CHAPTER 2.3.5.

INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

1. Scope

For the purpose of this chapter, infection with infectious salmon anaemia virus (ISAV) means infection with highly polymorphic region (HPR)-deleted ISAV or HPR0 ISAV (with a non-deleted HPR) of the genus *Isavirus* of the family *Orthomyxoviridae*.

Infection with HPR-deleted ISAV may cause infectious salmon anaemia (ISA) in Atlantic salmon (*Salmo salar*), which is a generalised and lethal condition characterised by severe anaemia, and variable haemorrhages and necrosis in several organs. The disease course is prolonged with low daily mortality (0.05–0.1%) typically only in a few cages. Cumulative mortality may become very high for a period lasting several months if nothing is done to limit disease dissemination (Rimstad et al., 2011).

Detection of HPR0 ISAV has never been associated with ISA in Atlantic salmon (Christiansen et al., 2011). This virus genotype replicates transiently and has mainly been localised to the gills. A link between non-pathogenic HPR0 ISAV and pathogenic HPR-deleted ISAV, with some outbreaks potentially occurring as a result of the emergence of HPR-deleted ISAV from HPR0 ISAV has been suggested (Cunningham et al., 2002; Mjaaland et al., 2002).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

ISAV is an enveloped virus, 100–130 nm in diameter, with a genome consisting of eight single-stranded RNA segments with negative polarity (Dannevig et al., 1995. The virus has haemagglutinating, receptor-destroying and fusion activity (Falk et al., 1997; Mjaaland et al., 1997; Rimstad et al., 2011).

The morphological, physiochemical 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. 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). 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 for a nuclear export protein (NEP). Whether the ORF1 gene product is nonstructural or a structural component of the virion remains to be determined. 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.

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas. According to sequence differences in the 5'-region of the HE gene, ISAV isolates have been divided into two major groups, one European and one North American group. 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 (HPR0) has been suggested to represent a precursor from which all ISAV HPR-deleted (pathogenic) variants of ISAV originate. The presence of nonpathogenic HPR0 ISAV genome has been reported in both apparently healthy wild and farmed Atlantic salmon, but has not been detected in

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1 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2014.
diseased fish with clinical disease and pathological signs consistent with ISA (Christiansen et al., 2011; Cunningham et al., 2002; Lyngstad et al., 2012; Markussen et al., 2008; McBeath A. et al., 2009; Nylund et al., 2007). A mixed infection of HPR-deleted and HPR0 ISAV variants has been reported (Kibenge et al., 2009). Recent studies show that HPR0 ISAV variants occur frequently in sea-reared Atlantic salmon. The HPR0 ISAV strain seems to be more seasonal and transient in nature and displays a tissue tropism with high prevalence in gills (Christiansen et al., 2011; Lyngstad et al., 2008). To date there has been no direct evidence linking the presence of HPR0 ISAV to a subsequent clinical ISA 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 (Christiansen et al., 2011; European Food Safety Authority, 2012; Lyngstad et al., 2012).

In addition to the variations seen in the HPR of the HE gene, other gene segments may also be of importance for development of ISA. A putative virulence marker has been identified in the fusion (F) protein. Here, a single amino acid substitution, or a 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). Aside from insertion/recombination, ISAV also uses gene segment reassortment in its evolution, with potential links to virulence (Devold et al., 2006; Markussen et al., 2008; Mjaaland et al., 2005).

2.1.2. Survival outside the host

ISAV has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in seawater sampled at farming sites with ISAV-positive Atlantic salmon (Kibenge et al., 2004). 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. Exposing cell culture-propagated ISAV to 15°C for 10 days or to 4°C for 14 days had no effect on virus infectivity (Falk et al., 1997).

2.1.3. Stability of the agent (effective inactivation methods)

ISAV is sensitive to UV irradiation (UVC) and ozone. A 3-log reduction in infectivity in sterile fresh water 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 infectivity. Incubation in the presence of chlorine (100 mg ml⁻¹) for 15 minutes also inactivated 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).

