CAG-CCC-TAA-GCT-CAA-CTC- (900nM) Probe: 6FAM-CTC-TCT-CAT-TGT-GAT-CCC-MGBNFQ (250nM)	1 × 2 minutes @ 50°C 1 × 10 minutes @ 95°C	7	155	Snow <i>et al.</i> , 2006
For: CTA-CAC-AGC-AGG-ATG-CAG-ATG-T (900 nM) Rev: CAG-GAT-GCC-GGA-AGT-CGA-T (900 nM) Probe: 6FAM-CAT-CGT-CGC-TGC-AGT-TC-MGBNFQ (250 nM)	45 × 15 seconds @ 95°C and 1 minute @ 60°C	8	104	Snow <i>et al.</i> , 2006

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal RT-PCR control. The positive control should be distinguishable from viral genomic sequence, thus allowing detection of any cross-contamination leading to false positive results.

4.4.2. Conventional RT-PCR

The primers described in Table 4.4.2 for RT-PCR will detect both European and North-American HPR-deleted ISAV and HPRO ISAV. RT-PCR may be used for detection of ISAV from total RNA (or total nucleic acid) extracted from recommended organs/tissues (see Section 3.2). However, the real-time RT-PCR (see Section 4.4.1.) for the detection of ISAV is recommended as it has increased specificity and, probably, also sensitivity.

Table 4.4.2.1. Primer sequences and cycling conditions for ISAV Segment 6 RT-PCR

Primer sequences (5'→3') (concentration)	Cycling conditions	Amplicon size (bp)	Reference
For: GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA (200 nM) Rev: GAT-GGT-GGA-ATT-CTA-CCT-CTA-GAC-TTG- TA (200 nM)	1 × 30 minutes @ 50°C 1 × 2 minutes @ 94°C 40 × 1 minute @ 94°C, 1 minute @ 50°C, 1 minute @ 68°C 1 × 7 minutes @ 68°C	304 if HPRO	Designed by OIE Ref. Lab.

4.5. Amplicon sequencing

There is evidence of the generation of complete amplicons for the eight segments of the viral genome that include the 5' and 3' ends of each one (Toro-Ascuy et al., 2015).

The segment 6 assay primers given in Section 4.4.2 are used for RT-PCR and amplicon sequencing.

4.6. *In-situ* hybridisation

Published methods are available but not recommended due to lack of validation.

4.7. Immunohistochemistry (IHC)

4.7.1. IHC on paraffin sections from formalin-fixed tissue

Antibody against HPR-deleted ISAV nucleoprotein is used on paraffin sections from formalin-fixed tissue. This IHC staining has given positive reactions in both experimentally and naturally infected Atlantic salmon. Preferred organs are mid-kidney and heart (transitional area including all three chambers and valves). Suspect cases due to pathological signs are verified with a positive IHC. Histological sections are prepared according to standard methods.

i) Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene or isopropanol and embedded in paraffin, according to standard protocols. Approximately 3 µm thick sections (for IHC sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for at least 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for pathomorphology and IHC as described below.

ii) Staining procedure for IHC

All incubations are carried out at room temperature on a rocking platform, unless otherwise stated.

- Antigen retrieval is achieved by boiling sections in 0.1 M citrate buffer pH 6.0 for 2 × 5 minutes followed by blocking with 5% non-fat dry milk and 2% goat serum in 50 mM TBS (TBS; Tris/HCl 50 mM, NaCl 150 mM, pH 7.6) for 20 minutes.
- Sections are then incubated overnight at 4°C with primary antibody (e.g. an antibody against ISAV nucleoprotein) diluted in TBS with 1% non-fat dry milk, followed by three washes in TBS, the last wash with 0.1% Tween 20.
- For detection of bound antibodies, sections are incubated with biotinylated species specific IgG (diluted in 2.5% BSA in Tris buffer) for 60 minutes, followed by ABC-AP (diluted in Tris buffer) for 45 minutes. Following a final wash, Fast Red (1 mg ml⁻¹) and Naphthol AS-MX phosphate (0.2 mg ml⁻¹) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) are added to develop for 20 minutes. Sections are then washed in tap water before counterstaining with Harris

haematoxylin and mounted in aqueous mounting medium. ISAV positive and ISAV negative tissue sections are included as controls in every setup.

iii) Interpretation

Negative control sections should not have any significant colour reactions. Positive control sections should have clearly visible red-coloured cytoplasmic and intranuclear staining of endothelial cells in blood vessels or heart endocardium. A test sample section should only be regarded as positive if clear, intranuclear red staining of endothelial cells is found. The intranuclear localisation is particular to the orthomyxovirus nucleoprotein during a stage of virus replication. Concurrent cytoplasmic staining is often dominant. Cytoplasmic and other staining patterns without intranuclear localisation must be considered as nonspecific or inconclusive.

