European Union Reference Laboratory
for Fish and Crustacean Diseases

NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

DIAGNOSTIC METHODS FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH HPR-DELETED INFECTIOUS SALMON ANEMIA VIRUS (ISAV)

In the front page, on the top left, collection of clinically affected specimen in a net pen during fish farm visit.

On the bottom right, necropsy of clinically affected Atlantic salmon during ISA outbreak, severe congestion of liver, enlargement of spleen, signs of anaemia of the heart.

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DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH HPR-DELETED INFECTIOUS SALMON ANEMIA VIRUS (ISAV)

Contents

I. Sampling procedures for the surveillance and control of infection with HPR-deleted ISAV

When sampling and laboratory examination are carried out, for the purpose of the surveillance or eradication programmes set out in Section 5 of Chapter 2 of Part II in Annex VI of Commission delegated regulation 2020/689, the detailed methods and procedures set out in points I.1, I.2 and I.3 of this Section shall apply.

I.1.Preparation of samples from fish.

For the purpose of laboratory examination for the presence of HPR-deleted ISAV, the pooling of up to 5 fish is accepted.

(a) Tissue for histological examination shall only be taken from freshly killed fish in good condition, exhibiting clinical signs, or post-mortem findings consistent with the presence of ISA. Any external or internal lesions shall be sampled and in any case samples of anterior-kidney, liver, heart, pancreas, intestine, spleen and gill shall be removed from individual fish using a scalpel and transferred to 10 % (vol:vol) phosphate buffered (pH -7,2) formalin. Thickness of tissue samples should not be more than 5 mm, and the ratio of fixative to tissue at least 10:1 with a minimum fixation time of 24 hours to allow sufficient fixation.

(b) Tissue for immunohistochemistry shall only be taken from freshly killed fish in good condition, exhibiting clinical signs, or post-mortem findings consistent with the presence of ISA. Any external or internal lesions shall be sampled and in any case samples of mid-kidney and heart including valves and bulbus arteriosus shall be removed from individual fish using a scalpel and transferred to 10 % (vol:vol) phosphate buffered (pH -7,2) formalin.

(c) For the purpose of examination by RT-PCR a piece of heart and mid-kidney shall be removed from the fish using a sterile instrument and transferred to a microfuge tube containing one ml of RNA*later*® or other preservative solution of proven efficacy. The ratio of tissue to RNA later should follow manufacturer instructions. When the fish are too small to obtain an adequate sample, pieces of kidney, heart, spleen may be taken, in that order of preference.

(d) Tissues for virological examination on cell culture shall be taken from mid-kidney, heart, and spleen. Whole or pieces of organs shall be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing virological transport medium with antibiotics (e.g. L-15 medium or Eagles MEM with 10% new-born calf serum (NCS) and antibiotics).

The quantity of fish material suitable for virological examination on cell culture and by RT-qPCR is dependent on fish size. Thus, whole alevin (body length < 4 cm), viscera including kidney (4 cm < body length < 6 cm) or, for larger size fish, kidney, spleen, heart shall be the tissues to be sampled. Ovarian or seminal fluid or organ pieces from a maximum of 5 fish may be collected in

one sterile tube containing at least 4 ml transport medium and represent one pooled sample. The tissue in each sample shall weigh a minimum of 0,5 gram (g).

I.2. Shipment of samples from fish

Whole fish may be transported to the laboratory if the temperature requirements during transportation, as described in paragraph 2 of this point can be fulfilled. Whole fish shall be shipped in a plastic bag, chilled as described in that paragraph. Live fish may also be shipped, but only under the supervision of the examining laboratory and taking into account the additional disinfection and biosecurity issues when transporting live fish.

Tubes containing fish tissues for virological examination or RT-PCR analysis shall be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or freezer blocks to ensure chilling of the samples during transportation to the laboratory. Freezing shall be avoided and ice must still be present in the transport box at receipt of the shipment or one or more of the 'freezer blocks' must still be partly or completely frozen. In exceptional circumstances, tissue samples for RT-PCR without RNA*later*® or tissue samples in virological transport medium maybe snap frozen and transported to the laboratory at – 20 °C or below.

For RT-PCR analysis of tissues preserved in RNA*later*® , RNA extraction shall be carried out within the following time frames depending on the temperature the samples are stored at: samples stored at 37 °C: 1 day; samples stored at 25 °C: 1 week; samples stored at 4 °C: 1 month; samples stored at – 20 °C: indefinitely.

If fish tissues are transported in fixative for histological examination, they shall be shipped in leak proof tubes within impact-resistant containers. Formalin-fixed tissues must not be frozen.

The virological examination on cell culture shall be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation were fulfilled.

All fish tissue samples shall be shipped in leak proof tubes within impact-resistant containers.

I.3. Collection of supplementary diagnostic material subject to the approval of the diagnostic laboratory.

Fish tissues other than those referred to in point I.1 may be collected and prepared for supplementary examination.

II. Detailed diagnostic methods and procedures for the surveillance and confirmation of the presence of or to rule out the suspicion of infection with HPR-deleted ISAV**.**

When laboratory examination for the purpose of obtaining or maintaining a certain health status with regard to ISA as set out in Chapter 2 of Part II of Annex VI in Regulation (EU) 2020/689, or for the purpose of the confirmation of the presence of or to rule out a suspicion of infection with HPR-deleted ISAV in accordance with Article 42 of Regulation (EU) 2016/429, are carried out, using the diagnostic methods set out in Section 5 of Part II of Annex IV, the detailed methods and procedures set out in the following points II.1 to II.5 shall apply.

