DIAGNOSTIC METHODS FOR THE CONFIRMATION AND SURVEILLANCE OF Category A disease Epizootic haematopoietic necrosis (EHN)

V2022.1

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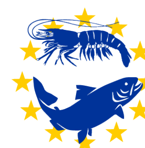

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I. Diagnostic methods and procedures for the surveillance and confirmation of infection with epizootic haematopoietic necrosis virus (EHNV)

When sampling and laboratory examination for the purpose of conducting surveillance in a surveillance zone as set out in Annex XV of **COMMISSION DELEGATED REGULATION (EU) 2020/687**, the detailed diagnostic methods and procedures described in the following points I.1 to I.7 shall apply.

I.1. Preparation and shipment of samples from fish

I.1.1. Fish for sampling

The sampling for laboratory examinations must include the susceptible species rainbow trout (*Oncorhynchus mykiss*), or redfin perch (*Perca fluviatilis*) or other fish that have recently died from the suspected/confirmed case of EHN or fish with an epidemiological link to a suspected or confirmed case of EHN

I.1.2. Tissues for virological examination on cell culture

Before shipment or transfer to the laboratory, pieces of the organs to be examined shall be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing transport medium.
Fish material suitable for virological examination on cell culture and for (q)PCR is dependent on fish size. When sampling fish too small in size to permit dissection of individual tissues, viscera including kidney shall be collected or whole fish homogenised after removal of the body behind the anal pore. For larger size fish, anterior kidney, spleen, shall be sampled. It is acceptable to pool these organs.
Organ pieces from a maximum of 10 fish may be collected in one sterile tube containing at least 4 ml of transport medium (i.e. cell culture medium with 10 % calf serum and antibiotics, the combination of 200 IU penicillin, 200 µg streptomycin, and 200 µg/ml kanamicin can be used, but other antibiotics of proven efficacy may be used as well) representing one pooled sample. The tissue in each sample shall weigh a minimum of 0.5 g.
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.

1.1.3. Samples for polymerase chain reaction (PCR or qPCR) analysis

Samples shall be taken from the fish in accordance with the procedure described in point I.1.1. using sterile instruments and transferred to sterile plastic tubes containing transport medium. Tissue from up to 10 fish may be collected in one tube and shall represent one pooled sample.
Alternatively, samples may be placed in at least five volumes of DNA/RNA stabilisation reagent (e.g. RNAlater) according to the manufacturer's recommendation. Samples in DNA/RNA stabilising reagents can be shipped on ice (<4°C) or at room temperature (<25°C). Sample size shall not exceed 5mm in any direction to allow quick penetration and proper stabilization of the viral DNA.

I.2. Shipment of fish samples

Tubes containing fish tissues in transport medium for cell cultivation or (q)PCR analysis shall be placed into insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples shall be avoided. The temperature of a sample during transit must never exceed 10 °C and ice must still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen or a recording of the temperature during the entire transport must be provided (e.g. with a datalogger placed inside the package).
Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish may be wrapped up in paper with absorptive capacity and shall finally be shipped in a plastic bag.

I.3. Collection of supplementary diagnostic material

When approved by the diagnostic laboratory, other fish tissues may be collected as well and prepared for supplementary examinations.
Records shall be made by the diagnostic lab with details on conditions of samples on receipt.

I.4. Preparation of samples for cell culture examination and qPCR

I.4.1. Freezing in exceptional cases

Where practical difficulties arise in the laboratory, which make it impossible to process the samples within 48 hours after the collection of fish tissues, it may be acceptable to freeze the tissue specimens in the transport medium or in the DNA/RNA stabilisation reagents at - 20 °C (or below) and to carry out virological examination within 14 days. However, the fish tissue shall only be frozen and thawed once before examination. Records shall be kept with details on the reason for each freezing of fish tissue samples.
Samples collected in DNA/RNA stabilization reagents shall not be used for cell cultivation.

I.4.2. Homogenisation of organs for virological examination on cell culture

In the laboratory, the fish tissue collected in tubes with transport medium shall be completely homogenised, either by stomacher, blender or mortar and pestle with sterile sand, and subsequently suspended in the original transport medium.
If a sample consists of whole fish too small in size to permit dissection of individual tissues, viscera including kidney shall be collected or whole fish homogenised after removal of the body behind the anal pore. In larger fish, the tissue specimens shall be collected as described in point I.1. The tissue specimens shall be minced with sterile scissors or scalpel and homogenised as described in the first paragraph of this point and suspended in transport medium.
The final ratio between tissue material and transport medium shall be adjusted in the laboratory to 1:10.

