INFECTIOUS SALMON ANAEMIA

1. Scope

Infectious salmon anaemia (ISA) is an orthomyxovirus infection of sea-farmed Atlantic salmon (*Salmo salar*) (28) inducing a systemic 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, but cumulative mortality may become very high. For the purpose of this chapter, ISA is considered to be infection with salmon anaemia virus (ISAV) (12).

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. The virus has haemagglutinating, receptor-destroying and fusion activity (1, 5, 14, 21, 24).

The morphological, physiochemical and genetic properties of ISAV are consistent with those of the *Orthomyxoviridae* (5, 21), and ISAV has recently been classified as the type species of the new genus *Isavirus* (12) within this virus family. The nucleotide sequences of all eight genome segments have been described. The viral genome encodes at least ten proteins (1, 14, 24). Four major structural proteins have been identified, including a 68 kDa nucleoprotein, a 22 kDa matrix protein, a 42 kDa haemagglutinin-esterase protein responsible for receptor-binding and receptor-destroying activity, and a 50 kDa surface glycoprotein with putative fusion activity, coded by genome segments 3, 8, 6 and 5, respectively. Segment 1, 2, and 4 encode the viral polymerases PB1, PB2) 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 nonstructural protein with interferon antagonistic properties, while ORF2 has been suggested to encode for a nuclear export protein. The possibility of a third ORF has been discussed. The smaller ORF1 of segment 8 encodes the matrix protein, while the larger ORF2 encodes an RNA-binding structural protein also with interferon antagonistic properties (1, 6, 13, 14, 24).

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas (1, 14, 24). According to differences in the 5'-terminal end of the HE gene, ISAV isolates have been divided into two major groups, one European and one North American group. The European group may be further divided into three major groups (1). A small, highly polymorphic region (HPR) of the haemagglutinin gene has been identified (18). This region is characterised by the presence of gaps rather than single-nucleotide substitutions. However, there is no direct correlation between phylogenetic groups and deletion patterns in the HPR. A full-length gene (HPR0) has been suggested to represent an ancient variant from which the variants with deletions in the HPR region have been derived. The presence of HPR0 has been reported in wild and farmed Atlantic salmon (1, 14), but has not been detected in diseased fish with clinical and pathological signs consistent with ISA. Variations in the HPR between virus isolates have been suggested to be important for virulence, as all diseased fish contain deletions in this region. However, other genes are most certainly also of importance in virulence because isolates with identical HPRs vary significantly in development and severity of disease (19). Recently, a potential virulence marker has been identified in segment 5 encoding the fusion protein and involving the protease recognition pattern at the cleavage site of the fusion protein (17). Furthermore, evidence for reassortment and nonhomologous recombination of ISAV has been provided (3, 17).

2.1.2. Survival outside the host

ISAV RNA has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in seawater sampled at farming sites with ISAV-positive Atlantic salmon (14). 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, UV irradiation and temperature. Exposing cell culture-propagated ISAV to 15°C for 10 days or to 4°C for 14 days had no effect on virus infectivity (5).

2.1.3. Stability of the agent (effective inactivation methods)

ISAV is sensitive to UV irradiation (UVC) and ozon (1). 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. Cell culture-isolated ISAV may survive for weeks at low temperatures, but virus infectivity is lost within 30 minutes exposure at 56°C (5). Incubation of tissue homogenate from ISA-diseased fish at pH 4 or pH 12 for 24 hours inactivated ISA infectivity. Incubation in the presence of chlorine (100 mg ml^{-1}) for 15 minutes also inactivated virus infectivity (1).

2.1.4. Life cycle

The main infection route is most likely through the gills but infection via the intestine cannot be excluded. Endothelial cells seem to be the primary target cells for ISAV by electron microscopy (1, 14, 24). This has recently been confirmed by immunohistochemical examination of several organs (National Veterinary Institute Norway, unpublished results) and by *in-situ* hybridisation (14). 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, virus replication may occur in several organs. *In-situ* hybridisation studies have indicated the most extensive and prolonged replication to occur in the heart tissue (14).

