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EURL FOR FISH DISEASES

SAMPLING AND DIAGNOSTIC PROCEDURES FOR EPIZOOTIC HAEMATOPOIETIC NECROSIS (EHN)

Mass mortality of redfin perch. Photo: Anonymous

Sampling and diagnostic procedures for epizootic haematopoietic necrosis (EHN)

PART I

Sampling and diagnostic procedures for epizootic haematopoietic necrosis (EHN)

I. Aetiology of EHN

Epizootic haematopoietic necrosis (EHN) is a disease of the susceptible species listed in Part II of Annex IV to Directive 2006/88/EC caused by epizootic haematopoietic necrosis virus (EHNV) a virus assigned to the genus *Ranavirus*, within the family *Iridoviridae*. For detailed description see OIE Diagnostic Manual EHN Chapter 2.3.1.

II. Preparation and shipment of samples from fish

Before shipment or transfer to the laboratory pieces of the organs to be examined must be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing 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 kanamycin per millilitre (ml) can be recommended, but other antibiotics of proven efficiency may be used as well.

Fish material suitable for virological examination 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, anterior kidney, liver, spleen, are suitable samples.

Ovarian fluid or organ pieces from a maximum of 10 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 should weigh a minimum of 0,5 gram (g).

The tubes should be placed in insulated containers (for instance thick-walled polystyrene boxes) together with sufficient ice or "freeze blocks" to ensure chilling of the samples during transportation to the laboratory. Freezing must be avoided. The temperature of a sample during transit should never exceed 10 °C and ice should still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen.

The virological examination must be started as soon as possible and not later than 48 hours after the collection of the samples. In exceptional[1] 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 transport medium and that the temperature requirements during transportation can be fulfilled.

Whole fish may be sent to the laboratory if the temperature requirements during transportation can be fulfilled. Whole fish may be wrapped up in paper with absorptive capacity and must finally be shipped in a plastic bag, chilled as mentioned. Live fish can be shipped as well.

All packaging and labelling must be performed in accordance with present national and international transport regulations as appropriate.

[1] In exceptional cases e.g. when fish are collected in very remote areas with no possibility of daily mailing.

III. Collection of supplementary diagnostic material

According to agreement with the involved diagnostic laboratory, other fish tissues may be collected as well and prepared for supplementary examinations.

IV. Preparation of samples for virological examination

IV.1. Freezing in exceptional cases

Where practical difficulties arise (e.g. bad weather conditions, non-working days, laboratory problems, etc.) which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples it is acceptable to freeze the tissue specimens in cell culture medium at - 20 °C or below and carry out virological examination within 14 days. The tissue, however, must be frozen and thawed only once before examination. Records must be kept with details on the reason for each freezing of tissue samples (such as storm, cell lines died, etc.)

IV.2. Homogenisation of organs

In the laboratory the tissue in the tubes must 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 less than 4 cm long, these should be minced with sterile scissors or scalpel after removal of the body behind the gut opening. If a sample consists of whole fish with body length between 4 cm and 6 cm, the viscera including kidney should be collected. If a sample consisted of whole fish more than 6 cm long, tissue specimens should be collected as described in Part I.III.1. The tissue specimens should be minced with sterile scissors or scalpel and homogenised as described above and suspended in transport medium.

The final ratio between tissue material and transport medium must be adjusted in the laboratory to 1:10.

IV.3. Centrifugation of homogenate

The homogenate is centrifuged in a refrigerated centrifuge at 2 °C to 5 °C at 2000 to 4000 x *g* for 15 minutes and the supernatant collected and treated for either four hours at 15 °C or overnight at 4 °C with antibiotics, e.g. gentamicin 1 mg/ml may be useful at this stage.

If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted.

The antibiotic treatment aims at controlling bacterial contamination in the samples and makes filtration through membrane filters unnecessary.

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 (e.g. incubator breakdown, problems with cell cultures, etc.) which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples, it is acceptable to freeze the supernatant at -80 °C and carry out virological examination within 14 days.

Prior to the inoculation of the cells the supernatant is mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPN virus and incubated with this for a minimum of one hour at 15 °C or a maximum of 18 hours at 4 °C. The titre of the antiserum must be at least 1/2000 in a 50 % plaque neutralisation test.

Treatment of all inocula with antiserum to IPN virus (a virus which in some parts of Europe occurs in 50 % of fish samples) aims at preventing CPE due to IPN virus from developing in inoculated cell cultures. This will 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 EHN.