2.1.4. Life cycle

The main infection route is most likely through the gills for both HPR0 and HPR-deleted ISAV, but infection via the intestine or skin cannot be excluded. HPR-deleted ISAV has been used in the studies referred to below. Endothelial cells lining blood vessels seem to be the primary target cells for ISAV 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). As endothelial cells are the target cells (see Section 2.2.4), virus replication may occur in any organ (Aamelfot et al., 2012; Rimstad et al., 2011).

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 alpha-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).

The route of shedding of ISAV from infected fish may be through natural excretions/secretions.

The HPR0 variant has not been isolated in cell culture, which hampers in-vivo and in-vitro studies of characteristics and the life cycle of this virus variant.
2.2. Host factors

2.2.1. Susceptible host species

Natural outbreaks of ISA have only been recorded in farmed Atlantic salmon, and in Coho salmon (*Onchorhynchus kisutch*) in Chile (Kibenge et al., 2001). Subclinically infected feral Atlantic salmon, brown trout and sea trout (*S. trutta*) have been identified by RT-PCR (Kibenge et al., 2004; Plarre et al., 2005). In marine fish, detection of ISAV by RT-PCR has been reported in tissues of pollock (*Pollachius virens*) and cod (*Gadus morhua*), but only in fish collected from cages with Atlantic salmon exhibiting ISA (MacLean et al., 2003). Following experimental infection by bath immersion, ISAV has been detected by RT-PCR in rainbow trout (*Onchorhynchus mykiss*) (Biacchesi et al., 2007) and herring (*Clupea harengus*), the latter in a subsequent transmission to Atlantic salmon. Attempts have been made to induce infection or disease in pollock, *Pollachius virens*, but with negative results.

2.2.2. Susceptible stages of the host

In Atlantic salmon, disease outbreaks are mainly reported in seawater cages, and only a few cases have been reported in the freshwater stage, including one case in yolk sac fry (Rimstad et al., 2011). ISA has been experimentally induced in both Atlantic salmon fry and parr kept in freshwater. Genetics may also play an important role in the susceptibility of Atlantic salmon to ISA, as differences in susceptibility among different family groups have been observed.

2.2.3. Species or subpopulation predilection (probability of detection)

ISA is primarily a disease of Atlantic salmon.

2.2.4. Target organs and infected tissue

For fish that have developed ISA: endothelial cells in all organs become infected (gills, heart, liver, kidney, spleen and others) (Aamelfot et al., 2012). HPR0 ISAV variants seem primarily to target the gills, but this variant has also been detected in kidney and heart (Christiansen et al., 2011; Lyngstad et al., 2011).

2.2.5. Persistent infection with lifelong carriers

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 HPR0 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 ISAV indicate that persistent infection in these species could be possible (Rimstad et al., 2011).

2.2.6. Vectors

Passive transfer of ISAV by salmon lice (*Lepeophtheirus salmonis*) has been demonstrated under experimental conditions. Although natural vectors have not been identified, several different vector groups could be possible vectors under certain defined conditions (reviewed in Rimstad et al., 2011).

2.2.7. Known or suspected wild aquatic animal carriers

Wild Atlantic salmon, brown trout and sea trout may be carriers of ISAV (Rimstad et al., 2011). The importance of wild marine fish (see Section 2.2.1) as virus carriers needs to be clarified. The results from a study from the Faroe Islands point to the potential presence of an unknown marine reservoir for this virus (Christiansen et al., 2011).

2.3. Disease pattern

2.3.1. Transmission mechanisms

Studies of recurrent epidemics of ISA in different salmon-producing areas conclude that the virus spreads locally between adjoining sites. Proximity to sites with ISA outbreaks is a risk of primary importance, and the risk for a susceptible farm increases the nearer it is to an infected farm. Sequence analysis of ISAV from ISA outbreaks in Norway shows a high degree of similarity between viruses isolated from neighbouring ISA affected sites, further supporting ISAV transmission between proximate sites. The risk of transmission of ISAV is dependent on the level of biosecurity measures in place. Suggested pathways for ISAV transmission are
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through sea water, shipment of live fish, transmission through sea lice, and via infected wild salmonids (Aldrin et al., 2011; Gustafson et al., 2007; Lyngstad et al., 2011; Mardones et al., 2011; Rimstad et al., 2011).