The haemagglutinin-esterase molecule of ISAV, like the haemagglutinin of other orthomyxoviruses, is essential for binding the virus to sialic acid residues on the cell surface. In the case of ISAV, the virus binds to glycoproteins containing 4-O-acetylated sialic acids, which also serve as substrates 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 from the cell surface (1, 14, 24).

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

2.2. Host factors

2.2.1. Susceptible host species

Natural outbreaks of ISA have only been recorded in farmed Atlantic salmon, but the virus has been isolated from rainbow trout in Ireland (7) and there is a report of isolation of ISAV from Coho salmon (*Oncorhynchus kisutch*) in Chile (14). Subclinically infected feral Atlantic salmon, brown trout and sea trout (*S. trutta*) have been identified by RT-PCR (14, 23). 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 (14). In these studies, only weak positive results were obtained, and gills were included in the tissue samples examined. Contamination of virus present in surrounding water cannot, therefore, be excluded, and corroborative studies are needed before these species can be identified as possible hosts for ISAV.

Following experimental infection, replication of ISAV has been demonstrated in several fish species, including brown trout, sea trout, rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), herring (*Clupea harengus*) (1, 14, 24) and Atlantic cod (*Gadus morhua*) (10). Mortality and histopathological changes have been introduced in rainbow trout by experimental infection, although the lesion characteristics were different from those in Atlantic salmon (16). Attempts have been made to induce infection or disease in *P. virens*, but with negative results (14).

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 (1). Furthermore, 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 (1). Furthermore, a functional association between disease resistance and major histocompatibility (MHC) class I and II polymorphism has been demonstrated (9). The use of MHC-compatible Atlantic salmon indicated that the ability to mount a strong proliferative response correlated to survival and virus clearance, while induction of a humoral response was less protective (19).

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

Endothelial cells in many organs (heart, liver, kidney, spleen and others).

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 cause any recognisable disease problems. Experimental infection of rainbow trout and brown trout indicates that persistent infection in these species could be possible (1, 14, 24). See Section 2.4.1 for the possible creation of virus carriers through vaccination.

2.2.6. Vectors

Passive transfer of ISA by salmon lice (*Lepeophtheirus salmonis*) has been demonstrated under experimental conditions. Although natural vectors have not been identified, several different vector groups could be possibel vectors under certain defined conditions.

2.2.7. Known or suspected wild aquatic animal carriers

Wild Atlantic salmon, brown trout and sea trout (*S. trutta*) may be carriers of ISAV (1, 23). The importance of wild marine fish (see Section 2.2.1) as virus carriers needs to be clarified.

2.3. Disease pattern

2.3.1. Transmission mechanisms

The disease is spread horizontally by water-borne transmission as shown by experimental infection studies. There is no strong evidence for vertical transmission through infected gonadal products. It has been suggested that ISAV is spread over long distances by transportation of smolt, either infected prior to shipping or by well boats contaminated with ISAV. Contamination of well boats may be due either to previous transport of infected fish or through intake water from areas with farms harbouring diseased fish.

Epidemiological studies have shown that the risk of ISA transmission is closely linked to husbandry practices in aquaculture and horizontal transmission. Geographical or hydrological (via prevailing currents) proximity (<5 km) to farms with ISA outbreaks or slaughterhouses/processing plants releasing contaminated water, numerous smolt deliveries and the use of well boats, and sharing staff and equipment are all considered significant risk factors (1, 11, 15, 25).

Other horizontal pathways have also been suggested, such as transmission through sea lice, infected wild fish and various harvesting methods (14, 24). According to Nylund *et al.* (22) vertical or transgenerational transmission may occur. Carryover or stocking of multiple year-classes on a given site, or within a region connected hydrologically, may also influence occurrence of ISA (11).

2.3.2. Prevalence

In a net pen containing diseased fish, the prevalence 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.