When samples come from production units, which are considered free from IPN, treatment of inocula with antiserum to IPN virus may be omitted.

V. Virological examination

V.1. Cell cultures and media

BF-2 or RTG-2 and either EPC or FHM cells are grown at 20 to 30 °C in suitable medium, e.g. Eagle's 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, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7,6 ± 0,2.

Cell cultures to be used for inoculation with tissue material should be young (4 to 48 hours old) and actively growing (not confluent) at inoculation.

V.2. Inoculation of cell cultures

Antibiotic-treated organ suspension is inoculated into cell cultures in two dilutions, i.e. the primary dilution and, in addition, a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively, (in order to prevent homologous interference). At least two cell lines have to be inoculated (See point V.1). The ratio between inoculum size and volume of cell culture medium should be about 1:10.

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 tray, has to be utilised. Use of cell culture trays is recommended, but other units of similar or bigger growth area are acceptable as well.

V.3. Incubation of cell cultures

Inoculated cell cultures are incubated at a temperature between 15°C and 22°C for 7 to 10 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 has to 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 is performed to verify the susceptibility of the cell cultures to infection. A recommended procedure is presented in Part II.

V.4. Microscopy

Inoculated cell cultures must 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 according to point IV have to be initiated immediately.

V.5. Subcultivation

If no CPE has developed after the primary incubation for 7 to 10 days, subcultivation is performed to fresh cell cultures utilising a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to cell line 7 to 10 days after inoculation. The pools are then inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:10 and 1:100, respectively, of the supernatant) as described in point VI.2. Alternatively aliquots of 10 % of the medium constituting the primary culture is inoculated directly into a well with fresh cell culture (well to well subcultivation). The inoculation may be preceded by preincubation of the dilutions with the antiserum to IPN virus at appropriate dilution as described in point IV.3.

The inoculated cultures are then incubated for 7 to 10 days at temperature between 15°C and 22°C with observation as in point V.4.

If toxic CPE occurs within the first three days of incubation, subcultivation may be performed at that stage, but the cells must then be incubated for seven days' and subcultivated again with a further seven days' incubation. When toxic CPE develops after three days, the cells may be passed once and incubated to achieve the total of 14 days from the primary inoculation. There should be no evidence of toxicity in the final seven days of incubation.

If bacterial contamination occurs despite treatment with antibiotics, subcultivation must be preceded by centrifugation at 2000 to 4000 x ***g*** for 15 to 30 min at 2 to 5 °C, and/or filtration of the supernatant through a 0,45 µm filter (low protein-binding membrane). In addition to this, subcultivation procedures are the same as for toxic CPE.

If no CPE occurs the test may be declared negative.

VI. Virus identification

VI.1. Virus identification tests

If evidence of CPE has been observed in a cell culture, medium (supernatant) is collected and examined by one or more of the following techniques: IF, PCR followed by sequencing. If these tests have not allowed definitive identification of the virus within one week, the supernatant must be forwarded to a national reference laboratory or to the EU reference laboratory for fish diseases for immediate identification.

VI.2. IF

For each virus isolate to be identified, at least eight coverglasses or equivalent are seeded with cells at a density leading to about 60 % to 90 % confluence after 24 hours of cultivation. EPC cells are recommended for this purpose because of their strong adherence to glass surfaces but other cell lines such as BF-2, RTG-2 or FHM may be used as well.

When the cells have sedimented onto the glass surface (about one hour after seeding), or when the cultures have been incubated for up to 24 hours, the virus to be identified is inoculated. Four cultures are inoculated at a volume-to-volume ratio to 1:10, and four cultures at a ratio of 1:1000. These are then incubated at a temperature between 15°C and 22°C for 20 to 30 hours.

After incubation, the cultures are rinsed twice in Eagle's MEM without serum, fixed in 80 % ice cold acetone and then stained by means of a two-layer IFAT. The first reagent layer consists of polyclonal antibodies of reference quality. The second reagent layer is a fluorochrome-conjugated antiserum to the immunoglobulin used in the first layer. For each of the antisera tested at least one high-dose and one low-dose inoculated culture have to be stained. Proper negative and positive controls have to be included in the test. Fluorochromes such as FITC or TRITC are recommended.

Mount stained cultures using glycerol saline. Examine under incident ultraviolet (UV) light. Use 10 x or 12 x eyepieces and x 25 or x 40 objective lens with numerical apertures > 0,7 and > 1,3, respectively.