II.1. Examination of samples by RT-PCR. The diagnostic method to be used for the screening of ISAV shall be RT-qPCR. As the results of the RT-qPCR can vary depending on the conditions under which it is performed, adequate positive and negative controls and amplicons shall be included to avoid any doubts. Positive and negative controls should be included with each stage of the assay: extraction, reverse-transcription (two-step assay only) and real-time PCR. An internal (endogenous) PCR control can be included. If an endogenous control is to be used, primers and probes have to be designed, optimised and validated for each fish species to be tested.

II.1.1. Total RNA extraction. Total RNA shall be extracted using validated RNA extraction and purification methods including RNA affinity spin columns or magnetic bead based, according to the manufacturer's instructions. It is recommended to include internal controls targeted against the host genome (e.g. ($EF1_{AA}$/*ELF-1α* according to the protocol provided by Olsvik et al., 2005) as referred to in point II.1.3.

II.1.2. RT-qPCR for ISAV detection. The assay described targeting segment 8 (Snow et al., 2006), shall be used. This assay should detect strains originating from the European Union, the European Free Trade Association, and South- and North America. The one-step method shall be used where possible, as the one tube assay minimises the risk of cross-contamination.

Forward primer: 5′- CTACACAGCAGGATGCAGATGT -3′;

Reverse primer: 5′- CAGGATGCCGGAAGTCGAT -3′;

and probe: 5′-FAM- CATCGTCGCTGCAGTTC – *MGBNFQ*-3′.

Negative template controls and positive controls shall be included on each plate run. Cycling conditions shall be adjusted according to the manufacturer’s instruction of the specific kit. Other validated RT-qPCR versions of proven similar diagnostic sensitivity and specificity may be used instead (Ref: OIE Manual of Diagnostic Tests for Aquatic Animals principles and methods of validation of diagnostic assays for infectious diseases). All controls have to yield expected results. Each laboratory should define cut-off values/criteria for positive, suspect and negative samples.

II.1.3. One Step-RT-PCR, electrophoresis and Sequencing of ISAV segment 6 including the highly polymorphic region (HPR)

With the widespread occurrence of ISAV-HPR0, including on farms with suspicion of infection with ISAV-HPR deleted, it is mandatory to follow up any real-time PCR positive results with RT-PCR amplification of the HPR followed by gel electrophoresis and sequencing to determine if the isolate is HPR0, HPR-deleted or a mixture of both. Therefore, all samples that test ISAV positive by real-time RT-PCR shall be amplified by one-step RT-PCR of ISAV segment 6 including the highly polymorphic region (HPR). Primers to be used are those published by OIE:

Forward primer:	5'-GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA-3'
Reverse primer:	5'-GAT-GGTGGA-AAT-CTA-CCT-CTA-GAC-TTG-TA-3'
Product size:	304 nt for HPR0

or primers validated and designed by the EURL in collaboration with Dr. Debes Christiansen (National Reference Laboratory for fish diseases, Faroese Food and Veterinary Authority FFVA, Torshavn, Faroe Islands)

Forward primer:	5'-TTG-ACC-AGA-CMA-GCT-TAG-GTA-ACA-3'
Reverse primer:	5'-GCA-ATC-CCA-AAA-CCT-GCT-ACA-CC-3'
Product size:	229 nt for HPR0

(manuscript in preparation)

Negative template controls, HPR0 positive controls and HPR-deleted positive controls shall be included in each run. The OneStep-RT-PCR cycling conditions include cDNA synthesis at 50 ºC for 30 minutes, one cycle of 94 ºC for 15 minutes followed by 40 cycles of 94 ºC for 30 seconds, 58 ºC for 30 seconds and 72 ºC for 30 seconds. Other OneStep RT-PCR kits of proven similar efficacy may be used and cycling conditions shall be adjusted according to manufacturer instructions.

The specificity and size of all amplified RT-PCR products shall be checked by gel electrophoresis. Any gel electrophoresis system can be used provided they can distinguish between HPR0 and HPR deleted. The minimum HPR deletion reported to dateis 21 nt.

All samples suspected to include HPR deleted bands shall be confirmed by sequencing using the amplification primers. The sequencing results shall be analysed with the search tool BLAST to confirm ISAV specificity and HPR deletion.

II.2. ISAV isolation on cell cultures

II.2.1. Preparation of samples.

