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FOR FISH
DISEASES

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTIOUS SALMON ANEMIA (ISA)

PART 3

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTIOUS SALMON ANEMIA (ISA)

I. **Sampling procedures for the surveillance and control of ISA**

When sampling and laboratory examination are carried out, for the purpose of the surveillance or eradication programmes set out in Part 3 of Annex I or to confirm or rule out the presence of ISA in accordance with Article 57(b) of Directive 2006/88/EC, 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 ISA, fish samples shall not be pooled where possible. However, for the purpose of surveillance for ISA, the pooling of 2 to be 5 fish is accepted.

Samples for Reverse Transcriptase polymerase chain reaction (RT-PCR) analysis shall be taken from all of the fish sampled. A piece of mid-kidney shall be removed from the fish using a sterile instrument and transferred to a microfuge tube containing one ml RNA preservative solution of proven efficacy. Tissue from up to five fish may be collected in one tube of transport solution and shall represent one pooled sample. The weight of tissue in one sample shall be 0,5 g. When the fish are too small to obtain a sample of the required weight, pieces of kidney, heart, spleen, liver or pyloric caeca may be taken, in that order of preference, to make up 0,5 g.

Tissue for histological examination shall only be taken from freshly killed fish with a normal constitution, 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, heart, liver, pancreas, gut, gills and spleen shall be removed from individual fish using a scalpel and transferred to 8 % to 10 % (vol:vol) buffered formol saline. The ratio of fixative to tissue shall be at least 20:1 to ensure satisfactory preservation of the tissues. For immunohistochemistry (IHC), samples from mid-kidney and heart shall be taken.

Tissues for virological examination on cell culture shall be taken from all of the fish sampled. Pieces of the liver, anterior or mid-kidney, heart and spleen shall be removed from the fish using a sterile instrument and transferred to plastic tubes containing 9 ml transport medium. Tissues from up to five fish may be collected in one tube containing transport solution and represent one pooled sample. The weight of tissue in one sample shall be 1,0 ± 0,5 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 3 of this point can be fulfilled. Whole fish shall be wrapped in absorbent paper and shipped in a plastic bag, chilled as described in that paragraph.

Live fish may also be shipped, but only under the supervision of the National reference laboratory for fish diseases and taking into account the additional disinfection and biosecurity issues when transporting live fish.

Blood samples and 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 freeze 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 'freeze blocks' must still be partly or completely frozen. In exceptional circumstances, RT-PCR samples and samples for virological examination may be snap-frozen and transported to the laboratory at – 20 °C or below.

For RT-PCR analysis of tissues preserved in Ribonucleic acid(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 in impact-resistant containers. Freezing of those samples shall be avoided.

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 can be fulfilled.

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 for the confirmation of the presence of or to rule out the suspicion of ISA**

When laboratory examination for the purpose of obtaining or maintaining a certain health status with regard to ISA as set out in Section I of Part 3 of Annex I, or for the purpose of the confirmation of the presence of or to rule out a suspicion of ISA in accordance with Article 57(b) of Directive 2006/88/EC, are carried out, using the diagnostic methods set out in Section II of Part 3 of Annex I, 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 for 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.

II.1.1. Total RNA extraction

All work with RNA shall be performed on ice, using gloves.

Total RNA shall be extracted using the phenol-chloroform method or RNA affinity spin columns, according to the manufacturer's instructions.

Purified RNA shall be re-suspended in distilled RNase-free water (namely water treated with 0,1 % diethyl pyrocarbonate).

The concentration and purity of the extracted RNA shall be estimated by measuring the optical density at 260 nm and at 280 nm. An alternative approach may be to include internal controls targeted against the virus genome as referred to in point II.1.3.

II.1.2. RT-PCR for ISAV detection

Several RT-PCR methods may be used for ISAV genome amplification. A two-step RT-PCR may be performed whereby the RT and the PCR reactions steps are run in two separate tubes. However, a one-step reaction, where the two reactions are run in one tube, may also be performed. The one-step method shall be used where possible, as the one tube assay minimises the risk of cross-contamination as no transfer of content have to be made and it is regarded to be as sensitive as the two-step method.