Many ISA outbreaks in Norway appear to be isolated in space and time from other outbreaks with unknown sources of infection (Aldrin et al., 2011). A suggested hypothesis for disease emergence is occasional transition of HPR0 ISAV into HPR-deleted ISAV variants causing solitary outbreaks or local epidemics through local transmission (Lyngstad et al., 2011; Lyngstad et al., 2012). The risk of emergence of HPR-deleted ISAV variants from a reservoir of HPR0 ISAV is considered to be low but not negligible (European Food Safety Authority, 2012). A direct link between HPR0 variants and HPR-deleted ISAV remains to be demonstrated.

As ISA has also been reported from smolt-producing sites with Atlantic salmon, transmission of ISAV from parent to progeny cannot be excluded. Even though there is no evidence of true vertical transmission, eggs and embryos could be a risk of transmission if ISAV biosecurity measures are not adequate (Mardones et al., 2014; Rimstad et al., 2011).

2.3.2. Prevalence

In a net pen containing diseased fish, the prevalence of HPR-deleted ISAV may vary widely, while in adjacent net pens ISAV may be difficult to detect, even by the most sensitive methods. Therefore, for diagnostic investigations it is important to sample from net pens containing diseased fish.

There is increasing evidence that the prevalence of the nonpathogenic HPR0 ISAV genotype may be high in Atlantic salmon production areas. HPR0 variants in Atlantic salmon appear to be a seasonal and transient infection (Christiansen et al., 2011). HPR0 variants of ISAV have also been detected in wild salmonids (reviewed in Rimstad et al., 2011).

2.3.3. Geographical distribution

Initially reported in Norway in the mid-1980s (Thorud & Djupvik, 1988), ISA in Atlantic salmon has since then been reported in Canada (New Brunswick in 1996; Mullins et al., 1998), the United Kingdom (Scotland in 1998), the Faroe Islands (2000), the USA (Maine in 2001) and in Chile (2007) (Cottet et al., 2011; Rimstad et al., 2011). The presence of the HPR0 ISAV variant has been reported in all countries where ISA has occurred.

2.3.4. Mortality and morbidity

During ISA outbreaks, morbidity and mortality may vary greatly within and between different net pens in a seawater fish farm, and between different fish farms. Morbidity and mortality within a net pen may start at very low levels. Typically, daily mortality ranges from 0.5 to 1% in affected cages. Without intervention, mortality increases and seems to peak in early summer and winter. The range of cumulative mortality during an outbreak is from insignificant to moderate, but in severe cases, cumulative mortality exceeding 90% may be recorded during several months. Initially, an outbreak of ISA may be limited to one or two net pens over a long time period. In such cases, if net pens with clinical ISA are slaughtered immediately, further development of clinical ISA at the site may be prevented. In outbreaks where smolts have been infected in well boats during transport, simultaneous outbreaks may occur.

HPR0 ISAV has not been associated with ISA in Atlantic salmon.

2.3.5. Environmental factors

Generally, outbreaks of ISA tend to be seasonal with most outbreaks in late spring and late autumn; however outbreaks may occur at any time of the year. 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 in advance (Lyngstad et al., 2008).

2.4. Control and prevention

2.4.1. Vaccination

Vaccination against ISA has been carried out in North America since 1999 and the Faroe Islands since 2005. In Norway vaccination against ISA was carried out for the first time in 2009 in a region with a high rate of ISA outbreaks. Chile started vaccinating against ISA in 2010. However, the currently available vaccines do not seem to offer complete protection in Atlantic salmon.
2.4.2. Chemotherapy

Most recently, it has been demonstrated that the broad-spectrum antiviral drug Ribavirin (1-beta-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).

2.4.3. Immunostimulation

Not applicable.

2.4.4. Resistance breeding

Differences in susceptibility among different family groups of Atlantic salmon in fresh water have been observed in challenge experiments and in field tests, indicating the potential for resistance breeding (Gjøen et al., 1997).

2.4.5. Restocking with resistant species

Not applicable.