The samples may be stored at – 80 °C, however, they must only be frozen and thawed once before examination. For surveillance and control purposes, the examination shall be undertaken as quickly as possible. Each sample (pool of tissues or a single tissue sample in viral transport solution) shall be completely homogenised using a validated homogeniser (e.g. mortar and pestle with sterile sand, or stomacher). The homogenate shall be centrifuged in a refrigerated centrifuge at 2 °C to 5 °C at 2 000 to 4 000 × g for 15 minutes, the supernatant collected and may be treated for either four hours at 15 °C or overnight at 4 to 8 °C with antibiotics, e.g Gentamicin 50µg/ml. If the sample has been shipped in a transport medium, the treatment of the supernatant with antibiotics may be omitted. Where practical difficulties arise, such as equipment malfunction or problems with cell cultures, which make it impossible to inoculate cells within 48 hours after the collection of the fish tissue samples, the supernatant may be frozen at – 80 °C and virological examination may be carried out within 14 days. If the collected supernatant is stored at – 80 °C within 48 hours after the sampling, it may be reused only once for virological examination. Prior to the inoculation of the cells, the supernatant shall be mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of infectious pancreatic necrosis (IPN) virus and incubated for a minimum of one hour at 15 °C or a maximum of 18 hours at 4 °C. Treatment of all inocula with antiserum to IPN virus aims at preventing cytopathic effect (CPE) due to IPN virus from developing in inoculated cell cultures. This will reduce the duration of the virological examination as well as the number of cases in which occurrence of CPE would have to be considered potentially indicative of HPR-deleted ISAV. When samples come from production units, which are considered free from IPN, the treatment of inocula with antiserum to IPN virus may be omitted.

If bacterial contamination occurs in the cell culture, sample supernatants should be filtered through a cell membrane filter 0.45 µm and treated again with antibiotic, e.g. Gentamycin 50µ/ml.

II.2.2. Inoculation on cell cultures Atlantic salmon kidney (ASK) cells shall be used for primary ISAV isolation. For maintenance of the ASK cell line, (pass 70-75 or lower) cells shall be grown in L-15 medium containing 10 % foetal bovine serum, 2 % (vol:vol) 200 mM L-glutamine and antibiotics in standard concentrations at an incubation temperature of 20 °C. ASK cell lines should be passaged every 3 weeks, with a split ration 1:2.

For preparation of ASK cell monolayer for virus isolation, cells (pass 70-75 or lower) shall be grown in T25 flasks or 24-well plates with L-15 medium containing 10 % foetal bovine serum, 2

% (vol:vol) 200 mM L-glutamine and antibiotics in standard concentrations. The pH must be 7,6 ± 0,2.

The cells must be actively growing at inoculation

For sample inoculation, the cell culture media is removed and the antibiotic treated inoculum absorbed for 1-2 hours at 15° C in 2 dilutions: the primary dilution and, in addition, a 1:10 dilution thereof (0.5 ml for T25 flask and 0.15 ml for 24-well trays). Following absorption, inoculum is removed (and washed if cytotoxic effect is expected) and L15 medium with 2% foetal bovine serum added (10 ml to T25 flasks or 1½ ml to 24-well trays)

Controls

To minimise the risk of cross-contamination, separate well plates shall be used for samples from different fish farm sites. At least one well in each plate should be left uninoculated or inoculated with virological transport medium to serve as a negative control. A separate plate shall be inoculated with a reference isolate of ISAV as a positive control, as follows. One hundred µl of a stock preparation of ISAV (minimum titre 10^5 Tissue culture infective dose at the 50 % end point (TCID50 ml^{-1})) shall be inoculated into the first well and mixed. A volume of this material shall be transferred from the first well to the second well to make a 1:10 dilution and mixed. This shall be repeated across the plate to make six 10-fold dilutions. Stock ISAV may be stored at – 80 °C for at least 2 years but once thawed must be used within 3 days. Care shall be taken to prevent cross-contamination of test plates with positive control material. To avoid that risk, positive controls shall be set up and handled separately from test plates.

Inoculation of ISAV as positive control can be omitted if replaced by a sensitivity test of ASK cells towards ISAV isolates as described in section II.2.4.

Incubation of cell cultures

Samples shall be incubated at 14 ± 2 °C for up to 14 days. Cell cultures shall be examined for CPE at least twice, between 5 to 7 and 13 to 15 days following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately in accordance with point II.2.4.

If no CPE is observed by day 15 following inoculation, a subcultivation shall be carried out.

II.2.3. Subcultivation

Culture supernatant shall be inoculated and absorbed onto fresh actively growing cells in T25 flasks or 24-well plates and incubated at 14 ± 2 °C for up to 14 days in L-15 medium with 2% FBS and following the same procedures as described in Section II.2.2.

Using a microscope, cell cultures shall be examined for CPE twice, between days 5 to 7 and days 13 to 15 following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately in accordance with point II.2.4.

If cytotoxicity occurs within the first 7 days of incubation, subcultivation shall be performed at that stage, and the cells shall be incubated for 14 days and sub cultivated again with a further period of 14 days incubation. If cytotoxicity occurs after 7 days, subcultivation shall be performed once and the cells shall be incubated to achieve the total period of minimum 28 days incubation from the primary inoculation. If bacterial contamination occurs in the primary culture, the test shall be set up again using the tissue homogenate stored at – 80 °C. Prior to inoculation, the tissue homogenate shall be centrifuged at 4 000 × *g* for 15 to 30 minutes at 2 to 5°C and the supernatant shall be filtered at 0,45 µm. If bacterial contamination occurs during the subcultivation step the supernatant shall be filtered at 0,45 µm, inoculated onto fresh cells and incubated for a further 14 days.

II.2.4. Procedure to verify the susceptibility of cell cultures to infection

A sensitivity test every 6 months of ASK cells towards ISAV isolates may replace the use of a positive control at each inoculation; a sensitivity test should be conducted if it is suspected that the susceptibility of a cell line has decreased.

