

INFECTION WITH HPR-DELETED OR HPR0 INFECTIOUS SALMON ANAEMIA VIRUS

1. Scope

Infection with infectious salmon anaemia virus (ISAV) means infection with the pathogenic agent highly polymorphic region (HPR)-deleted infectious salmon anaemia virus (ISAV), or the non-pathogenic HPR0 (non-deleted HPR) ISAV of the Genus *Isavirus* of the Family *Orthomyxoviridae*.

HPR-deleted ISAV may cause disease 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 clinical signs of disease 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 (Cardenas *et al.*, 2014; Christiansen *et al.*, 2017; Cunningham *et al.*, 2002; Gagné & Leblanc, 2017; 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 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 (Gagné & LeBlanc, 2017). 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 non-pathogenic HPR0 ISAV genome has been reported in both apparently healthy wild and farmed Atlantic salmon, but has not been detected in fish with clinical disease and pathological signs consistent with infection with HPR-deleted ISAV (Christiansen *et al.*, 2011; Cunningham *et al.*, 2002; Lyngstad *et al.*, 2012; Markussen *et al.*, 2008; McBeath *et al.*, 2009; Nylund *et al.*, 2007). A mixed infection with HPR-deleted and HPR0 ISAV variants has been reported (Cardenas *et al.*, 2014; Kibenge *et al.*, 2009). Recent studies show that HPR0 ISAV variants occur frequently in sea-

reared Atlantic salmon. 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; 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 clinical disease. 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 (Cardenas *et al.*, 2014; Devold *et al.*, 2006; Gagné & Leblanc, 2017; 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. 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).

2.1.4. Life cycle

The main route of infection 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 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).

Shedding of ISAV from infected fish may occur through natural excretions/secretions.

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.

2.2. 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 the Aquatic Animal Health Code (Aquatic Code) include: 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 for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: Atlantic herring (*Clupea harengus*) and amago trout (*Oncorhynchus masou*).

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

2.2.3. Susceptible stages of the host

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 only 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 ISA has been experimentally induced in both Atlantic salmon fry and parr kept in freshwater.

2.2.4. Species or subpopulation predilection (probability of detection)

HPR-deleted ISAV is only known to cause clinical disease in Atlantic salmon.

2.2.5. Target organs and infected tissue

For fish that have developed infection with HPR-deleted ISAV endothelial cells in all organs become infected (gills, heart, liver, kidney, spleen and others) (Aamelfot *et al.*, 2012). HPR0 ISAV seem primarily to target epithelial cells of the gills (Aamelfot *et al.*, 2016), but has also been detected in kidney and heart (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011).

2.2.6. Persistent 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 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).

2.2.7. Vectors

Transmission of ISAV by salmon lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi* (Oelkers *et al.*, 2014)) 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 (Rimstad *et al.*, 2011).

2.2.8. Known or suspected wild aquatic animal carriers

Wild Atlantic salmon and brown 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 infection with HPR-deleted ISAV in different salmon-producing areas conclude that the virus spreads locally between adjacent sites. Proximity to sites with disease 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 outbreaks in Norway shows a high degree of similarity between viruses isolated from neighbouring 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 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 outbreaks of clinical disease caused by HPR-deleted ISAV 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 causing solitary outbreaks or local epidemics through local transmission (Lyngstad *et al.*, 2011; 2012). The risk of emergence of HPR-deleted ISAV from HPR0 ISAV is considered to be low but not negligible (EFSA, 2012). A direct link between HPR0 and HPR-deleted ISAV remains to be demonstrated (Cardenas *et al.*, 2014; Gagné & Leblanc, 2017).

As infection with ISAV 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 biosecurity measures are not adequate (Mardones *et al.*, 2014; Marshall *et al.*, 2014; Rimstad *et al.*, 2011).

2.3.2. Prevalence

In net pens containing diseased fish, the prevalence of HPR-deleted ISAV may vary widely, while in adjacent net pens (without diseased fish) 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 non-pathogenic HPR0 ISAV variants may be high in Atlantic salmon production areas. HPR0 ISAV in Atlantic salmon appears to be a seasonal and transient (Christiansen *et al.*, 2011). HPR0 ISAV have also been detected in wild salmonids (Rimstad *et al.*, 2011).

2.3.3. Geographical distribution

Initially reported in Norway in the mid-1980s (Thorud & Djupvik, 1988), infection with ISAV 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 infection with HPR-deleted ISAV has occurred, with the known exception of Iceland.

2.3.4. Mortality and morbidity

During outbreaks of infection with HPR-deleted ISAV, morbidity and mortality may vary greatly within and between net pens in a seawater fish farm, and between 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 often peaks 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 over several months. Initially, a clinical disease outbreak may be limited to one or two net pens over a long time period. In such cases, if net pens with clinical disease are slaughtered immediately, further development of clinical infection with HPR-deleted ISAV 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 clinical disease in Atlantic salmon.

2.3.5. Environmental factors

Generally, outbreaks of infection with HPR-deleted ISAV tend to be seasonal with most outbreaks in late spring and late autumn; however outbreaks can 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 infection with ISAV has been carried out in North America since 1999 and the Faroe Islands since 2005. In Norway, vaccination against infection with ISAV was carried out for the first time in 2009 in a region with a high rate of infection with HPR-deleted ISAV outbreaks. Chile started vaccinating against infection with ISAV in 2010.