2.4.6. Blocking agents

Not applicable.

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs according to standard procedures is suggested as an important control measure.

2.4.8. General husbandry practices

The incidence of ISA 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. The experience from the Faroe Islands, where the prevalence of HPR0 is high, demonstrates that the combination of good biosecurity and husbandry reduces the risk of ISA outbreaks substantially.

3. Sampling

3.1. Selection of individual specimens

The following is primarily for verification of suspected cases based on clinical signs and gross pathology or positive RT-PCR for HPR-deleted ISAV.

For detection of HPR0 ISAV, gill tissue should be sampled in randomly selected individuals at different points of time through the production cycle. Only detection using RT-PCR is possible for this genotype.
3.2. Preservation of samples for submission

<table>
<thead>
<tr>
<th>Haematology:</th>
<th>Heparin or EDTA (ethylene diamine tetra-acetic acid)</th>
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</thead>
<tbody>
<tr>
<td>Cell culture:</td>
<td>Virus transport medium</td>
</tr>
<tr>
<td>Histology and immunohistochemistry:</td>
<td>Fixation in neutral phosphate-buffered 10% formalin</td>
</tr>
<tr>
<td>Immunofluorescence (smears):</td>
<td>Either submitted dried, or dried and fixed in 100% acetone</td>
</tr>
<tr>
<td>Molecular biology (RT-PCR and sequencing):</td>
<td>Appropriate medium for preservation of RNA</td>
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3.3. Pooling of samples

Pooling of samples may be acceptable, however, the impact on sensitivity and design prevalence must be considered.

3.4. Best organs or tissues

3.4.1. Detection of HPR-deleted ISAV

Blood is preferred for non-lethal sampling. Generally, as ISA is a generalised infection, internal organs not exposed to the environment should be used for diagnostic testing.

Virological examination (cell culture and PCR): heart (should always be included) and mid-kidney;

Histology (prioritised): mid-kidney, liver, heart, pancreas/intestine, spleen;

Immunofluorescence (smears): mid-kidney;

Immunohistochemistry: mid-kidney, heart (including valves and bulbus arteriosus).

3.4.2. Detection of HPR0 ISAV

Gills should be tested by RT-PCR.

3.5. Samples/tissues that are not suitable

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4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

The most prominent external signs of ISA 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.

4.2. Pathological evaluation

4.2.1. 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 ISA, but anaemia and circulatory disturbances are always present. The
following findings have been described to be consistent with ISA though all changes are seldom observed in one single fish.

- Yellowish or blood-tinged fluid in peritoneal and pericardial cavities.
- Oedema of the swim bladder.
- Small haemorrhages of the visceral and parietal peritoneum.
- Focal or diffusely dark red liver. A thin fibrin layer may be present on the surface.
- Swollen, dark red spleen with rounded margins.
- Dark redness of the intestinal wall mucosa in the blind sacs, mid- and hind-gut, without blood in the gut lumen of fresh specimens.
- Swollen, dark red kidney with blood and liquid effusing from cut surfaces.
- Pinpoint haemorrhages of the skeletal muscle.

### 4.2.2. Clinical chemistry

- Haematocrit <10 in end stages (25–30 often seen in less advanced cases).
  
  Haematocrit <10 should always be followed up by investigation for ISA in sea-water 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.

### 4.2.3. Microscopic pathology

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

- Numerous erythrocytes in the central venous sinus and lamellar capillaries where erythrocyte thrombi also form in the gills.

- 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.

- Accumulation of erythrocytes in blood vessels of the intestinal lamina propria and eventually haemorrhage into the lamina propria.

- Spleen stroma distended by erythrocyte accumulation.

- Slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney.

- Erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

### 4.2.4. Wet mounts

Not applicable.
4.2.5. Smears
See Section 4.3.1.1.2

4.2.6. Fixed sections
See Section 4.3.1.1.3

4.2.7. Electron microscopy/cytopathology
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.

4.2.8. Differential diagnoses
Other anaemic and haemorrhagic conditions, including erythrocytic inclusion body syndrome, winter ulcer and septicaemias caused by infections with *Moritella viscosa*. Disease cases in Atlantic salmon with haematocrit values below 10 is not a unique finding for ISA, however cases with such low haematocrit values without any obvious explanation should always be tested for the presence of ISAV.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods
With the exception of molecular techniques (see Section 4.3.1.2.3), these direct detection methods are only recommended for fish with clinical signs of infection with HPR-deleted ISAV.