General procedure:

a. Prepare a seeding stock by inoculating reference virus isolate in low MOI (<1) onto ASK cells in flasks according to the method described in II.2.2. At total CPE, the virus shall be harvested by centrifugation of cell culture supernatant at 2 000 × g for 15 minutes, filter sterilised through 0,45 µm membrane filter and aliquoted in labelled cryotubes. This virus can be stored at -80°C for at least 10 years.

b. Cell sensitivity test: The ASK cell lineage to be tested is seeded in 96-well plate with L-15 medium containing 10 % foetal bovine serum, 2 % (vol:vol) 200 mM L-glutamine and antibiotics in standard concentrations and incubated for 24 hrs at a temperature of 20°C.

Prepare stock virus in 10- or 5 fold dilutions, remove the cell culture media from the ASK cells and absorb with 25µl virus stock dilutions per well, 6 replicates per dilution, for one hour, then add L15 medium with 2% FBS and incubate at 15°C. Final reading for CPE 8 days post inoculum. Antibody-based assay (IFAT or HPR-based) as described in II.2.5 shall be used to detect infected wells as CPE in 96 well plate may be difficult to interpret. Titration procedures must be described in detail and the same procedure followed each time. The titres shall be compared with previously obtained titres. If the titre of the virus isolates drops by a factor of 2 logs or more, compared with the initial titre, the cell line shall no longer be used for surveillance purposes. If different cell lines are kept in the laboratory each line shall be examined separately. Records shall be kept for a period of at least 10 years.

II.2.5. Virus identification tests If evidence of CPE is observed at any stage, virus identification shall be carried out. The methods of choice for the identification of ISAV shall be RT-qPCR, RT-PCR in accordance with II.1 and/or antibody-based detection in accordance with point II.2.6. If it is considered that other viruses may be present, supplementary virus identification tests shall be carried out. If those tests have not resulted in a definitive identification of the virus within 1 week, the supernatant shall be forwarded for immediate identification to: (a) the World Organisation for Animal Health (OIE) reference laboratory for ISA, or; (b) a National reference laboratory or the EU reference laboratory for fish diseases as referred to in Article 94 point 2 (i) of Regulation 2017/625 .

II.2.6 Antibody-based detection and identification of ISAV in cell culture.

Prepare ASK cells in 96-, or 24-well tissue culture plates with 150 µl or 0.8 ml tissue culture media, respectively (in 24 well plates, cells can be cultivated on 13 mm standard cover glasses.) When cells are outgrown (usually after 4-7 days), add 50 µl or 200 µl of putatively virus-infected cell culture supernatant to the cultures, respectively. Mock infected- and controls infected with an ISAV reference isolate are prepared on a separate plate. The plates are incubated at 15°C for 5 - 7 days, washed gently with PBS and fixed with 80% acetone for 10 minutes at room temperature (acetone is diluted in aqua dest. to prevent acetone to attack the plastic plates). Following fixation, the acetone is removed and the cells air-dried for one hour. Fixed cells can be stored dry for up to4 days in a fridge, or for several months at -80 °C. We recommend storing the cell culture plates in zip lock plastic bags with silica gel as desiccant.

The staining procedure starts with a blocking step using 5% non-fat dry milk in PBS/0.1% Tween20, for 30 minutes. The blocking solution is poured off and primary detection antibody diluted in diluent (PBS/0.1% Tween20 with 1% non-fat dry milk) is added and incubated for 45 minutes. For primary detection, use anti ISAV HE Mab mix 3H6F8/10C9F5 (Falk et al., 1998) or

other Mab or rabbit antibody of proven efficacy and specificity. These reagents can be obtained by NRLs through the OIE reference laboratory currently hosted at Norwegian Veterinary Institute (NVI) in Oslo. The cells are then washed three times with PBS/0.1% Tween20, and a secondary visualization antibody diluted in diluent is added and incubated for another 45 minutes, followed by three washes in PBS/0.1% Tween 20. All incubations are performed at room temperature. Both fluorochrome-labelled, or HRP-labelled secondary antibodies can be used for visualization of bound primary detection antibody. Use of fluorochrome-labelled antibodies require access to an inverted fluorescence microscope unless cells are cultivated on coverslips. If a HRP-labelled antibody is used, we recommend using the TrueBlue™ (www.seracare.com) substrate which is at least 100 times more sensitive compared to other commonly used substrates.

A test shall be considered positive if stained cells are observed. For a test to be valid, the positive controls shall score positive and the negative controls shall score negative.

II.3. Gross pathology, Histology and Immunohistochemistry (IHC)

Relevant tissues to be examined are mid-kidney, heart, liver, spleen. Additionally; gill and intestine including pancreas can be collected; any external or internal lesions shall be sampled. The tissues shall be removed from individual fish using a scalpel and transferred to fixative. Thickness of tissues should be a maximum 5 mm, ratio of fixative to tissue at least 10:1 (v:w) and fixation time minimum 24 hours to ensure proper preservation of the tissues. Gill and intestine should be fixated in formalin within 10 minutes from the euthanasia to preserve epithelial integrity.