I.4.3. Centrifugation of homogenate for virological examination on cell culture

The homogenate shall be clarified in a refrigerated centrifuge at 2 °C to 5 °C at 2.000 to 4.000 x *g* for 15 minutes. The collected supernatant may be treated with antibiotics for either four hours at 15 °C or overnight at 4 to 8°C. If the sample has been shipped in transport medium supplemented with antibiotics, the treatment of the supernatant with additional antibiotics may be omitted.

If the collected supernatant is stored at -80 °C within 48 hours after the sampling, it may be reused only once for virological examination.
Where practical difficulties arise, such as incubator breakdown 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.
Prior to the inoculation of the cells, the supernatant shall be mixed with equal parts of a suitably diluted pool of antisera produced against the indigenous serotypes of infectious pancreatic necrosis virus (IPNV) and incubated for a minimum of one hour at 15 °C or a maximum of 18 hours at 4 °C. The antiserum shall reduce the titre of IPNV by at least 3 log steps (e.g. at least 1/2.000 in a 50 % plaque neutralisation test).
Treatment of all inocula with antisera to IPNV aims at preventing cytopathic effect (CPE) due to IPNV from developing in inoculated cell cultures.
When samples come from production units, which are considered free from IPN, the treatment of inocula with antisera to IPNV may be omitted.

I.4.4. Sample preparation for detection of Ranavirus by qPCR

If samples were collected in transport medium, the procedure set out in points I.4.2. and I.4.3. shall be carried out. After centrifugation, supernatants shall be collected and DNA extracted. If further examination is not to be undertaken directly after centrifugation, the samples shall be immediately frozen at -20 °C or below. For the analysis of fish tissues preserved in DNA/RNA stabilization reagent, subsequent work shall be carried out within the following time scales for samples stored at different temperatures:

- samples stored at 25 °C: one week;
- samples stored at 4 °C: one month;
- samples stored at –20 °C: indefinitely.

Disruption and homogenization of tissues for qPCR-based surveillance, can be performed either on the total amount of tissue (the whole pool) or by aliquots of each tissue of every specimen included in the pool (e.g. by tissue lyzer, Fast Prep or similar, or methods given in I.4.2.).

I.4.5. Pooling of samples for qPCR

Pooling of samples for qPCR testing can be done according to one of the following options:

- Use the supernatant from homogenised fish tissue material of pooled organs from up to 10 fish in cell culture medium for qPCR.
- Pools of aliquoted material representing each and every organ from up to 10 fish can be disrupted and homogenized and tested for qPCR.
- Single fish samples can be individually disrupted, homogenized and then pooled for qPCR testing.

I.5. Virological examination on cell culture

I.5.1. Cell cultures and media

Bluegill fry cell line -2 (BF-2) (Wolf et al., 1988) and *Epithelioma papulosum cyprini* (EPC) (Fijan et al., 1983) cells shall be grown between 20 °C and 30 °C in suitable medium, namely Eagle's Minimum essential medium (MEM) or modifications thereof, with a supplement of 10 % foetal bovine serum and antibiotics in standard concentrations.

When the cells are cultivated in closed vials, the medium shall be buffered with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) (23 mM) and Natrium bicarbonate (6 mM). The pH must be 7.6 ± 0.2. The cell cultures to be used for inoculation with fish tissue material shall be young, normally one day old cell culture monolayers where possible; however, a range between 4 to 48 hours after the last passage may be accepted. The cells must be actively growing at inoculation.

I.5.2. Inoculation of cell cultures

Antibiotic-treated organ suspensions shall be inoculated into cell cultures in at least two dilutions, namely a primary dilution of 1:100 and, in addition, a 1:10 dilution thereof (final dilution 1:1.000). At least two cell lines shall be inoculated as referred to in point I.5.1.
For each dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well cell culture plate, shall be utilised. Cell culture plates shall be used where possible.

I.5.3. Incubation of cell cultures

The inoculated cell cultures shall be incubated at 15-20 ± 2 °C for seven to ten days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances shall be performed to ensure cell susceptibility to virus infection.