2.3.3. Geographical distribution

Initially reported in Norway in the mid-1980s, ISA in Atlantic salmon has since then been reported in Canada (New Brunswick in 1996 and Nova Scotia in 2000), the United Kingdom (Scotland in 1998), the Faroe Islands (2000, report to OIE), USA (Maine in 2001) and in Chile (1, 8, 14, 24). The virus has been reported from rainbow trout in Ireland in 2002 (7) and from Coho salmon in Chile (14).

2.3.4. Mortality and morbidity

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 experienced during a period of, for example, 3 months. Initially, an outbreak of ISA may be limited to one or two net pens over a long period and the spread to other net pens may take months but is slowed by

early detection and depopulation of infected pens. In outbreaks where smolts have been infected in well boats during transport, simultaneous outbreaks may occur.

2.3.5. Environmental factors

Generally, outbreaks of ISA have been recorded at various times during 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 (15).

2.4. Control and prevention

2.4.1. Vaccination

Vaccination against ISA has been carried out in North America and the Faroe Islands during the past 5 years, but the currently available vaccines do not seem to offer complete protection in Atlantic salmon. The vaccines, which are inactivated, whole virus vaccines, do not give virus clearance in immunised fish, and they may thus become virus carriers (14).

2.4.2. Chemotherapy

Not applicable.

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.

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.

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 ISAV.

3.2. Preservation of samples for submission

Haematology: Heparin or EDTA (ethylene diamine tetra-acetic acid)
Cell culture: Virus transport medium
Histology and immunohistochemistry: Fixation in neutral phosphate-buffered 10% formalin
Immunocytochemistry (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 is not recommended for verification of ISAV as it is usually of interest to compare results from the various examinations for each individual. For surveillance purposes, pooling of samples for virological examination (PCR and/or cell culture) may be accepted. However, the number of fish to be pooled may depend on the suggested prevalence of ISAV in the population and of the method used.

3.4. Best organs or tissues

Blood for non-lethal sampling;
Virological examination (cell culture and PCR): heart (should always be included) and mid-kidney. For surveillance purposes using PCR, gills should also be included;
Histology (prioritised): mid-kidney, liver, heart, pancreas/intestine, spleen, gills, skin/muscle;
Immunocytochemistry (smears): mid-kidney;
Immunohistochemistry: mid-kidney, heart (including valves and bulbus arteriosus).

3.5. Samples/tissues that are not suitable

For virus isolation, gills are not recommended as it is quite difficult to avoid microbial contamination of cell cultures inoculated with gill tissue homogenate even after appropriate filtration.

4. Diagnostic methods

The diagnosis of ISA was initally based on clinical and pathological findings only (14, 24). Following the isolation of the causative agent, a number of direct methods for detection of virus and confirmation of the diagnosis have been established. These include isolation of the virus in cell culture followed by immunological identification, immunological demonstration of ISAV antigen in tissues and PCR techniques. Differential diagnoses are: other anaemic and haemorrhagic conditions, and winter ulcer and septicaemias caused by infections with *Moritella viscosa.*

4.1. Field diagnostic methods

4.1.1. Clinical signs

The most prominent external signs 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.

Nutritional status is usually quite normal, but diseased fish has no feed in the digestive tract.

4.1.2. Behavioural changes

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

4.2. Clinical methods

4.2.1. Gross pathology

Fish infected with 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 but anemia and circulatory disturbances are always present. The following findings have been described to be consistent with ISA.

- 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

A haematocrit value below 10 is not a unique finding for ISA. Fish with disease conditions such as ulcerations and erythrocytic inclusion body syndrome, may regularly demonstrate haematocrit values this low.