Paraffin-embedded sections shall be cut at 3-5 µm and stained using haematoxylin and eosin according to standard operating procedures in the laboratory. Examples of gross pathology, histopathological changes and immunohistochemical stainings of ISAV HPRΔ antigen, observed in clinically affected specimens of Atlantic salmon during ISA outbreak, are given in Annex 1

Histological changes in clinically diseased Atlantic salmon are variable, these include changes representing circulatory disturbances (e.g. exophtalmia, oedema, ascites, congestion), anaemia, bleedings and may also include signs of necrosis in different tissues. The exact location of the changes vary; a common pattern is blood in the anterior eye chamber, skin petechial bleeding on abdominal skin and fin bases, while in the internal organs severe bleeding can occur in liver, kidney or intestinal walls. The spleen is always enlarged and dark. Note that a significant anaemia is a key finding with ISA.

Main findings can include the following (in an order suggesting importance for the examination of HE-stained tissues):

Cardiovascular erythrophagocytosis; Destruction of red blood cells (RBC) by phagocytosis is significantly increased in the spleen (normal RBC turnover is usually rare to observe). Particular for ISA is erythrophagocytosis in organs not associated with erythrocyte turnover especially liver,

heart, kidney, but macrophages containing RBC remains can be observed in any part of the vascular system.

Spleen: Depending on the stage of disease, less erythrophagocytosis and more hemosiderosis is found. The stroma can appear edematous.

Liver: subtle (early) changes are sinusoids dilated by erythrocyte accumulations (often a correlate of widespread endothelial infection as confirmed by IHC), later bleeding and then haemorrhagic necrosis in a multifocal to zonal pattern at some distance from larger vessels may develop.

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

Intestine: Accumulation of erythrocytes in blood vessels of the intestinal lamina propria and haemorrhage into the lamina propria in fish preserved when fresh: transverse section of red intestines show the redness to be an outline of lamina propria, while no blood is visible in the lumen.

Heart: At peak of endothelial infection (moderate to low haematocrit), RBC may appear to stick to the endothelium, more common is a reactive endothelium of the bulbus with an increased number of eosinophilic granular cells (mast cells): both changes are subtle by HE-stain while IHC is revelatory and corroborative.

Gills: Numerous erythrocytes may fill the central venous sinus, but it is difficult to separate from agonal circulatory disturbances giving the same effect. Erythrocyte accumulations may also form in lamellar capillaries, sometimes with necrosis of pillar cells. Gill changes need corroboration by IHC.

II.4. Immunohistochemistry (IHC)

Monoclonal antibody or Polyclonal antibody against ISAV nucleoprotein (Norwegian Veterinary Institute – NVI or © Aquatic Diagnostics Ltd) shall be used on paraffin sections from formalin-fixed tissue. The organs to be examined shall be primarily mid-kidney and heart. Suspected cases due to pathological signs shall be verified with a positive IHC. Histological sections shall be prepared in accordance with standard methods.

(1) Preparation of tissue sections; as minimum heart (including the valve and bulbus parts) and kidney tissues should be examined. The tissues shall be fixed in neutral phosphate-buffered 10 % formalin for at least 1 day, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 5 µm thick sections (for IHC placed on poly-L-lysine-coated slides) shall be heated at 56 °C to 58 °C (maximum 60 °C) for 20 minutes, dewaxed in xylene, rehydrated through a graded ethanol series, and stained with haematoxylin and eosin for pathomorphology and IHC in accordance with point (2).

(2) Staining procedure for IHC: All incubations shall be carried out at room temperature on a rocking platform, unless otherwise provided for in this description:

(a) antigen retrieval shall be done by microwave oven treatment of the sections in 0,1 M citrate buffer pH 6,0 for 2 × 6 minutes. The treatment is 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 shall then be incubated overnight (at 4°C) with primary antibody (monospecific rabbit antibody against ISAV nucleoprotein or the Aquatic Diagnostics Ltd monoclonal antibody) 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, any preferred Alkaline phosphatase based visualisation system can be applied following manufacturer's instructions. Sections shall then be washed in tap water before counterstaining with haematoxylin (e.g. Harris) and mounted in appropriate mounting medium depending on the choice of substrate/chromogen. ISAV positive and ISAV negative tissue sections shall be included as controls in every setup.

Comments to the procedure:

1) Do not use PBS when using an Alkaline phosphatase visualization system
2) Do not use non-fat dry milk associated with the visualization system if an avidin-biotin system is used.

(3) Interpretation of the result of IHC

The interpretation of the result of the IHC test shall be as set out in points (a) and (b):

(a) control sections shall be considered as positive, when it is observed that the control sections have clearly visible red-coloured (reddish) cytoplasmic staining of endothelial cells. Intranuclear staining of these endothelia cells will strengthen the specificity of the test. Endothelial staining might not be found in areas with extensive haemorrhage and necrosis.