At least every six months or if decreased cell susceptibility is suspected, titration of frozen stocks of EHNV or other Ranaviruses (e.g. ECV or ESV) shall be performed to verify the susceptibility of the cell cultures to infection. The procedure set out in point III shall be used, if possible. Where susceptibility testing of cell cultures according to point III is not performed routinely in the laboratory, it is necessary to include a positive control.

I.5.4. Microscopy

Inoculated cell cultures shall be inspected regularly, at least three times a week, for the occurrence of CPE at 40 to 150 x magnification. If obvious CPE is observed, virus identification procedures in accordance with point I.7. shall be initiated immediately.

I.5.5. Subcultivation

If no CPE has developed after the primary incubation for 7 to 10 days, subcultivation shall be performed to fresh cell cultures utilising a cell area similar to that of the primary culture.
Aliquots of medium (supernatants) from all cultures or wells constituting the primary culture shall be pooled according to the respective cell line 7 to 10 days after inoculation. The pools shall then be inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:100 and 1:1000, respectively, of the supernatant) as described in point I.5.2. Alternatively, aliquots of 10 % of the medium constituting the primary culture shall be inoculated directly into a well with 24h old cell culture (namely, well to well subcultivation). The inoculation may be preceded by pre-incubation of the dilutions with the antiserum to IPNV at appropriate dilution as described in point I.4.3.
The inoculated cultures shall then be incubated for another 7 to 10 days at 15 °C -20 ± 2°C and inspected in accordance with point I.5.4.

If toxic CPE occurs within the first three days of incubation, subcultivation shall be performed at that stage, but the cells shall then be incubated for seven days and subcultivated again for further seven days. When toxic CPE develops after three days, the cells shall be passaged once and incubated to achieve the total of 14 days from the primary inoculation. There must be no evidence of toxicity in the final seven days of incubation.
If bacterial contamination occurs despite treatment with antibiotics, subcultivation shall be preceded after centrifugation at 2.000 to 4.000 x g for 15 to 30 minutes at 2 to 5 °C, or filtration of the supernatant through a 0.45 µm filter (low protein-binding membrane) or both. In addition to this, subcultivation shall follow the same procedures as described for toxic CPE in the fourth paragraph of this point.
If no CPE occurs, the test may be declared negative.

I.6. Examination of samples by qPCR

Currently there is one molecular method approved by the EURL for detection of Ranavirus DNA directly on fish tissue. This method is the qPCR described in I.6.2. (Stilwell et al., 2018) As the results of the qPCR can vary depending on the conditions under which it is performed, adequate positive and negative controls shall be included in each stage of the assay (extraction and q-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.

I.6.1 Total DNA extraction

Total DNA shall be extracted using validated DNA extraction and purification methods including DNA affinity spin columns or magnetic bead-based methods, according to the manufacturer's instructions. It is recommended to include internal controls targeted against the host genome (e.g. EF1AA/*ELF-1α* according to the protocol provided by Jonstrup et al., 2013 for rainbow trout) as referred to in point I.6.

I.6.2. qPCR for Ranavirus detection

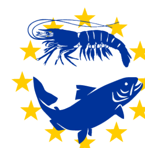
The assay described targeting the MCP gene (Stilwell et al., 2018) shall be used. This assay should detect all ranaviruses, including the causative agent of the list A disease EHN and other Ranavirus species endemic in Europe.

- Forward primer: 5′- CCA GCC TGG TGT ACG AAA ACA -3′;
- Reverse primer: 5′- ACT GGG ATG GAG GTG GCA TA-3′ and
- Probe: 5′ 6-FAM-TGG GAG TCG AGT ACT AC-MGB-3′.

Negative template controls and positive controls shall be included on each plate run.
Standard thermocycling conditions are 95°C for 10 minutes, followed by 45 cycles of 95°C for 30 seconds and 60°C for 45 seconds.
Cycling conditions shall be adjusted according to the manufacturer’s instruction of the specific kit.
Other validated 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.

I.7. Virus identification

If evidence of CPE has been observed in a cell culture or Ranavirus DNA is detected by qPCR, the cell supernatant from cell culture or the purified DNA shall be examined by PCR followed by sequencing.



Supplementary testing of the isolate may include Indirect Fluorescence Antibody Test (IFAT) and or Restriction Enzyme Analysis (REA) of the DNA template obtained by conventional PCR.

