European Union Reference Laboratory
for Fish and Crustacean Diseases

NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH VHSV AND IHNV

v2021.2

Authors

EURL for Fish and Crustacean Diseases DTU Aqua: Niels Jørgen Olesen, Argelia Cuenca, Niccolò Vendramin

We highly acknowledge the inputs and support of colleagues from:

- IZSVe (Anna Toffan)
- ANSES (Lenaig Loubotin, Thierry Morin and the whole French NRL team)
- FLI (Uwe Fisher, Heike Schütze and Sven M. Bergmann)

Contents

MAJOR CHANGES FROM THE PREVIOUS VERSION (V.1 21-04-2021)

Amendment of the probe sequence and reference for the detection of IHNV RNA by RT-qPCR in Paragraph 1.6.3.3; furthermore the two steps RT-qPCR for detection of IHNV RNA (Purcell et al., 2013) has been removed.

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH VHSV AND IHNV

I. Diagnostic methods and procedures for the surveillance of infection with VHSV and IHNV

When sampling and laboratory examination for the purpose of obtaining or maintaining disease-free health status with regard to IHN or VHS as set out in Part II chapter 1 section 2 of **COMMISSION DELEGATED REGULATION (EU) 2020/689** are carried out, using the diagnostic methods set out in Annex VI Part II chapter 1 Section 5 of **(EU) 2020/689**, the detailed diagnostic methods and procedures set out in following points I.1 to I.6 shall apply.

I.1. Preparation and shipment of samples from fish

I.1.1. 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 RT-qPCR 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, heart and/or *encephalon*, and ovarian and seminal fluid from broodfish at the time of spawning shall be sampled.

Ovarian or seminal 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 shall weigh a minimum of 0,5 gram (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.

1.1.2. Samples for reverse transcriptase polymerase chain reaction (RT-PCR or RT-qPCR) analysis

Samples shall be taken from the fish in accordance with the procedure described in point I.1.1. using a sterile instrument and transferred to a sterile plastic tube 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 RNA stabilisation reagents (eg. RNAlater) according to the recommendation from the manufacturers. Samples in 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 proper stabilization of the viral RNA.

I.2. Shipment of fish samples

Tubes containing fish tissues in transport medium for cell cultivation or RT-PCR / RT-qPCR analysis shall be placed in 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 able to 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.

I.4. Preparation of samples for cell culture examination and RT-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 the fish tissues; it may be acceptable to freeze the tissue specimens in the transport medium or in the RNA stabilisation reagents (only for RT-qPCR) 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.

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 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 may be treated for either four hours at 15 °C or overnight at 4 to 8°C with antibiotics. 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 to the indigenous serotypes of infectious pancreatic necrosis virus (IPNV) and incubated with this for a minimum of one hour at 15 °C or a maximum of 18 hours at 4 °C. The antiserum shall reduce of at least 3 log the titre of IPNV (eg. at least 1/2 000 in a 50 % plaque neutralisation test).

Treatment of all inocula with antiserum to IPNV aims at preventing cytopathic effect (CPE) due to IPNV 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 VHSV or IHNV.

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

I.4.4. Sample preparation for RT-qPCR based surveillance programmes

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 RNA 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 RNA stabilization reagent, subsequent work shall be carried out within the following time scales for samples stored at different temperatures:

- samples stored at 37 °C: 24 hours;
- 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 RT-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 (eg. by tissue lyzer, Fast Prep or similar, or methods given in I.4.2.)

Samples collected in RNA stabilization reagents shall not be used for cell cultivation.

I.4.5. Pooling of samples for RT-qPCR

Pooling of samples for RT-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 RT-qPCR.
- Pools of aliquoted material representing each and every organ from up to 10 fish can be disrupted and homogenized and tested for RT-qPCR
- Single fish samples can be individually disrupted and homogenized and then pooled for RT-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) or Rainbow trout gonad cell line - 2 (RTG-2) (Wolf et al., 1962) and either *Epithelioma papulosum cyprini* (EPC) (Fijan et al., 1983) or Fathead minnow (FHM) (Gravell and Malsberger 1965) 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 old may be accepted. The cells must be actively growing at inoculation.