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. Electron microscopy/cytopathology

Virus has been observed in endothelial cells throughout the body 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

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) Preparations of cryosections

Tissue samples from kidney, liver and heart are collected from moribund fish, frozen in iso-pentane, chilled in liquid nitrogen, and stored at –80°C. Sections are cut on a cryostat, placed on poly-L-lysine-coated slides, fixed in chilled 100% acetone for 10 minutes and stored at –80°C until use.

iii) 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 approriate dilution of anti-ISAV MAb, followed by three washes. For the detection of bound antibodies, the preparations are incubated with fluoescein 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 Naphtol 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

SHK-1 (2), ASK (4) or other susceptible cell lines, such as TO and CHSE-214 (14), may be used but strain variability and the ability to replicate in different cell lines should be taken into consideration. The SHK-1 and ASK cells seem to support 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-mercaptoethanol (40 µM) (this latter may be omitted).

For virus isolation, cells grown in 25 cm^2 tissue culture flasks or multi-well cell culture plates, which may be sealed with parafilm 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 (1–3 day old cultures or cultures of 70–80 % confluency) grown in 25 cm^2 tissue culture flasks or 24-well plates. Inoculate monolayers with 0.1 ml of the 2% tissue homogenate. Allow 3–4 hours incubation at 15°C followed by removal of the inoculum, and addition of fresh, fully supplemented growth medium. 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 a CPE. Typical CPE due to ISAV appears as vacuolated cells that subsequently round up and loosen from the growth surface. If a 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, subcultivate 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.

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 plastic 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 of 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 containing 0.5% dry skimmed milk for 1 hour. and rinse twice with PBS/0.05% Tween 20.

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 decribed rinsing in between the additional step. Rinse once with PBS/0.05% Tween 20, as described above. The nuclei can be stained with propidiumiodid (100 µg ml^{-1} in sterile distilled water. If the plates cannot be examined immediately, add a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent as an anti-fade solution. Examine under UV light.

4.3.1.2.3. Molecular techniques

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

RT-PCR may be used for detection of ISAV in tissue samples or in samples from cell cultures. Care should be taken for the interpretation of results from cell culture, as detected virus does not necessarily indicate that virus replication has taken place, but may represent virus remaining in the cell culture after inoculation with a tissue sample.

Total RNA (or total nucleic acid) is extracted from tissues, tissue homogenates or from infected cell layers. The concentration and purity of the extracted nucelic acid can be estimated by measuring the optical density at 260 nm and at 280. An alternative approach is to include internal controls directed against host RNAs. For detection of viral nucleic acids in fish tissue, primers against 18s rRNA, elongation factor 1 alpha (ELF-1A) or RNA polymerase 1 have been used successfully as internal controls.

Since the first RT-PCR for ISAV was reported in 1997, several attempts have been made to optimise the method (see ref. 20 for a review). A two-step RT-PCR can be performed whereby the RT and PCR steps are run in separate tubes. The introduction of one-step procedures, where the two reactions are run in a single tube, has been successful regarding sensitivity of the test. However, in this case, no cDNA is left for use in additional amplifications, which may be a disadvantage if several primer sets need to be included in the examination.

Several primer sets for ISAV RT-PCR have been reported and some are presented in the table below. The primer sets derived from genomic segment 8 (ILA1/ILA2 and FA3/RA3) have been used by several laboratories and have been found suitable for detection of ISAV during disease outbreaks and in carrier fish. The ILA2 reverse primer does not match isolates from North America and alternative primer sets should be used. In these cases, a modified FA3 primer together with RA3 can be used. The segment 6 primers may be useful for verification of PCR results based on segment 8 primers as an alternative to sequencing the PCR product.

RT-PCR: Primer sequences	Named	Genomic segment	Product size	Reference
5'-GGC-TAT-CTA-CCA-TGA-ACG-AAT-C-3' 5'-GCC-AAG-TGT-AAG-TAG-CAC-TCC-3'	ILA1 ILA2	8	155	(20)
5'-GAA-GAG-TCA-GGA-TGC-CAA-GAC-G-3' 5'-GAA-GTC-GAT-GAA-CTG-CAG-CGA-3'	FA3 RA3	8	211	(4)
5'-GGA-ATC-TAC-AAG-GTC-TGC-ATT-G-3' 5'-CTT-CAA-AGG-TGT-CTG-ACA-CGT-A-3'	Seg6U Seg6L	6	130	Designed by OIE Ref. Lab.