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts
Not applicable.

4.3.1.1.2. Smears

4.3.1.1.2.1. Indirect fluorescent antibody test
An indirect fluorescent antibody test (IFAT) using validated monoclonal antibodies (MAbs) against ISAV haemagglutinin-esterase (HE) on kidney smears (imprints) or on frozen tissue sections of kidney, heart and liver has given positive reactions in both experimentally and naturally infected Atlantic salmon. Suspected cases (see Section 7.1) may be confirmed with a positive IFAT.

i) Preparations of tissue smears (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 fixed 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.

4.3.1.1.3. Fixed sections

4.3.1.1.3.1. Immunohistochemistry (IHC)
Polyclonal antibody against 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). Suspected 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 and embedded in paraffin, according to standard protocols. Approximately 5 µm thick sections (for IHC sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for 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.

a) Antigen retrieval is done by boiling sections in 0.1 M citrate buffer pH 6.0 for 2 × 6 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.

b) Sections are then incubated overnight with primary antibody (monospecific rabbit antibody against ISAV nucleoprotein) diluted in TBS with 1% non-fat dry milk, followed by three washes in TBS with 0.1% Tween 20.

c) For detection of bound antibodies, sections are incubated with Alkaline phosphatase-conjugated antibodies to rabbit IgG for 60 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) is 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.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture

ASK cells (Devold et al., 2000) are recommended for primary 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 for ISAV with increasing passage level.

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 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 ISAV-positive controls should be
inoculated in parallel with the tissue samples as a test for cell susceptibility to ISAV (this should be performed in a separate location from that of the test samples).

i) **Inoculation of cell monolayers**

Prepare a 2% suspension of tissue homogenate using L-15 medium without serum or other medium with documented suitability. Remove growth medium from actively growing monolayers (1- to 3-day old cultures or cultures of 70–80% confluency) grown in 25 cm² tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers (25 cm² tissue culture flasks) with 1.5 ml of the 2% tissue homogenate. Adjust volume to the respective surface area in use. Allow 3–4 hours incubation at 15°C followed by removal of the inoculum, and addition of fresh, L-15 medium supplemented with 2–5% FCS. Alternatively, a 1/1000 dilution and direct inoculation without medium replacement can be used.

When fish samples come from production sites where infectious pancreatic necrosis virus (IPNV) is regarded as endemic, the tissue homogenate supernatant should be incubated (for a minimum of 1 hour at 15°C) with a pool of antisera to the indigenous serotypes of IPNV prior to inoculation.

ii) **Monitoring incubation**

Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of CPE. Typical CPE due to ISAV appears as vacuolated cells that subsequently round up and loosen from the growth surface. If CPE consistent with that described for ISAV or IPNV appears, an aliquot of the medium for virus identification, as described below, must be collected. In the case of an IPNV infection, re-inoculate cells with tissue homogenate supernatant that has been incubated with a lower dilution of IPNV antisera. If no CPE has developed after 14 days, subculture to fresh cell cultures.

iii) **Subcultivation procedure**

Aliquots of medium (supernatant) from the primary cultures are collected 14 days (or earlier when obvious CPE appears) after inoculation. Supernatants from wells inoculated with different dilutions of identical samples may be pooled for surveillance purposes.

Supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 3–4 hours before addition of fresh medium. Alternatively, add supernatants (final dilutions 1/10 and higher) directly to cell cultures with growth medium.

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures with no CPE should always be examined for the presence of ISAV by immunofluorescence (IFAT), haemadsorption or by PCR because virus replication may occur without development of apparent CPE.

The procedure described below has been successful for isolation of HPR-deleted ISAV from fish with clinical signs or from suspected cases. HPR0 has hitherto not been isolated in cell culture.