I.5.2. Inoculation of cell cultures

Antibiotic-treated organ suspension shall be inoculated into cell cultures in at least two dilutions, namely 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 shall be inoculated as referred to in point I.5.1. The ratio between inoculum size and volume of cell culture medium shall 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, shall be utilised. Cell culture trays shall be used where possible.

I.5.3. Incubation of cell cultures

The inoculated cell cultures shall be incubated at 15 °C ± 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 VHSV and IHNV 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. A traceability of these controls must be carried out. In case susceptibility test of cell culture 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.6. 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 (supernatant) from all cultures or wells constituting the primary culture shall be pooled according to 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 1:10 and 1:100, 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 fresh 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 7 to 10 days at 15 °C ± 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 by centrifugation at 2000 to 4000 x g for 15 to 30 minutes at 2 C to 5 C, or filtration of the supernatant through a 0.45 µm filter or both (low protein-binding membrane). 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. Virus identification

If evidence of CPE has been observed in a cell culture, medium (supernatant) shall be collected and examined by one or more of the following techniques: Enzyme-linked immunosorbent assay (ELISA), Indirect Fluorescence Antibody Test (IFAT), RT-PCR or RT-qPCR.

I.6.1. ELISA

A double antibody sandwich ELISA shall be performed according to Mortensen et al., 1999 in order to identify the virus isolate. Microwell plates shall be coated with 50 µl/well (0.9 pg) of proven quality protein-A purified immunoglobulins(Ig) from rabbit antisera against IHNV or VHSV diluted in carbonate buffer (pH 9.6) containing 15 mM sodium azide and incubated from 18 hours to *2* weeks at 4° C.

On a dilution plate, each sample containing 1% Triton X-100 and the positive controls shall be diluted with buffer solution (namely, phosphate buffered saline (PBS)-T-BSA, 1 % BSA) in a 4-fold dilution: undiluted, 1:4, 1:16, 1:64. The ELISA plates shall be washed in PBS containing 0.05% Tween-20 (PBS-T) and 50 µl of each dilution shall be transferred from the dilution plate to the washed and coated ELISA-plate.

ELISA plates shall then be incubated for 30 minutes at 37° C. Subsequently plates shall be washed and incubated for 30 minutes at 37° C with specific monoclonal antibodies (namely for VHSV identification MAb IP5B11 (Lorenzen et al., 1988) and for IHNV Hyb

136-3 (Fregeneda-Grandes et al., 2007), respectively, available through request to the EURL for Fish and Crustacean diseases. 50µl of horseradish-peroxydase (HRP) conjugated rabbit anti mouse antibodies diluted 1:1000 in PBS-T-BSA shall be transferred to the ELISA plate.

Finally, after renewed washing the reactions shall be developed adding 50 µl/well of Tetramethylbenzidin (TMB) or similar colorimetric substrate (e.g. ortho-phenylenediamine OPD). The Elisa plates shall be incubated for 20 minutes at room temperature in the dark and the reaction shall be stopped by adding 100 µl/well 0.5 M H_2SO_4.

The absorbance shall be monitored at a wavelength of 450 nm and 620 nm in an ELISA reader. Samples shall be designated positive or negative after comparing the test results to the absorbance values for the positive and negative controls. In general, samples with combined absorbance (A) < 0.5 for undiluted material shall be considered negative, samples with A values between 0.5 and 1.0 shall be considered suspicious and samples with A values > 1.0 shall be considered positive.

Other ELISA versions with a proven similar efficiency may be used instead of those referred to in this point.