I.7.1 Sequencing of the MCP gene for discrimination of EHNV from other ranaviruses

With the occurrence of endemic fish ranaviruses in Europe, it is mandatory to follow up any qPCR positive results or isolation on cell culture with PCR amplification (Hyatt et al.,2000) and sequencing to determine if the isolate is EHNV or one of the endemic ranaviruses (e.g. ECV, ESV). Therefore, all samples that test Ranavirus positive by real-time PCR shall be amplified by PCR of the MCP gene region. Once an EHNV is detected from one farm, no further sequencing of Ranavirus positive samples from that farm is required. Primers to be used are those published by OIE (Aquatic Manual Chapter 2.3.2.):

- Forward primer: 5'- CGC-AGT-CAA-GGC-CTT-GAT-GT -3'
- Reverse primer: 5'- AAA-GAC-CCG-TTT-TGC-AGC-AAA-C -3'
- Product size: 580 bp.

Negative template controls and PCR positive controls shall be included in each run.
The PCR cycling conditions include, one cycle of 94 ºC for 3 minutes followed by 40 cycles of 94 ºC for 30 seconds, 50 ºC for 30 seconds and 72 ºC for 60 seconds. Cycling conditions shall be adjusted according to manufacturer instructions of the PCR kit used.
The specificity and size of all amplified PCR products shall be checked by gel electrophoresis. Any gel electrophoresis system can be used.
All samples suspected to yield PCR products of ranavirus DNA shall be confirmed by sequencing. The obtained clean consensus sequence shall be analyzed with BLASTnt in NCBI, matching (>99 % identity) with a reference sequence of EHNV.
Other PCR methods of proven similar efficiency and specificity may be used instead.

I.8 Additional identification methods

I.8.1 Protocol for the Restriction Endonuclease Assay (REA) for the discrimination of EHNV from other ranaviruses

The PCR products to be analysed by Restriction Endonuclease Assay (REA) are obtained using the MCP-2 primers described in the OIE manual (Aquatic manual Chapter 2.3.2.):

- Forward primer (M153): 5'-ATG-ACC-GTC-GCC-CTC-ATC-AC-3'
- Reverse primer (M154) : 5'-CCA-TCG-AGC-CGT-TCA-TGA-TG-3'

PCR conditions are as follows: 1 cycle of denaturation at 94°C for 3 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; final extension at 72°C for 5 minutes, and cooling to 4°C.
In a total volume of 10 µl of a suitable cleavage buffer, 2 µl of the amplified DNA from the MCP-2 PCR is incubated with two units of the restriction endonuclease Acc I, following the instructions of the manufacturer. The products of the reaction are then separated in a suitable agarose gel electrophoresis system.

Cleavage products of amplified DNA from the MCP-2 PCR using Acc I

Virus	Cleavage products (bp)
EHNV	238 / 387
BIV, ESV, ECV, WV	625
FV3, GV	164 / 461

I.8.2. Indirect Fluorescence Antibody Test - IFAT

The identification of ranaviruses can be performed by infecting cells in adapted plates ("Black" or conventional 96-well plates, conventional 24 or 4-well plates or on cover slips placed into 24-well plates) followed by IFAT. This method can distinguish ranaviruses from other viruses (i.e. *Rhabdoviridae, Aquabirnaviridae*, etc.) but is not capable to differentiate EHNV from the other ranaviruses.

The following protocol shall apply:

(a) 24-wells or cover slips shall be seeded with cells (e.g. EPC incubated at the optimal temperature of 25-26°C) so that a density of between 60 % and 90 % confluency is reached after 24 hours of cultivation. EPC cells shall be used where possible for this purpose because of their strong adherence to glass surfaces, but other cell lines such as BF-2 may be used as well. 150 µl of infected cell culture supernatant in two different dilutions (1:10 and 1:1.000) shall be inoculated in duplicate onto the monolayers and incubated at 15-20°C ± 2 °C for 24-48 hours;

(b) subsequently, cell culture medium shall be removed, and the infected cell monolayers fixed with 0.5 ml ice-cold, aqueous acetone solution (80% v/v). Fixation shall take place in fume hood for 15 minutes at room temperature, then the acetone solution shall be removed and the fixed cells shall be air dried. At this stage, the plates with the cover slips shall either be processed immediately or stored at –20°C for further use;