(b) control sections shall be considered as negative if they don't have any significant colour reaction

III Acronyms and abbreviations

ASK Atlantic salmon kidney cells
CPE Cytopathic effect
DEPC Diethylpyrocarbonate
ELF Elongation factor 1α
FITC Fluorescein isothiocyanate
HPR Highly polymorphic region
IF Immunofluorescence
IFAT Indirect fluorescent antibody test
IPN(V) Infectious pancreatic necrosis (virus)
ISA(V) Infectious salmon anaemia (virus)
mRNA Messenger Ribonucleic acid
MOI Multiplicity of infection
OIE Office international des epizooties/ world animal health organization
PBS Phosphate buffered saline
PCR Polymerase Chain Reaction
RNA Ribonucleic acid
RT-(PCR) Reverse transcriptase (polymerase chain reaction)
RT-qPCR Real time RT-PCR (q for quantitative)
TCID50 Tissue culture infective dose at the 50 % end-point

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ANNEX 1 Gross pathology, histopathological findings and immunohistochemistry staining of clinically affected Atlantic salmon during ISA outbreak

Fig 1. Atlantic salmon parr experimentally infected with ISAV HPRΔ by immersion. Extensive haemorrhage in the right eye.

Fig 2. Atlantic salmon parr experimentally infected with ISAV HPRΔ by immersion. Haemorrhage in the left eye, gill anaemia and severe liver congestion.

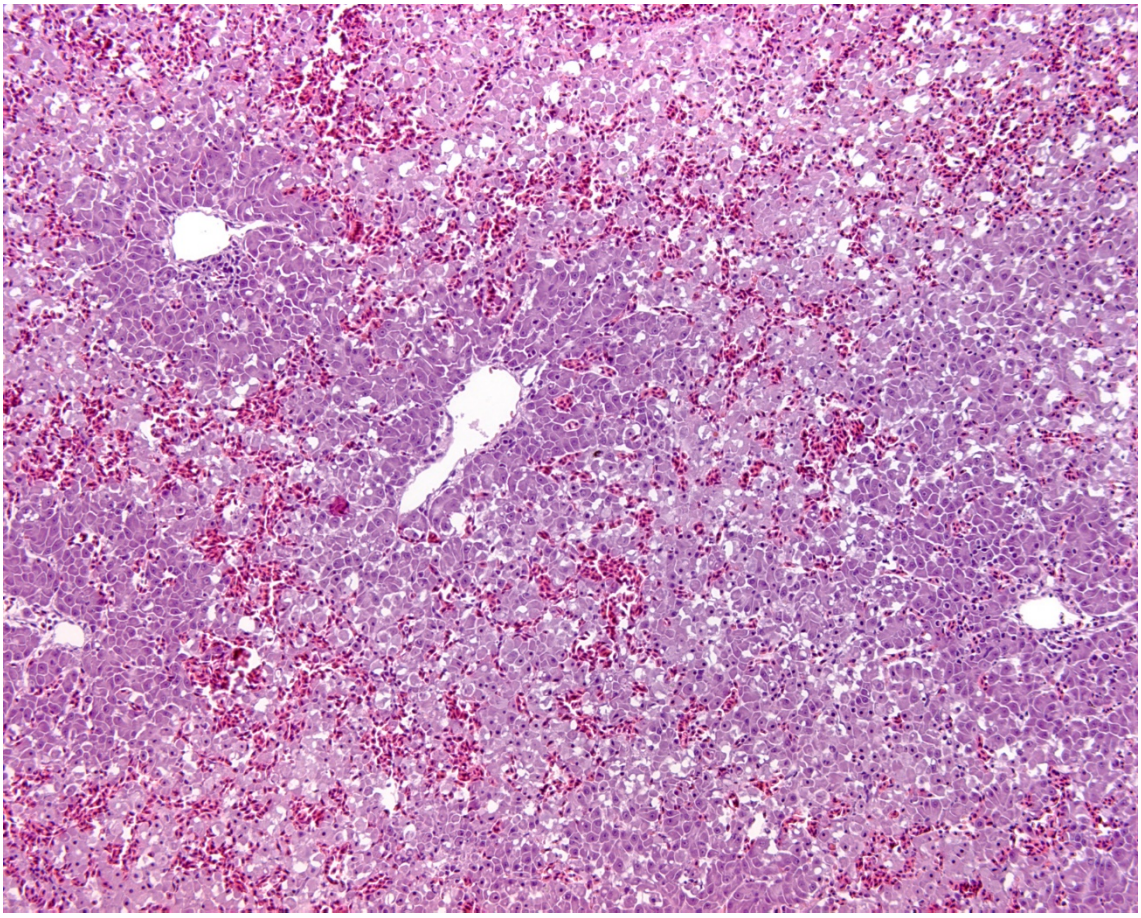

Fig 3. Hematoxylin and Eosin stained section of liver tissue of Atlantic salmon during ISA clinical outbreak. Extensive haemorrhagic liver necrosis in a zonal pattern at some distance from larger vessels