The strongest positive staining reactions are usually obtained in endothelial cells of heart and kidney. Endothelial staining reactions within very extensive haemorrhagic lesions can be slight or absent, possibly because of lysis of infected endothelial cells.

4.7.2. IFAT on tissue imprints and blood smears

An indirect fluorescent antibody test (IFAT) using validated MABs against ISAV haemagglutinin-esterase (HE) on kidney imprints, on blood smears or on frozen tissue sections of kidney, heart and liver has given positive reactions in both experimentally and naturally infected Atlantic salmon. Suspect cases (see Section 6.1) may be confirmed with a positive IFAT.

i) Preparations of tissue imprints

A small piece of the mid-kidney is briefly blotted against absorbent paper to remove excess fluid, and several imprints in a thumbnail-sized area are made on poly-L-lysine-coated microscope slides. The imprints are air-dried, fixed in chilled 100% acetone for 10 minutes and stored either at 4°C for a few days or at –80°C until use.

ii) Staining procedure

After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS) for 30 minutes, the preparations are incubated for 1 hour with an appropriate dilution of anti-ISAV MAB, followed by three washes. For the detection of bound antibodies, the preparations are incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig for 1 hour. PBS with 0.1% Tween 20 is used for washing. All incubations are performed at room temperature.

iii) Preparation of blood smear

Blood fraction is obtained using a discontinuous Percoll gradient. A small fraction is smeared on poly-L-lysine-coated microscope slide. The smear is air-dried, fixed in chilled 100% acetone for 10 minutes and stored either at 4°C for a few days or at –80°C until use.

iv) Staining procedure

After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS) for 30 minutes, the preparation is incubated for 1 hour with appropriate dilution of anti-ISAV MAB, followed by three washes. For the detection of bound antibodies, the preparation is incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig for 1 hour. PBS with 0.1% Tween 20 is used for washing. All incubations are performed at room temperature.

4.8. Bioassay

Not available.

4.9. Antibody- or antigen-based detection methods

4.9.1. Virus identification by IFAT

All incubations are carried out at room temperature unless otherwise stated.

- i) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well or 24-well plates), in slide flasks or on cover-slips dependent on the type of microscope available (an inverted fluorescent microscope is necessary for monolayers grown on tissue culture plates). SHK-1 cells grow rather poorly on glass cover-slips. The necessary monolayers for negative and positive controls must be included.
- ii) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 7 days or, if CPE appears, for a shorter time.
- iii) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. The fixed cell cultures may be stored dry for less than 1 week at 4°C or at –20°C for longer storage.
- iv) Incubate the cell monolayers with anti-HPR-deleted ISAV MAb in an appropriate dilution in PBS for 1 hour, and rinse twice with PBS/0.05% Tween 20. If non-specific binding is observed, incubate with PBS containing 0.5% dry skimmed milk.
- v) Incubate with FITC-conjugated species specific immunoglobulin antibody for 1 hour or if antibody raised in rabbits is used as the primary antibody, use FITC-conjugated antibody against rabbit immunoglobulin, according to the instructions of the supplier. To increase the sensitivity, FITC-conjugated goat anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with the described rinsing in between the additional step. Rinse once with PBS/0.05% Tween 20, as described above. The nuclei can be stained with propidium iodide (100 µg ml⁻¹ in sterile distilled water). Add PBS (without Tween 20) and examine under fluorescent microscope. To avoid fading, the stained plates should be kept in the dark until examination. To reduce photobleaching of FITC due to exposure to excitation light during microscopy, a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

4.10. Other methods

None published or validated.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time RT-PCR is validated for surveillance to demonstrate freedom in apparently healthy populations.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status¹

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with HPR0 or HPR-deleted ISAV shall be suspected if at least one of the following criteria is met:

- ii) Positive result by conventional RT-PCR
- iii) Positive result by real-time RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to OIE.