I.6.2. Indirect Fluorescence Antibody Test - IFAT

The identification of listed pathogens VHSV and IHNV shall be performed by infecting cells in adapted plates ("Black" or conventional 96-well plates, conventional 24 or 4-well plates or on round cover slips placed into 24-well plates). When IHNV or VHSV or both are to be identified by infecting cells on cover slips, the following protocol shall apply:

(a) Cover slips shall be seeded with cells so that a density of between 60 % and 90 % confluence 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, RTG-2 or FHM may be used as well. 150 µl cell culture supernatant in two different dilutions (1:10 and 1:1000) shall be inoculated in duplicate onto one-day-old monolayers and incubated at 15°C ± 2 °C for 24 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 cover glasses shall be air dried for at least 30 minutes. At this stage, the plates with the cover slips shall either be processed immediately or stored at –20°C for further use;

(c) specific monoclonal antibodies (namely for VHSV identification, Mab IP5B11 and for IHNV, Hyb 136-3 respectively) shall be diluted in 0.01 M PBS-T, pH 7.2

in the dilution recommended by the provider of the MAbs; 50 to 100 µl/well 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. The cells shall subsequently be incubated for one hour at 37°C with fluorescein isothiocyanate (FITC) - or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibodies against mouse immunoglobulin used as the primary antibody, diluted according to the supplier instructions, washed again in PBS-T, and the excess of buffer removed. Stained cultures shall be mounted onto glass slides 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 objective 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.

I.6.3 RT-qPCR protocols for surveillance of infection with VHSV and IHNV

Currently there is one molecular method approved by the EURL for conducting surveillance of infection with VHSV directly on fish tissue. This method is the RT-qPCR described at 1.6.3.2 (Jonstrup et al., 2013) targeting a 77 nt region within the nucleoprotein gene (positions 532-608).

To conduct surveillance of infection with IHNV directly testing fish tissue, one molecular method is available and approved by the EURL. This method is a one-step RT-qPCR as described at 1.6.3.3 (Cuenca et al., 2020;) targeting a 97 nt region of the nucleoprotein gene (positions 796 -893). The probe to be used shall be replaced with the one described in Hoferer et al., 2019 spanning from nucleotide 826 to 849.

Other RT-qPCR methods of proven similar efficiency may be used instead.

I.6.3.1. Preparation of viral RNA

RNA shall be extracted using the phenol-chloroform method or by RNA affinity spin columns or by adsorption on magnetic beads, according to the manufacturer's instructions. Commercially available RNA extraction kits that will produce high quality RNA suitable for use with the RT-PCR protocols detailed in the points below may be used.

RNA shall be re-suspended in distilled RNAse-free water, (namely water treated with 0,1% diethyl pyrocarbonate) or an appropriate elution buffer.

I.6.3.2. RT-qPCR for detection of VHSV RNA

The protocol is described in the scientific publication from Jonstrup et al., 2013.

For VHSV, amplification shall be performed using the following primers and probe:

- Forward primer: 5'-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3';
- Reverse primer: 5'-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3';
- and probe: 5' 6 FAM-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-BHQ1.

One-step RT-qPCR

Negative template controls and positive controls both for purification phase and PCR phase shall be included on each plate run. In order to prevent issues with contamination, it is suggested to include positive controls yielding Ct values close to the limit of detection. Additionally, internal controls can be included to rule out sample degradation and ensure successful purification procedure.

Cycling conditions: 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 40 seconds, 72°C for 20 seconds; adjust if necessary the thermal profile. Other RT-qPCR versions of proven similar efficiency may be used instead.

I.6.3.3 RT-qPCR for detection of IHNV RNA

This procedure can be performed as one-step method. The primers to be used are according to the protocol described by Purcell et al.,2013 and susbsequently referred to in Cuenca et al., 2020, while the probe shall be the one described in Hoferer et al., 2019.

For IHNV, amplification shall be performed using the following primers and probe:

- Forward primer: 5'- AGA-GCC-AAG-GCA-CTG-TGC-G-3';
- Reverse primer: 5'- TTCTTTGCGGCTTGGTTGA – 3';
- and probe: 5' 6 FAM-AGC GGG ACA GGR ATG ACA ATG GTG BHQ1

One-step RT-qPCR:

Negative template controls and positive controls both for purification phase and PCR phase shall be included on each plate run. In order to prevent issues with contamination, it is suggested to include positive controls yielding Ct values close to the limit of detection. Additionally, internal controls can be included to rule out sample degradation and ensure successful purification procedure.