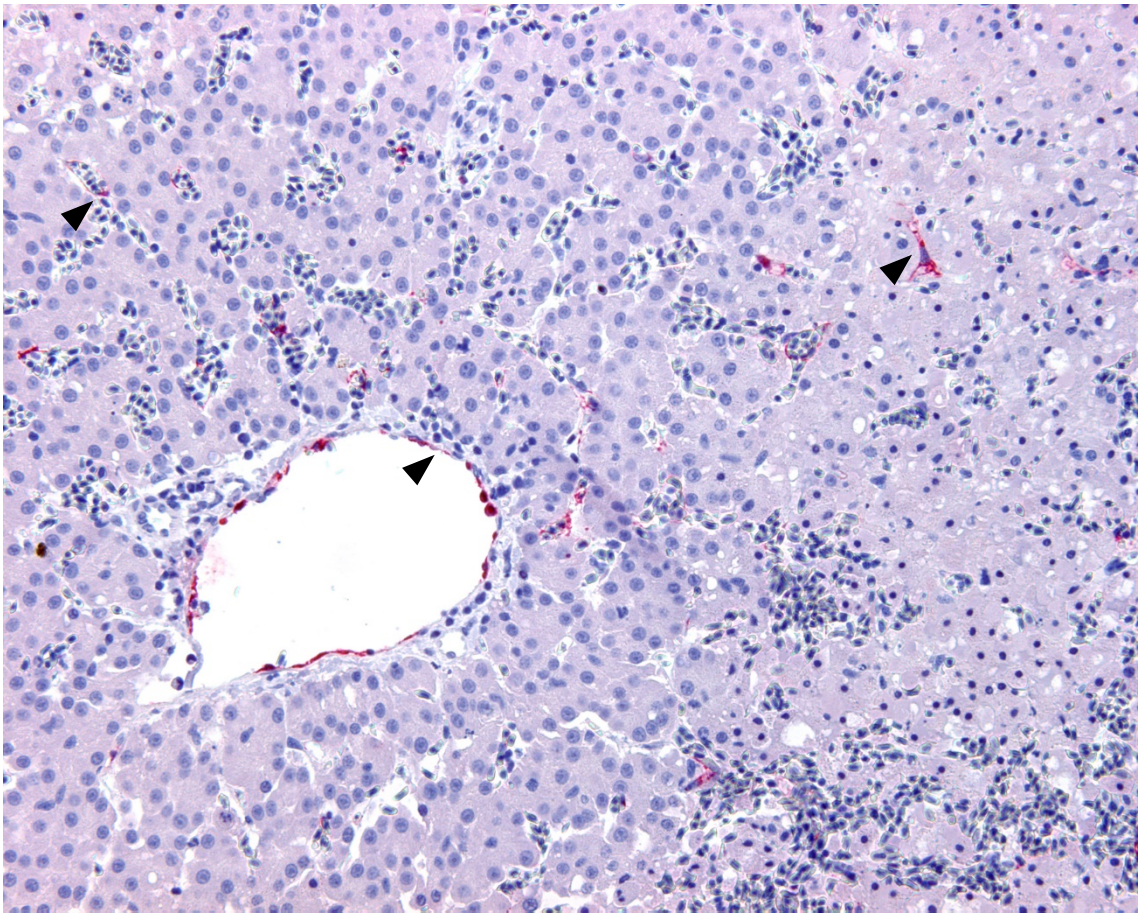

Fig 4. Same specimen of figure 3. Immunohistochemistry staining of ISAV nucleoprotein antigen in liver section of Atlantic salmon during ISA clinical outbreak. Immunohistochemistry showing red staining of endothelial cells only (arrows), note that necrotic liver cells are not stained in keeping with the narrow tropism of the ISA-virus to endothelial cells in this tissue. Severe anemia and hypoxia appear to cause this kind of liver necrosis and may explain why this classical ISA change is a late stage pathology.