Definition of confirmed case of infection with HPR-deleted ISAV

The presence of infection with HPR-deleted ISAV is considered to be confirmed if, in addition to the criteria in Section 6.1.1, at least one of the following points is met:

- i) Detection of ISAV in tissue samples by RT-PCR (conventional or real-time) and detection of ISAV in histological sections of internal organs by immunoassay using specific anti-ISAV antibodies (IFAT or immunohistochemistry)
- ii) Detection of ISAV in tissue samples by RT-PCR (conventional or real-time) and conventional PCR of segment 6 and sequencing of the amplicon to verify HPR-deletion

Definition of confirmed case of infection with HPR0 ISAV

The presence of infection with HPR0 ISAV is considered to be confirmed if the following criterion is met:

- i) Detection of ISAV in tissue samples by real-time RT-PCR and detection of ISAV by conventional RT-PCR of segment 6 and sequencing of the amplicon to verify HPR0-deletion

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with HPR-deleted ISAV shall be suspected if at least one of the following criteria is met:

- i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality
- ii) Histo- or cytopathological changes consistent with the presence of the pathogen or the disease
- iii) ISAV-typical CPE in cell culture

¹ For example transboundary commodities.

- iv) Positive result by a real-time RT-PCR
- v) Positive result of a conventional RT-PCR
- vi) Positive result by immunohistochemistry
- vii) Positive result by IFAT on tissue imprints

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with HPR-deleted ISAV is considered to be confirmed if at least one of the following criteria is met:

- i) Virus isolation with ISAV-typical CPE in cell culture and virus identification by RT-PCR (conventional or real-time) followed by sequencing of the amplicon
- ii) Detection of ISAV in tissue samples by RT-PCR (conventional or real-time) and detection of ISAV in histological sections by immunoassay using specific anti-ISAV antibodies (IFAT or immunohistochemistry)
- iii) Detection of ISAV in tissue samples by RT-PCR (conventional or real-time) and conventional RT-PCR of segment 6 and sequencing of the amplicon to verify HPR-deletion

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with ISAV are provided in Table 6.3. This information can be used for the design of surveys for infection with ISAV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals [under study]

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (<i>n</i>)	DSp (<i>n</i>)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study.

6.3.2. For surveillance of apparently healthy animals [under study]

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (<i>n</i>)	DSp (<i>n</i>)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study.