(c) specific antibodies against ranaviruses (either produced in house or purchased) shall be diluted in 0.01 M PBS, pH 7.2 in the dilution recommended by the producer of the antibodies. In case the polyclonal serum is produced in house, this shall be tested for specificity and absence of cross reactions against VHSV, IHNV, IPNV and SVCV of the antibody solution shall be added to the fixed monolayers and plates shall be incubated for one hour at 37°C in a humid chamber;

(d) cover glasses shall be washed gently three times with PBS containing 0.05% Tween-20 (PBS-T), and the buffer shall be removed completely after the last rinse but drying of the monolayer shall be avoided. The cells shall subsequently be incubated for one hour at 37°C with fluorescein isothiocyanate (FITC) - or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated species specific antibodies against the primary antibody (either monoclonal or polyclonal), diluted according to the supplier instructions, washed again in PBS-T, and the excess of buffer removed. Stained cultures shall be mounted using an aqueous fluorescence mounting medium and examined under a fluorescence microscope. Use 10 x or 12 x eyepieces and a x 25 or x 40 lens.

Other IFAT techniques, with regard to cell cultures, fixation, time and temperature requirements and antibodies of reference quality, of proven similar efficiency may be used instead.

II. Diagnostic procedures for "the confirmation of" or "to rule out" the suspicion of infection with EHNV

When sampling and laboratory examination for the purpose of confirming or ruling out a suspicion of infection with EHNV, the following health visit, sampling and testing procedures must comply with the requirements laid down in **COMMISSION DELEGATED REGULATION (EU) 2020/687** Annex XII.

The confirmation of the first case of EHN in Member States, zones or compartments must be based on either :

- conventional virus isolation in cell culture followed by EHNV identification by sequencing of the MCP PCR product

or

- Ranavirus genome detection by qPCR on organ material followed by EHNV identification by sequencing of the MCP PCR product

Selection of samples

At least 10 fish, when clinical signs or post-mortem lesions consistent with infection with EHNV are observed or a minimum of 30 fish, when clinical signs or post-mortem lesions are not observed, shall be selected for examination.

(i) if weak, abnormally behaving or freshly dead but not decomposed fish are present, those fish must be selected. If such animals are not present, the fish selected must include fish of listed species, belonging to different year classes, proportionally represented in the sample;

(ii) if more than one water source is used for fish production, listed species representing all water sources must be included for sampling to ensure that all parts of the establishment are proportionally represented in the sample;

(iii) if rainbow trout (*Oncorynchus mykiss*) or European perch (*Perca fluviatilis*) are present, only fish of those species may be selected for sampling. If neither rainbow trout nor European perch are present, the sample must be representative of all other listed species present, following the criteria in points (a) to (d);

(iv) when collection of samples from wild populations of listed species is required under Article 102(a) of Regulation 2020/697, the number and geographical distribution of the sampling points must be determined in a way that ensures a reasonable coverage of the area suspected to be infected.

The sampling points must also be representative of the different ecosystems where the wild populations of susceptible species are located such as marine, estuary, river and lake systems.

III. Procedure for titration to verify the susceptibility of the cell cultures to infection

When titration to verify the susceptibility of the cell cultures to infection as referred to in point I.5.3. is carried out, the procedures set out in the following paragraphs of this point shall be followed.

Well-defined isolates of the genus Ranavirus made available to Member States from the EURL for fish diseases shall be used. Batches of Ranavirus shall be propagated in cell culture flasks on BF-2 or on EPC cells. Cell culture medium with at least 10 % serum shall be used. Low MOI for inoculation (< 1) shall be used.

At total CPE, the virus shall be harvested by centrifugation of cell culture supernatants at 2.000 x g for 15 minutes, sterile filtered through a 0.45 µm membrane filter and aliquoted into labelled cryotubes. The virus shall be kept at -80 °C.

Three replicate vials with each virus shall be thawed under cold water and subsequently titrated. Once thawed the tube shall not be frozen and reused again. At least every six months, or if it is suspected that the susceptibility of a cell line has decreased the susceptibility test shall be repeated.

Titration procedures must be described in detail and the same procedure followed each time.

Titration by end point dilution shall include at least six replicates at each dilution step. The titres shall be compared with previously obtained titres. If the titre of any of the three 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.

Records shall be kept for a period of at least 5 years.

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