The primers and assay described in this point, namely the ILA1 or ILA2 primer pair that target segment 8 and which have been found suitable for detection of ISAV in outbreaks and in carrier fish, shall be used. The ILA2 reverse primer does not match isolates from North America and an alternative primer set shall be used in those cases.

Forward primer (ILA1): 5′-GGCTATCTACCATGAACGAATC-3′;

Reverse primer (ILA2): 5′-GCCAAGTGTAAGTAGCACTCC-3′.

Cycling conditions: one cycle of 50 °C for 30 minutes, 1 cycle of 94 °C for 15 minutes, 40 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 60 seconds; one cycle of 72 °C for 5 min. Product size 155 bp.

The results of the PCR may vary depending on the conditions under which it is performed, namely the thermal protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results may occur because of false primer annealing or laboratory contamination. Negative template controls and positive controls shall be included on each plate run. However, other RT-PCR versions of proven similar efficacy may be used instead.

II.1.3. RT-qPCR for ISAV detection

The use of RT-qPCR may increase specificity and probably also sensitivity. The method can be performed more rapidly as no gel electrophoresis step is required and it reduces the risk of cross contamination as it is possible to estimate the amount of viral genomic RNA within the sample tube. A drawback of the RT-qPCR assay is that it is often not possible to sequence amplified products. However, if there is doubt on the specificity of the amplified product, another ISAV specific assay must be run to verify the result.

The assay described in this point, which is an assay that target segment 8, shall be used. This assay shall cover isolates from the European Union, the European Free Trade Association and North America. The one-step method shall be used where possible, because 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: one cycle of 50 °C for 30 minutes, one cycle of 95 °C for 15 minutes, 40 cycles of 94 °C for 15 seconds, 60 °C for 60 seconds; it shall be adjusted if necessary. Other RT-PCR or RT-qPCR versions of proven similar efficacy may be used instead.

II.1.4. Sequencing of amplified PCR products

Forward primer (ILAs6-3F): 5′-ATGAGGGAGGTAGCATTGCA -3′;

Reverse primer (ILAs6-2R): 5′-CATGCTTTCCAACCTGCTAGGA -3′.

Negative template controls and positive controls shall be included on each plate run. Cycling conditions (One-step RT-PCR): one cycle of 50 °C for 30 minutes, one cycle of 94 °C for 15 minutes, 40 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 60 seconds, one cycle of 72 °C for 5 minutes; it shall be adjusted if necessary. Other RT-PCR or RT-qPCR versions of proven similar efficacy may be used instead.

Alternatively, the following method for sequencing HPR in segment 6 may be used:

Forward primer: 5′-GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA-3′;

Reverse primer: 5′-GAT-GGT-GGA-ATT-CTA-CCT-CTA-GAC-TTG-TA-3′;

Product size: 304 nt if HPR0.

RT-PCR assays with similar sensitivities and specificities to the assays described in this point may also be used.

The purity of the amplified RT-PCR product shall be checked by gel electrophoresis before sequencing. If only one pure fragment appears, it shall be purified directly from the PCR reaction. If multiple amplified fragments are present, the fragment of interest shall be purified by gel electrophoresis. The purification of PCR fragments from solutions or agarose gels shall be made using PCR fragment affinity spin columns, according to the manufacturer's instructions.

Sequencing shall be performed using amplification primers at external specialised sequencing companies. The results shall be analysed with the search tool BLAST and the sequences shall be compared with other known sequences in the US National Centre for Biotechnical Information (NCBI) nucleotide database.

Sequencing must eliminate any doubt on the specificity of an amplified RT-PCR product.

II.2. ISAV isolation on cell cultures

II.2.1. Preparation of samples

The tissue may be kept at – 80 °C. The tissue shall only be frozen and thawed once before examination. For surveillance and control purposes, the examination shall be undertaken as fast as possible.

Each sample (tissue pool in transport solution) shall be completely homogenised using a validated homogeniser, centrifuged at 2 000 to 4 000 × *g* for 15 minutes at 0 to 6 °C, and the supernatant shall be filtered (0,45 µm) and incubated with an equal volume of a suitably diluted pool of antisera to the indigenous serotypes of IPNV. The titre of the antiserum must be at least 1:2 000 in a 50 % plaque neutralisation test. The mixture shall be incubated for one hour at 15 °C. This shall represent the inoculum.