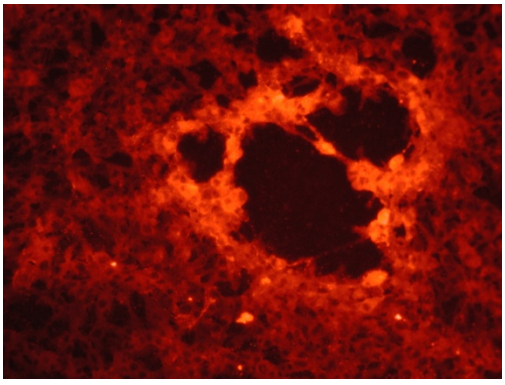

Photo: National Veterinary Institute, Technical University of Denmark

The above IF technique is given as an example. Other IF techniques (with regard to cell cultures, fixation and antibodies of reference quality) of proven efficiency may be used alternatively. Beware that the available antibodies cannot differentiate EHNV from other ranaviruses.

VI.3. PCR followed by sequencing

Preparation of viral DNA

Collect aliquots of culture medium from infected monolayer cells exhibiting CPE.

DNA can be extracted using DNA affinity spin columns according to the manufacturer’s instructions. DNA must be resuspended in distilled DNAse-free water.

OIE recommended primers for EHNV:
Reverse primer 5’-AAA-GAC-CCG-TTT-TGC-AGC-AAA-C-3’;

Forward primer 5'-CGC-AGT-CAA-GGC-CTT-GAT-GT-3'.
The following cycles are recommended: 35 cycles at 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and finally held at 72°C for 15 minutes.
Quantity and specificity of the PCR reactions can be evaluated by gel electrophoresis in 1.5% agarose gel with ethidium bromide and observed using UV transillumination. A 580 bp PCR amplicon can be observed for ranaviruses. As the PCR primers are not specific for EHNV, it is necessary to sequence the amplicon and identify the viral species by its unique DNA sequence available from GenBank Acc. No AY187045.1.

The PCR can vary depending on the conditions under which it is performed, e.g. the thermal protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results can occur because of, for example, false primer annealing or laboratory contamination. It is therefore important to include adequate positive and negative controls.

The above PCR versions are given as examples. Other PCR versions of proven efficiency may be used instead.

PART II

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

Recommended procedures for titration referred to in Part I.IV.3 are given below.

EHNV reference isolate is used. Reference isolate is available from the EU reference laboratory for fish diseases.

Batches of virus in low cell culture passage numbers are propagated in cell culture flasks on e.g. BF-cells. Cell culture medium with at least 10 % serum should be used. Use low MOI for inoculation (< 1).

At total CPE, virus is harvested by centrifugation of cell culture supernatant at 2000 x ***g*** for 15 minutes, filter sterilised through 0,45 µm membrane filter and distributed in labelled cryotubes. The virus is kept at -80 °C.

One week after freezing, three replicate vials with virus are thawed under cold water and titrated on their respective cell lines. At least every six months, or if it is suspected that the susceptibility of a cell line has decreased, the isolate is thawed and titrated.

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

Titration by end point dilution should include at least six replicates at each dilution step. The titres are compared with previously obtained titres. If the titre drops by a factor of 2 logs or more, compared with the initial titre, the cell line should no longer be used for surveillance purposes.

If different cell lines are kept in the laboratory each line should be examined separately.

Records should be kept for at least 10 years.

PART III

Acronyms and abbreviations

BF-2	Bluegill fry -2 (cell line)
CPE	Cytopathic effect
CRL	Community reference laboratory for fish diseases
ELISA	Enzyme-linked immunosorbent assay
EPC	*Epithelioma papulosum cyprini* (cell line)
EHN(V)	Epizootic haematopoietic necrosis (virus)
FHM	Fathead minnow (cell line)
FITC	Fluorescein isothiocyanate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HRP	Horseradish peroxidase
IF	Immunofluorescence
IFAT	Indirect fluorescent antibody test
IPN	Infectious pancreatic necrosis (virus)
MEM	Minimum essential medium
MOI	Multiplicity of infection (ratio of number of infectious virus particles added to a known number of cells in a culture)
PBS	Phosphate buffered saline
RTG-2	Rainbow trout gonad (cell line)
PCR	Polymerase chain reaction
Tris-HCl	Tris (hydroxymethyl) aminomethane - HCl
TRITC	Tetramethyl-rhodamine-isothiocyanate