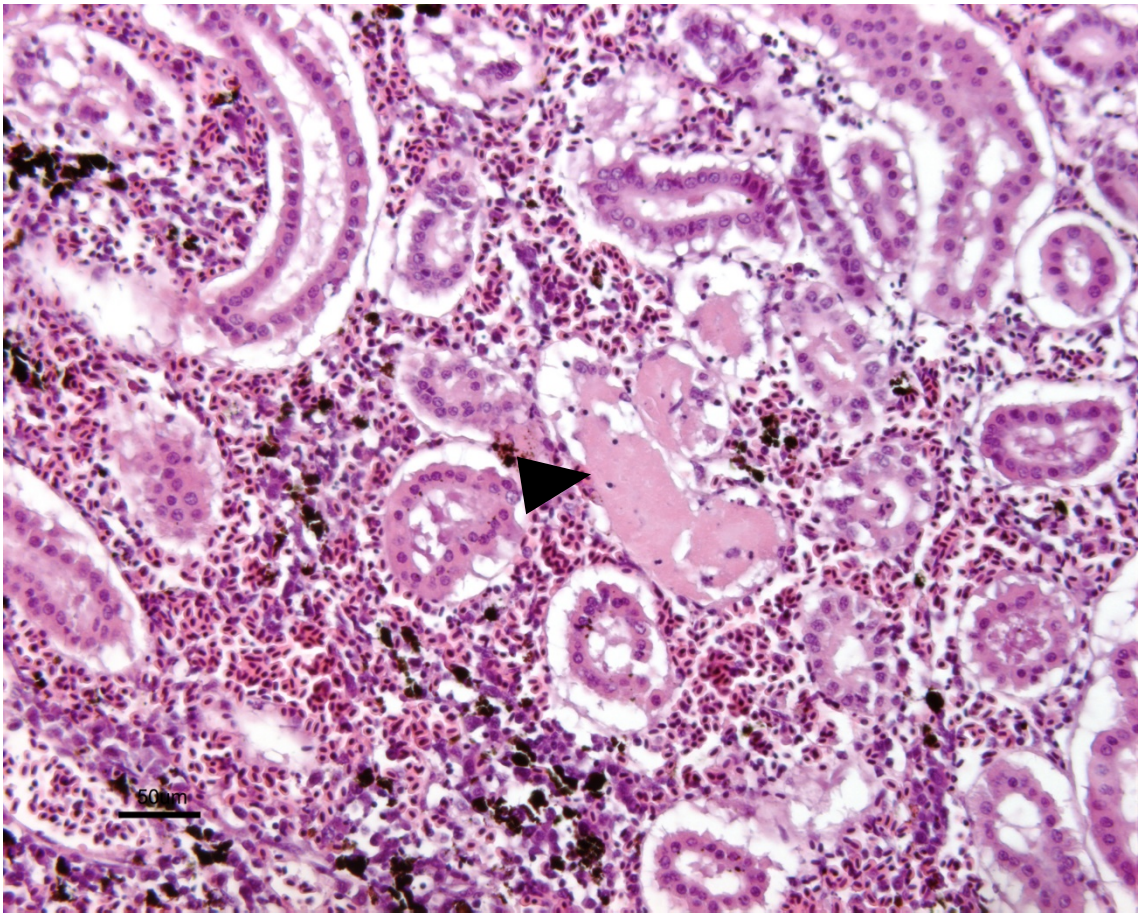

Fig 5 Hematoxylin and Eosin stained section of kidney tissue of Atlantic salmon during ISA clinical outbreak. Diffuse haemorrhage almost completely replacing the hematopoietic part of the kidney, some necrotic kidney tubules (full arrow) can be found especially if the bleeding is severe.

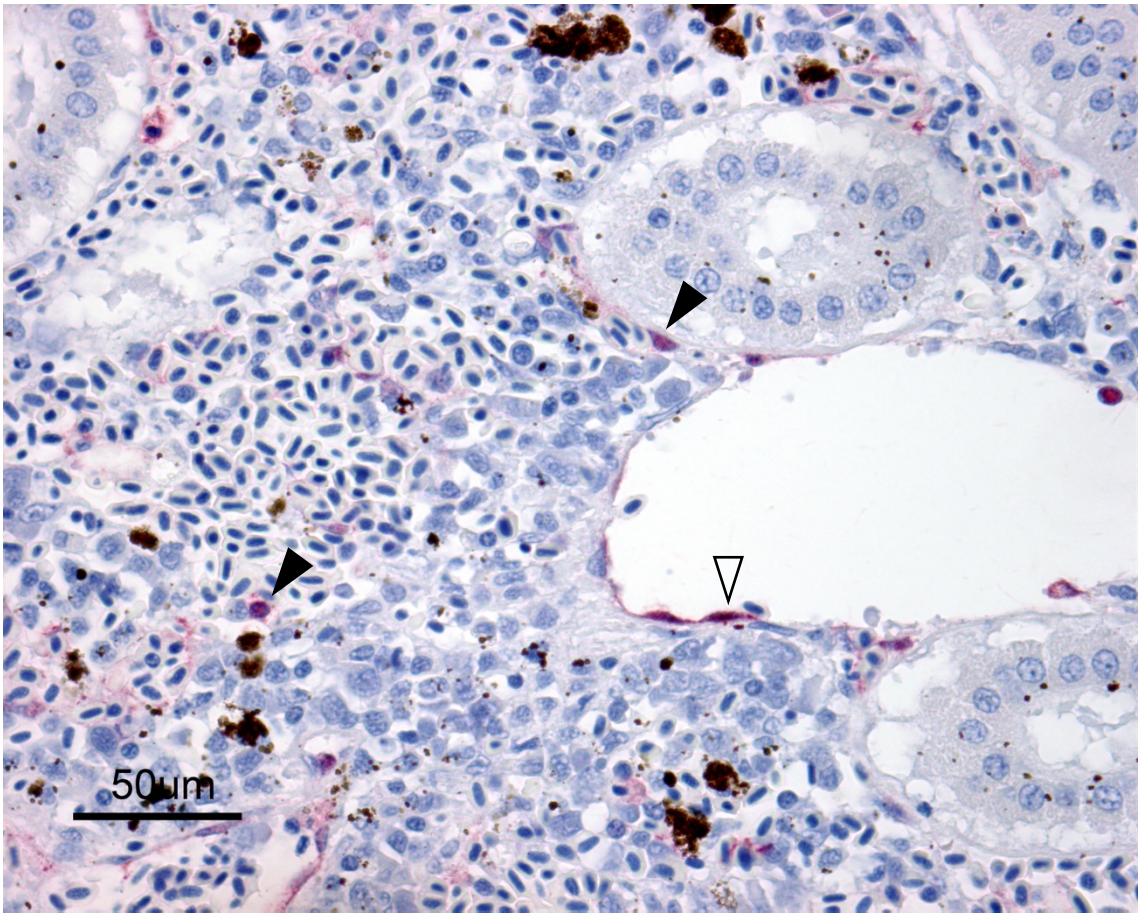

Fig 6. Same specimen of figure 45. Immunohistochemistry staining of ISAV nucleoprotein antigen in kidney section of Atlantic salmon during ISA clinical outbreak. Immunohistochemistry showing red staining of endothelial cells (empty arrow) and some intranuclear staining (full black arrow) can be observed. There are no staining of kidney tubule cells.