Treatment of all inocula with antiserum to Infectious pancreatic necrosis virus (a virus which in some parts of the Europe occurs in 50 % of fish samples) aims at preventing cytopatic effect (CPE) due to IPN virus from developing in inoculated cell cultures. To reduce the duration of the virological examinations as well as the number of cases in which occurrence of CPE would have to be considered potentially indicative of ISAV, such treatment may be carried out. When samples come from production units which are considered free from IPN, the treatment of inocula with antiserum to IPN virus may be omitted.

II.2.2. Inoculation on cell cultures

Atlantic salmon kidney (ASK) cells shall be used for primary ISAV isolation. Other cell lines of proven efficacy and sensitivity in isolating ISAV may be used, taking into consideration strain variability and the ability of different strains to replicate in different cell lines. The ASK cells seem to support isolation and growth of the hitherto known virus isolates, as long as a low passage level is used. A more distinct cytopathic effect (CPE) may appear in ASK cells than in other susceptible cell lines like SHK-1 (Salmon head kidney-1).

ASK (pass 65 or lower) cells shall be grown in L-15 medium containing 10 % foetal bovine serum, 2 % (vol:vol) 200 mM L-glutamine, and 0,08 % (vol:vol) 50 mM 2-mercaptoethanol in multi-well plates. Antiserum-treated organ suspension shall be inoculated into young actively growing cell cultures to give a final dilution of tissue material to culture medium of 1:1 000. For each organ, suspension 40 µl of inoculum shall be added to one well containing 2 ml of culture medium. To minimise the risk of cross-contamination, separate 12- or 24-well plates shall be used for samples from different fish farm sites.

One plate shall be left uninoculated 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 10^7 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. A sensitivity test every 6 months of ASK cells towards ISAV isolates may replace the use of including a positive control at each inoculation.

Samples shall be incubated at 15 ± 2 °C for up to 15 days. Using a microscope, cell cultures shall be examined for CPE twice, between 5 to 7 and 12 to 14 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 14, an Indirect fluorescent antibody test (IFAT), haemadsorption or RT-PCR shall be performed.

II.2.3. Subcultivation

Subcultivation shall be carried out between days 13 to 15. Culture supernatant shall be added to wells containing fresh actively growing cells in appropriate dilution (1/10) in multi-well plates and incubated at 14 ± 2 °C for up to 18 days. Using a microscope, cell cultures shall be examined for CPE twice, between days five to seven and days 14 to 18 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 days 14 to 18, a haemadsorption or an RT-PCR test shall be performed.

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 to 18 days and sub cultivated again with a further period of 14 to 18 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 28 to 36 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 0 to 6 °C and the supernatant shall be filtered at 0,22 µm. If bacterial contamination occurs during the subcultivation step the supernatant shall be filtered at 0,22 µm, inoculated onto fresh cells and incubated for a further 14 to 18 days.

II.2.4. Virus identification tests

If evidence of CPE is observed at any stage, or if a haemadsorption test is positive, virus identification shall be carried out. The methods of choice for the identification of ISAV shall be RT-PCR in accordance with point II.1 and Immunofluorescence (IF) 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 Annex VI to Directive 2006/88/EC.

II.2.5. Haemadsorption

As replication of ISAV in cell cultures does not always result in a CPE, every well shall be subject to an RT-PCR test or a haemadsorption test in accordance with this point, or an IF test in accordance with point II.2.6.

Cell culture medium shall be removed from each well, including those of positive and negative controls, and placed in labelled sterile tubes. 500 µl of a 0,2 % (vol:vol) suspension of washed rabbit or horse red blood cells, or a 0,05 % (vol:vol) suspension of washed rainbow trout or Atlantic salmon red blood cells, shall be added to each well and incubated at room temperature for 45 minutes. The red blood cells shall be removed and each well shall be washed twice with L-15 medium. Each well shall be examined using a microscope.

The presence of clusters of red blood cells attaching to the surface of ASK cells shall be indicative of presumptive infection with an orthomyxovirus. If a haemadsorption test is positive, a virus identification test shall be performed immediately in accordance with point II.2.4.