2.4.2. Chemotherapy

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).

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 (chapter 4.4 of the *Aquatic Code*).

2.4.8. General husbandry practices

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. The experience from the Faroe Islands, where the prevalence of HPR0 ISAV is high, demonstrates that the combination of good biosecurity and husbandry reduces the risk of outbreaks of infection with HPR-deleted ISAV substantially.

3. Sampling

3.1. Selection of individual specimens

For detection of HPR-deleted ISAV, fish displaying clinical signs or gross pathology should be sampled.

For detection of HPR0 ISAV, randomly selected individuals should be sampled at different time points throughout the production cycle.

3.2. Preservation of samples for submission

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| Haematology: | Heparin or EDTA (ethylene diamine tetra-acetic acid) |
| Cell culture: | Virus transport medium |
| Histology and immunohistochemistry: | Fixation in neutral phosphate-buffered 10% formalin |
| Immunofluorescence (smears): | Either submitted dried, or dried and fixed in 100% acetone |
| Molecular biology (RT-PCR and sequencing): | Appropriate medium for preservation of RNA |

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

Virological examination (cell culture and real-time or conventional RT-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

Gill tissue

3.5. Samples/tissues that are not suitable

None known.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

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.

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 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 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 infection with HPR-deleted ISAV 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.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

With the exception of molecular techniques (see 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 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). 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 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 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-mercaptoethanol (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 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).

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 (1–3 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 enzootic serotypes of IPNV prior to inoculation to neutralise any IPNV that may be present.

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 HPR-deleted ISAV appears as vacuolated cells that subsequently round up and loosen from the growth surface. If CPE consistent with that described for HPR-deleted 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 HPR-deleted 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 ISAV 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 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-HPR-deleted 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 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 if the isolate is either HPR-deleted or HPR0 ISAV 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 HPR0 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.

| Real-time and conventional RT-PCR: Primer and probe sequences | Named | Genomic segment | Product size | Reference |
|---|--|-----------------|----------------|---------------------------|
| 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-TGT-GAT-CCC-MGBNFQ-3' | forward primer reverse primer Taqman@probe | 7 | 155 nt | Snow <i>et al.</i> , 2006 |
| 5'-CTA-CAC-AGC-AGG-ATG-CAG-ATG-T-3' 5'-CAG-GAT-GCC-GGA-AGT-CGA-T-3' 5'-6FAM-CAT-CGT-CGC-TGC-AGT-TC-MGBNFQ-3' | forward primer reverse primer Taqman@probe | 8 | 104 nt | Snow <i>et al.</i> , 2006 |
| 5'-GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA-3' 5'-GAT-GGT-GGA-ATT-CTA-CCT-CTA-GAC-TTG-TA-3' | forward primer reverse primer | 6 (HPR) | 304 nt if HPR0 | Designed by OIE Ref. Lab. |

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

None published or validated.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance for HPR-deleted ISAV and diagnosis of infection with HPR-deleted ISAV are listed in Table 5.1. For surveillance for HPR0 ISAV, a real-time RT-PCR followed by conventional RT-PCR and sequencing are the only recommended methods (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 5.1. Methods for targeted surveillance and diagnosis*

| Method | Targeted surveillance for infection with HPR-deleted ISAV | | | | Presumptive diagnosis | Confirmatory diagnosis |
|---|---|------|-------|--------|-----------------------|------------------------|
| | Fry | Parr | Smolt | Adults | | |
| Gross signs | d | d | d | d | c | b |
| Histopathology | d | d | d | d | b | b |
| IFAT on kidney imprints | d | d | d | d | b | a |
| Immunohistochemistry | d | d | d | d | b | a |
| Isolation in cell culture with virus identification | b | b | b | b | b | a |
| RT-PCR | c | c | c | c | b | c |
| Real-time RT-PCR | a | a | a | a | a | b |
| Sequencing | d | d | d | d | d | a |

*As the diagnosis of infection with HPR-deleted ISAV 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 infection with HPR-deleted ISAV diagnosis.

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 ISAV

For infection with ISAV, real-time RT-PCR is the recommended test for surveillance.

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

Infection with HPR0 or HPR-deleted ISAV shall be suspected if the following criterion is met:

- i) Positive conventional RT-PCR or real-time RT-PCR result

In addition, infection with HPR-deleted ISAV shall be suspected if one of the following criteria is met:

- ii) Clinical signs or pathological changes consistent with ISA;
- ii) CPE typical of ISAV in cell cultures;
- iii) Positive IFAT on tissue imprints

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

The presence of ISAV is considered to be confirmed if, in addition to the criteria in Section 7.1, one or more of the following criteria are met:

- i) ISAV isolation is carried out in cell culture followed by virus identification by either an antibody-based test (IFAT) and/or conventional PCR followed by sequencing of the amplicon;
- ii) ISAV is detected in histological sections by immunoassay using specific anti-ISAV antibodies;
- iii) Detection of ISAV in tissue preparations by conventional PCR followed by sequencing of the amplicon

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 amplification and sequencing of the HPR region of segment 6 to confirm the presence of HPR0 only.

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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: <https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>). Please contact the OIE Reference Laboratory 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 2018.