7. References

- AAMELFOT M., CHRISTIANSEN D.H., DALE O.B., McBEATH A., BENESTAD S.L. & FALK K. (2016) Localised Infection of Atlantic Salmon Epithelial Cells by HPR0 Infectious Salmon Anaemia Virus. *PLoS ONE*, **11**(3): e0151723. <https://doi.org/10.1371/journal.pone.0151723> 2015.
- AAMELFOT M., DALE O.B., WELI S., KOPPANG E.O. & FALK K. (2012). Expression of 4-O-acetylated sialic acids on Atlantic salmon endothelial cells correlates with cell tropism of Infectious salmon anemia virus. *J. Virol.*, **86**, 10571–10578.
- CARDENAS C., CARMONA M., GALLARDO A., LABRA A. & MARSHALL S.H. (2014). Coexistence in field samples of two variants of the infectious salmon anemia virus: a putative shift to pathogenicity. *PLoS One*, **9**, e87832. doi: 10.1371/journal.pone.0087832.
- CARDENAS C., GUZMÁN F., CARMONA M., MUÑOZ C., NILO L., LABRA A. & MARSHALL S.H. (2020). Synthetic Peptides as a Promising Alternative to Control Viral Infections in Atlantic Salmon. *Pathogens*, **9**, 600.
- CARDENAS C., OJEDA N., LABRA Á. & MARSHALL S.H. (2019). Molecular features associated with the adaptive evolution of Infectious Salmon Anemia Virus (ISAV) in Chile. *Infect. Genet. Evol.*, **68**, 203–211. doi: 10.1016/j.meegid.2018.12.028.
- CHRISTIANSEN D.B., McBEATH A.J.A., AAMELFOT M., MATEJUSOVA I., FOURRIER M., WHITE P., PETERSEN P.E. & FALK K. (2017). First field evidence of the evolution from a non-virulent HPR0 to a virulent HPR-deleted infectious salmon anaemia virus. *J. Gen. Virol.*, **98**, 595–606. doi: 10.1099/jgv.0.000741. PMID: 28475029.
- CHRISTIANSEN D.H., ØSTERGAARD P.S., SNOW M., DALE O.B. & FALK K. (2011). A low-pathogenic variant of infectious salmon anemia virus (ISAV1 - HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (*Salmo salar* L.) in the Faroe Islands. *J. Gen. Virol.*, **92**, 909–918.
- COTTET L., RIVAS-ARAVENA A., CORTEZ-SAN MARTIN M., SANDINO A.M. & SPENCER E. (2011). Infectious salmon anemia virus – genetics and pathogenesis. *Virus Res.*, **155**, 10–19.
- CLOUTHIER S.C., RECTOR T., BROWN N.E.C. & ANDERSON E.D. (2002). Genomic organization of infectious salmon anaemia virus. *J. Gen. Virol.*, **83**, 421–428.
- CUNNINGHAM C.O., GREGORY A., BLACK J., SIMPSON I. & RAYNARD R.S. (2002). A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. *Bull. Eur. Assoc. Fish Pathol.*, **22**, 366–374.
- DANNEVIG B.H., FALK K. & NAMORK E. (1995). Isolation of the causal virus of infectious salmon anemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *J. Gen. Virol.*, **76**, 1353–1359.
- DITLECADET D., GAUTREAU C., BOSTON L., LISTON R., JOHNSEN E. & GAGNE N. (2022). First report of successful isolation of a HPR0-like variant of the infectious salmon anaemia virus (ISAV) using cell culture. *J. Fish Dis.*, **45**, 479–483. doi: 10.1111/jfd.13556. Epub 2021 Nov 29.
- DEVOLD M., KARLSEN M. & NYLUND A. (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.
- DEVOLD M., KROSSOY B., ASPEHAUG V. & NYLUND A. (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.
- EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2012) EFSA Panel on Animal Health and Welfare (AHAW); Scientific Opinion on infectious salmon anaemia. *EFSA Journal*, **10**, 2971.
- FALK K., NAMORK E., RIMSTAD E., MJAALAND S. & DANNEVIG B.H. (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *J. Virol.*, **71**, 9016–9023.
- GAGNE N. & LEBLANC F. (2017). Overview of infectious salmon anaemia virus (ISAV) in Atlantic Canada and first report of an ISAV North American-HPR0 subtype. *J. Fish Dis.*, doi: 10.1111/jfd.12670