4.3.1.2.2. Antibody-based antigen detection methods

4.3.1.2.2.1. Virus identification by IFAT

All incubations are carried out at room temperature on a rocking platform, 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 microscope equipped with UV light 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-ISAV MAb in an appropriate dilution in PBS for 1 hour and rinse twice with PBS/0.05% Tween 20. If unspecific binding is observed, incubate with PBS containing 0.5% dry skimmed milk.

v) Incubate with FITC-conjugated goat anti-mouse immunoglobulin 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 UV light. To avoid fading, the stained plates should be kept in dark until examination. For long periods of storage (more than 2–3 weeks) 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.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Reverse-transcription polymerase chain reaction (RT-PCR)

The primers described below for RT-PCR and real-time RT-PCR will detect both European and North-American HPR-deleted ISAV, and also HPR0 ISAV.

RT-PCR may be used for detection of ISAV from total RNA (or total nucleic acid) extracted from recommended organs/tissues (see Section 3.4). The real-time RT-PCR for the detection of ISAV is recommended as it increases the specificity and, probably, also the sensitivity of the test. Though several primer sets for ISAV real-time RT-PCR have been reported, recommended primer sets are presented in the table below. 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 HPR0 ISAV variants, it is essential to follow up any positive PCR results based on segment 7 or 8 primer sets by sequencing the HPR of segment 6 in order to determine the ISAV HPR variant present (HPR-deleted or HPR0 or both). Adequate primers, designed and validated by the OIE Reference Laboratory are given in the table below. Validation of the HPR primer set for the North American isolates is restricted by the limited sequence data available in the Genbank for the 3' end of ISAV segment 6.

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>
<thead>
<tr>
<th>Real-time RT-PCR: Primer sequences</th>
<th>Named</th>
<th>Genomic segment</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-CAG-GGT-TGT-ATC-CAT-GGT-TGA-AAT-G-3' 5-GTC-CAG-CCC-TAA-GCT-CAA-CTC-3' 5'-6FAM-CTC-TCT-CAT-GAT-CAT-CCC-MGBNFQ-3'</td>
<td>forward primer reverse primer Taqman® probe</td>
<td>7</td>
<td>155 nt</td>
<td>Snow et al., 2006</td>
</tr>
<tr>
<td>5'-CTA-CAC-AGC-AGG-ATG-CAG-ATG-T-3' 5'-CAG-GAT-GGC-GGA-AGT-CGA-T-3' 5'-6FAM-CAT-CTG-TCG-AGT-TC-MGBNFQ-3'</td>
<td>forward primer reverse primer Taqman® probe</td>
<td>8</td>
<td>104 nt</td>
<td>Snow et al., 2006</td>
</tr>
<tr>
<td>5'-GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA-3' 5'-GAT-GGT-GGA-ATT-CTA-CCT-CGA-3'</td>
<td>forward primer reverse primer</td>
<td>6 (HPR)</td>
<td>304 nt if HPR0</td>
<td>Designed by OIE Ref. Lab.</td>
</tr>
</tbody>
</table>

4.3.1.2.4. Agent purification

ISAV propagated in cell culture can be purified by sucrose gradient centrifugation (Falk et al., 1997) or by affinity purification using immunomagnetic beads coated with anti-ISAV MAb.
4.3.2. Serological methods

Both Atlantic salmon and rainbow trout develop a humoral immune response to the ISAV infection. Enzyme-linked immunosorbent assays (ELISAs) with either purified virus or lysates from ISAV-infected cell cultures have been established for detection of ISAV-specific antibodies. ELISA titres can be very high and appear to be quite specific for the nucleoprotein in Western blots (K. Falk, pers. comm.). The test is not standardised for surveillance or diagnostic use, but may be used as a supplement to direct virus detection and pathology in obscure cases. Furthermore, the level and distribution of seroconversion in an ISAV-infected population may give some information about the spread of infection, particularly in cases where vaccination is not practised, and in wild fish.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance for infection with HPR-deleted ISAV and diagnosis of ISA are listed in Table 5.1. For surveillance of infection with HPR0 ISAV, real-time RT-PCR followed by sequencing is the only recommended method (not included in the table). The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance for infection with HPR-deleted ISAV</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fry</td>
<td>Parr</td>
<td>Smolt</td>
</tr>
<tr>
<td>Gross signs</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Histopathology</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>IFAT on kidney imprints</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Isolation in cell culture with virus identification</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>RT-PCR or real-time RT-PCR followed by sequencing</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
</tbody>
</table>