The use of real-time RT-PCR may increase the specificity and, probably, also the sensitivity of the test, especially when including a sequence-specific probe (23, 26, 27). This method is more rapid compared with conventional one-tube RT-PCR, the risk of contamination may be reduced and it is possible to estimate the relative amount of viral RNA in the sample. Primer and probe sequences that have been used for screening for ISAV by real-time RT-PCR are presented in the table below. Both primers and probes listed in this table target conserved regions of and ensure detection of all documented ISAV strains (26).

Real-time RT-PCR: Primer and probe sequences	Named	Genomic segment	Reference
5'- CAGGGTTGTATCCATGGTTGAAATG -3' 5'- GTCCAGCCCTAAGCTCAACTC -3' *5'-6FAM-* CTCTCTCATTGTGATCCC-MGB *MGBNFQ-3'*	forward primer reverse primer Taqman®probe	 7	 (26)
5'- CTACACAGCAGGATGCAGATGT -3' 5'- CAGGATGCCGGAAGTCGAT -3' *5'-6FAM-* CATCGTCGCTGCAGTTC *-TAMRA-3'*	forward primer reverse primer Taqman®probe	 8 	(26)

As an alternative, ILAS7-probe listed in the table below has been shown to be effective at detecting European ISAV isolates.

Real-time RT-PCR: Primer and probe sequences	Named	Genomic segment	Reference
5'-TGG-GAT-CAT-GTG-TTT-CCT-GCT-A-3' 5'-GAA-AAT-CCA-TGT-TCT-CAG-ATG-CAA-3' *5'-6FAM-CACATGACCCCTCGTC-MGBNFQ-3'*	ILAS7-F1 ILAS7-R1 ILAS7-probe	 7 	 (23)

4.3.1.2.4. Agent purification

ISAV propagated in cell culture can be purified by sucrose gradient centrifugation (5) 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 and diagnosis of infectious salmon anaemia are listed in Table 5.1. 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 ISAV				Presumptive ISA diagnosis	Confirmatory ISA diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	c	b
Histopathology	d	d	d	b	b	b
IFAT on kidney imprints	c	c	c	c	b	a
Immunohistochemistry	c	c	c	c	b	a
Transmission EM	d	d	d	d	c	d
Isolation in cell culture with virus identification	a	a	a	a	a	a
RT-PCR or real-time RT-PCR (Sequencing for genotyping)	a	a	a	a	b	a

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 infectious salmon anaemia

None of the methods described above have been evaluated for the purpose of declaration of freedom of virus or disease, as data on prevalence and distribution of ISAV in subclinically infected fish population are lacking. 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 may be an efficient way of obtaining data on the prevalence of ISA in farmed populations. Alternatively, testing for ISAV, preferentially by PCR, at certain intervals may be carried out, in addition to regular health inspections. However, 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 carefully interpreted.

7. Corroborative diagnostic criteria

Reasonable grounds to suspect fish of being infected with ISAV 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

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

i) Clinical signs consistent with ISA 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 IFAT on tissue imprints (Section 4.3.1.1.2);

iv) Detection of antibodies to ISAV.

7.2. Definition of confirmed case

The following criteria in i) should be met for confirmation of ISA. The criteria given in ii) and iii) should be met for the confirmation of ISAV infection.

i) Mortality, clinical signs and pathological changes consistent with ISA (Section 4.2), and detection of ISAV in tissue preparations by means of specific antibodies against ISAV (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:

   a) 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

   b) detection of ISAV by RT-PCR by the methods described in Section 4.3.1.2.3;

ii) Isolation and identification of ISAV in cell culture from at least two independent samples (targeted or routine) from any fish on the farm tested on separate occasions as described in Section 4.3.1.2.1;

iii) 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 (Sections 4.3.1.1.2 and 4.3.1.1.3).

8. References

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NB: There are OIE Reference Laboratories for Infectious salmon anaemia (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).