II.2.6. Immunofluorescence (IF)

ASK (pass 65 or lower) shall be grown in a L-15 medium containing 10 % foetal bovine serum, 2 % (vol:vol) 200 mM L-glutamine, and 0,08 % (vol:vol) 50 mM 2-mercaptoethanol in multi-well plates and used at greater than 50 % confluence. Other cell lines or growth medium of proven efficacy may also be used. 225 µl of putative virus-infected culture supernatant shall be added to each of two wells, mixed and 225 µl transferred to two further wells, namely a 1:5 dilution. Two additional wells shall be left uninoculated to act as controls. Samples from each fish farm site shall be handled on separate plates, as shall the virus control. A virus control shall be established using a reference isolate of ISAV.

Plates shall be incubated at 14 ± 2 °C and examined microscopically for up to 7 days. When early CPE is observed, or if no CPE is observed within 7 days, the next step shall be fixation. Wells shall be washed with Phosphate buffered saline (PBS) and fixed by incubation with 80 % acetone for 20 minutes at room temperature. Plates shall be air-dried and stained immediately or stored at 0 to 6 °C for no more than 24 hours prior to staining.

Replicate wells shall be stained with a mix of monoclonal antibodies (MAb) 3H6F8 and 1OC9F5 against ISAV, or other MAb of proven efficacy and specificity, diluted in PBS and incubated at 37 ± 4 °C for 30 minutes. MAb shall be removed and plates washed three times with 0,05 % Tween 20 in PBS. Anti-mouse IgG Fluorescein isothiocyanate (FITC) conjugate diluted in PBS shall be added to each well and incubated at 37 ± 4 °C for 30 minutes. The dilutions of different batches of MAb and FITC conjugate shall be optimised in each laboratory. Antibody shall be removed and plates shall be washed three times with 0,05 % Tween 20 in PBS.

Wells shall be examined immediately using an inverted microscope set up for fluorescence microscopy with a suitable filter for excitation of FITC. A test shall be considered positive if fluorescent cells are observed. For a test to be valid, the positive controls shall score positive and the negative controls shall score negative.

II.3. Examination of other tissues

The technique referred to in point II.2.6 may be applied to other fish tissues such as liver, spleen and heart providing a reasonable quantity of endothelial cells, leucocytes or lymphocytes can be deposited on the slide. The staining procedure shall remain the same for each tissue, although for some tissues it may be preferable to omit the propidium iodide staining and rely on the phase illumination to identify the cell types present in the imprint.

II.4. Histology

Paraffin-embedded sections shall be cut at 5 µm and stained using haematoxylin and eosin.

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

(a) numerous erythrocytes in the central venous sinus and lamellar capillaries of the gills, where erythrocyte thrombi also may be formed;

(b) multifocal to confluent petechiae or hepatocyte necrosis or both at some distance from larger vessels in the liver; multifocal accumulation of erythrocytes in dilated hepatic sinusoids;

(c) accumulation of erythrocytes in blood vessels of the intestinal lamina propria and eventually haemorrhage into the lamina propria;

(d) spleen stroma distended by erythrocyte accumulation;

(e) slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney;

(f) erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

II.5. Immunohistochemistry (IHC)

Polyclonal antibody against ISAV nucleoprotein shall be used on paraffin sections from formalin-fixed tissue. The organs to be examined shall be mid-kidney and heart (transitional area including all three chambers and valves). 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

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 decision:

(a) antigen retrieval shall be 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 shall then be 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 shall be 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) shall be added to develop for 20 minutes. Sections shall then be washed in tap water before counterstaining with Harris haematoxylin and mounted in aqueous mounting medium. ISAV positive and ISAV negative tissue sections shall be included as controls in every setup.

(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 and intranuclear staining of endothelia cells in blood vessels of endocardium. A test sample section shall only be regarded as positive if such clear, intranuclear red staining of endothelial cells is found;

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

Since the intranuclear localisation is particular to the orthomyxovirus nucleoprotein during a stage of virus replication but concurrent cytoplasmic staining is often dominant, cytoplasmic and other staining patterns without intranuclear localisation shall 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 may be slight or absent, possibly because of lysis of infected endothelial cells.