*As the diagnosis of ISA is not based on the results of a single method, the information in this Table should be used with care. See Section 7 for the criteria for ISA diagnosis. PLs = postlarvae; IFAT = indirect fluorescent antibody test; EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with infectious salmon anaemia virus

Regular health inspections combined with investigation for ISA when increased mortality is associated with one of the given clinical signs and/or pathological changes consistent with ISA is an efficient way of obtaining data on the occurrence of ISA in farmed populations. In addition to regular health inspections, testing for HPR-deleted ISAV, preferentially by PCR-based methodology, at certain intervals may be carried out. However, due to the expected low prevalence in apparently healthy populations and the uneven spread of infection within a farm, statistically appropriatenumbers of samples need to be tested. The significance of positive findings of ISAV by PCR alone for the risk of developing ISA disease is not clear, and therefore any positive findings would have to be followed up by either further testing and/or surveillance of the production site.

Because of the transient nature of HPR0 ISAV, statistically appropriate sample sizes need to be tested at time points through the production cycle to be able to document freedom of this infection.
7. Corroborative diagnostic criteria

Reasonable grounds to suspect fish of being infected with ISAV (HPR-deleted or HPR0) are outlined below. The Competent Authority should ensure that, following the suspicion of fish infected with ISAV on a farm, an official investigation to confirm or rule out the presence of the disease will be carried out as quickly as possible, applying inspection and clinical examination, as well as collection and selection of samples and using the methods for laboratory examination as described in Section 4.

7.1. Definition of suspect case (HPR-deleted ISAV)

ISA or infection with HRP-deleted ISAV would be suspected if at least one of the following criteria is met:

i) Clinical signs consistent with ISA and/or pathological changes consistent with ISA (Section 4.2) whether or not the pathological changes are associated with clinical signs of disease;

ii) Isolation and identification of ISAV in cell culture from a single sample (targeted or routine) from any fish on the farm, as described in Section 4.3.1.2.1;

iii) Evidence for the presence of ISAV from two independent laboratory tests such as RT-PCR (Section 4.3.1.2.3) and/or IFAT on tissue imprints (Section 4.3.1.1.2.1) or IHC (Section 4.3.1.1.3.1);

7.2. Definition of confirmed case (HPR-deleted ISAV)

7.2.1. Definition of confirmed ISA

The following criteria should be met for confirmation of ISA: detection of ISAV in tissue preparations by means of specific antibodies against ISAV (IHC on fixed sections [Section 4.3.1.1.3.1] or IFAT on tissue imprints [Section 4.3.1.1.2] or fixed sections as described in Section 4.3.1.1.3) in addition to either:

i) Isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm, as described in Section 4.3.1.2.1.

or

ii) Detection of ISAV by RT-PCR by the methods described in Section 4.3.1.2.3.

7.2.2. Definition of confirmed HPR-deleted ISAV infection

The criteria given in i) or ii) should be met for the confirmation of infection with HPR-deleted ISAV.

i) Isolation and identification of ISAV in cell culture from any fish sample on the farm as described in Section 4.3.1.2.1.

ii) 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 (Section 4.3.1.2.3) or IFAT/IHC (Sections 4.3.1.1.2 and 4.3.1.1.3).

7.3. Definition of confirmed infection with HPR0 ISAV

7.3.1. Definition of confirmed infection with HPR0 ISAV

The criteria given in i) should be met for the confirmation of HPR0 ISAV infection.

i) 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.

8. References


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


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**NB:** There are OIE Reference Laboratories for Infection with infectious salmon anaemia virus (see Table at the end of this Aquatic Manual or consult the OIE web site for the most up-to-date list: [http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on Infection with infectious salmon anaemia virus.