- GARCIA-ROSADO E., MARKUSSEN T., KILENG O., BAEKKEVOLD E.S., ROBERTSEN B., MJAALAND S. & RIMSTAD E. (2008). Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. *Virus Res.*, **133**, 228–238. doi: 10.1016/j.virusres.2008.01.008.
- GIRAY C., OPITZ H.M., MACLEAN S. & BOUCHARD D. (2005). Comparison of lethal versus non-lethal sample sources for the detection of infectious salmon anemia virus (ISAV). *Dis. Aquat. Org.*, **66**, 181–185.
- GJOEN H.M., REFSTIE T., ULLA O. & GJERDE B. (1997). Genetic correlations between survival of Atlantic salmon in challenge and field tests. *Aquaculture*, **158**, 277–288.
- HAMMELL K.L. & DOHOO I.R. (2005). Mortality patterns in infectious salmon anaemia virus outbreaks in New Brunswick, Canada. *J. Fish Dis.*, **28**, 639–650. doi: 10.1111/j.1365-2761.2005.00667.x.
- KAWAOKA Y., COX N.J., HALLER O., HONGO S., KAVERIN N., KLENK H.D., LAMB R.A., MCCAULEY J., PALESE P., RIMSTAD E. & WEBSTER R.G. (2005). Infectious Salmon Anaemia Virus. In: *Virus Taxonomy – Eight Report of the International Committee on Taxonomy Viruses*, Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A., eds. Elsevier Academic Press, New York, USA, pp 681–693.
- KIBENGE F.S.B., GODOY M.G., WANG Y., KIBENGE M.J.T., GHERARDELLI V., MANSILLA S., LISPERGER A., JARPA M., LARROQUETE G., AVENDAÑO F., LARA M. & GALLARDO A. (2009). Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. *Viol. J.*, **6**, 88.
- KIBENGE F.S.B., KIBENGE M.J.T., WANG Y., QIAN B., HARIHARAN S. & MCGEACHY S. (2007). Mapping of putative virulence motifs on infectious salmon anaemia virus surface glycoprotein genes. *J. Gen. Virol.*, **88**, 3100–3111.
- KULSHRESHTHA V., KIBENGE M., SALONIUS K., SIMARD N., RIVEROLL A. & KIBENGE F. (2010). Identification of the 3' and 5' terminal sequences of the 8 RNA genome segments of European and North American genotypes of infectious salmon anaemia virus (an orthomyxovirus) and evidence for quasispecies based on the non-coding sequences of transcripts. *Viol. J.*, **7**, 338.
- LOVDAL T. & ENGER O. (2002). Detection of infectious salmon anaemia virus in seawater by nested RT-PCR. *Dis. Aquat. Org.*, **49**, 123–128.
- LYNGSTAD T.M., HJORTAAS M.J., KRISTOFFERSEN A.B., MARKUSSEN T., KARLSEN E.T., JONASSEN C.M. & JANSEN P.A. (2011). Use of molecular epidemiology to trace transmission pathways for infectious salmon anaemia virus (ISAV) in Norwegian salmon farming. *Epidemics*, **3**, 1–11.
- LYNGSTAD T.M., JANSEN P.A., SINDRE H., JONASSEN C.M., HJORTAAS M.J., JOHNSEN S. & BRUN E. (2008). Epidemiological investigation of infectious salmon anaemia (ISA) outbreaks in Norway 2003–2005. *Prev. Vet. Med.*, **84**, 213–227.
- MARKUSSEN T., JONASSEN C.M., NUMANOVIC S., BRAAEN S., HJORTAAS M., NILSEN H. & MJAALAND S. (2008). Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus. *Virology*, **374**, 515–527.
- MCBEATH A., AAMELFOT M., CHRISTIANSEN D.H., MATEJUSOVA I., MARKUSSEN T., KALDHUSDAL M., DALE O.B., WELI S.C. & FALK K. (2015). Immersion challenge with low and highly virulent infectious salmon anaemia virus reveals different pathogenesis in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.*, **38**, 3–15.
- MARSHALL S.H., RAMÍREZ R., LABRA A., CARMONA M. & MUÑOZ C. (2014). Bona Fide Evidence for Natural Vertical Transmission of Infectious Salmon Anemia Virus in Freshwater Brood Stocks of Farmed Atlantic Salmon (*Salmo salar*) in Southern Chile. *J. Virol.*, **88**, 6012–6018. doi: 10.1128/JVI.03670-13.
- MJAALAND S., HUNGNES O., TEIG A., DANNEVIG B.H., THORUD K. & RIMSTAD E. (2002). Polymorphism in the infectious salmon anemia virus hemagglutinin gene; importance and possible implications for evolution and ecology of infectious salmon anemia disease. *Virology*, **302**, 379–391.
- MJAALAND S., MARKUSSEN T., SINDRE H., KJOGLUM S., DANNEVIG B.H., LARSEN S. & GRIMHOLT U. (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.

- MJAALAND S., RIMSTAD E., FALK K. & DANNEVIG B.H. (1997). Genomic characterisation 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.
- OELCKERS K., VIKE S., DUESUND H., GONZALEZ J., WADSWORTH S. & NYLUND A. (2014). *Caligus rogercresseyi* as a potential vector for transmission of Infectious Salmon Anaemia (ISA) virus in Chile. *Aquaculture*, **420–421**, 126–132.
- RAMIREZ R. & MARSHALL S.H. (2018). Identification and isolation of infective filamentous particles in Infectious Salmon Anemia Virus (ISAV). *Microb. Pathogenesis*, **117**, 219–224. <https://doi.org/10.1016/j.micpath.2018.02.029>
- RAMLY R.B., OLSEN C.M., BRAAEN S. & RIMSTAD E. (2013). Infectious salmon anemia virus nuclear export protein is encoded by a spliced gene product of genomic segment 7. *Virus Res.*, **177**, 1–10. doi: 10.1016/j.virusres. 2013.07.001.
- RIMSTAD E., DALE O.B., DANNEVIG B.H. & FALK K. (2011). Infectious Salmon Anaemia. In: Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections, Woo P.T.K. & Bruno D., eds. CAB International, Oxfordshire, UK, 143–165.
- RIVAS-ARAVENA A., VALLEJOS-VIDAL E., MARTIN M.C., REYES-LOPEZ F., TELLO M., MORA P., SANDINO A.M., SPENCER E. (2011). Inhibitory effect of a nucleotide analog on ISAV infection. *J. Virol.*, **85**, 8037–8045.
- SANDVIK T., RIMSTAD E. & MJAALAND S. (2000). The viral mRNA transcription and the structure of the 3'- and 5'-ends of viral RNA of infectious salmon anaemia virus resemble that of influenza viruses. *Arch. Virol.*, **145**, 1659–1669.
- SMAIL D.A. & GRANT R. (2012). The stability of infectious salmon anaemia virus infectivity at –80°C in tissue homogenate and dry-stored tissue from clinically diseased Atlantic salmon, *Salmo salar* L. *J. Fish Dis.*, **35**, 789–792. doi.org/10.1111/j.1365-2761.2012.01402.x
- SNOW M., MCKAY P., McBEATH A. J. A., BLACK J., DOIG F., KERR R., CUNNINGHAM C. O., NYLUND A. & DEVOLD M. (2006). Development, application and validation of a taqman® real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*), Vannier P. & Espeseth D., eds. New Diagnostic Technology: Applications in Animal Health and Biologics Controls. *Dev. Biol.*, Basel, Karger. **126**, 133–145.
- THORUD K.E. & DJUPVIK H.O. (1988). Infectious salmon anaemia in Atlantic salmon (*Salmo salar* L). *Bull. Eur. Assoc. Fish Pathol.*, **8**, 109–111.
- THUKRAL V., VARSHNEY B., RAMLY R., PONIA S., KARJEE S., OLSEN C., BANERJEA A., MUKHERJEE S., ZAIDI R., RIMSTAD E. & LAL S.K. (2018). s8ORF2 protein of ISAV is an RNAi Suppressor and interacts with SsMov10 of host RNAi machinery. *Virus Genes*, **54**, 199–214 doi: 10.1007/s11262-017-1526-z
- TORO-ASCUY D., TAMBLEY C., BELTRAN C., MASCAYANO C., SANDOVAL N., OLIVARES E., MEDINA R.A., SPENCER E. & CORTEZ-SAN MARTÍN M. (2015). Development of a reverse genetic system for infectious salmon anemia virus: rescue of recombinant fluorescent virus by using salmon internal transcribed spacer region 1 as a novel promoter. *Appl. Environ. Microbiol.*, **81**, 1210–1224. <https://doi.org/10.1128/AEM.03153-14>.
- TOTLAND G.K., HJELTNES B. & FLOOD P.R. (1996). Transmission of infectious salmon anaemia (ISA) through natural secretions and excretions from infected smolts of Atlantic salmon *Salmo salar* during their presymptomatic phase. *Dis. Aquat. Org.*, **26**, 25–31.
- VIKE S., OELCKERS K., DUESUND H., ERGA S.R., GONZALEZ J., HAMRE B., FRETTE O. & NYLUND A. (2014). Infectious salmon anemia (ISA) virus: infectivity in seawater under different physical conditions. *J. Aquat. Anim. Health*, **26**, 33–42. doi: 10.1080/08997659.2013.864720.

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NB: There is a OIE Reference Laboratory for infection with infectious salmon anaemia virus
(please consult the OIE Web site for the most up-to-date list:
<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
Please contact OIE Reference Laboratories for any further information on
infection with infectious salmon anaemia virus

NB: FIRST ADOPTED IN 1995 AS INFECTIOUS SALMON ANAEMIA. MOST RECENT UPDATES ADOPTED IN 2022.