Cycling conditions: 50°C x 30 minutes; 95°C x 15 minutes, 40 cycles of 95°C x 15 seconds and 60°C x 1 minute, and fluorescence signal is read at the end of each cycle. Cycle condition are kit specific and should be adjusted if necessary.

Other RT-qPCR versions of proven similar efficiency may be used instead. Please note that probe recommended in Purcell et al., 2013 show low sensitivity in detecting some of the IHNV variants circulating in Europe and should not be used.

I.6.4. RT-PCR for detection of VHSV RNA

The RT-PCR for detection of VHSV RNA can be used in the following circumstances:

- identification of viral isolate from cell culture
- diagnostic case, meant as disease outbreak with clinical signs consistent to VHS
- corroboration of positive finding of VHSV RNA described in I.1.6.3.2

The recommended protocol is described by Kim et al., 2018 targeting 320 nt of the viral nucleoprotein gene (positions 658-977)

The following primers shall be used:

- Forward primer 3F (nt. 658–677 in N gene) 5'- GGGACAGGAATGACCATGAT
- Reverse primer 2R (nt. 955–977 in N gene) 5'-TCTGTCACCTTGATCCCCTCCAG

Cycling conditions: 50 °C for 30 minutes; 95 °C for 15 minutes; 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 68 °C for 60 seconds; and finally, 68 °C for 7 minutes; adjust if necessary.

Negative template controls and positive controls both for purification phase and PCR phase shall be included on each run.

The results of the RT-PCR reactions shall be evaluated by agarose gel electrophoresis using appropriate DNA staining (e.g. ethidium bromide). An amplicon of 320 bp shall be observed in positive VHSV samples and positive controls.

The results of the RT-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.

Other RT-PCR versions of proven similar efficiency may be used instead.

1.6.5 RT-PCR for detection of IHNV RNA

The RT-PCR for detection of IHNV RNA can be used in the following circumstances:

- identification of viral isolate from cell culture
- corroboration of positive finding of IHNV RNA described in I.1.6.3.3

One protocol is described by Emmenegger et al. 2000 targeting a 301 nt of the viral glycoprotein gene (positions 688 to 989)

The following primers shall be used for detection of IHNV:

- Forward Primer 5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3';
- Reverse Primer 5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'.

Negative template controls and positive controls both for purification phase and PCR phase shall be included on each run.

The following cycles shall be used (one-step RT-PCR): 1 cycle: 50°C for 30 minutes; 1 cycle 95°C for 2 minutes; 30 cycles: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; 1 cycle: 72°C for 7 minutes and 4°C indefinetely; adjust if necessary.

The results of the RT-PCR reactions shall be evaluated by agarose gel electrophoresis - using appropriate DNA staining (e.g. ethidium bromide). An amplicon of 301 bp shall be observed in positive IHNV samples and positive controls.

Other RT-PCR versions of proven similar efficiency may be used instead.

The results of the RT-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.

II. Diagnostic procedures for "the confirmation of" or "to rule out" the suspicion of infection with VHSV and IHNV in suspected outbreaks

When a suspicion of VHS or IHN is required to be confirmed or ruled out, the following health visit, sampling and testing procedures must comply with the following requirements:

i) the presence of VHS must be considered as confirmed, if one or more of the following diagnostic methods are positive for VHSV;

ii) the presence of IHN must be considered as confirmed, if one or more of the following diagnostic methods are positive for IHNV;

The confirmation of the first case of VHS or IHN in Member States, zones or compartments previously not infected must be based on conventional virus isolation in cell culture with subsequent immunochemical or molecular identification or with genome detection including confirmation by sequencing of the amplification (RT-PCR) product;

II.1. Virus isolation with subsequent virus identification

II.1.1. Selection of samples

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

II.1.2. Preparation and shipment of samples from fish

The preparation and shipment for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.2.

II.1.3. Collection of supplementary diagnostic material

The collection of supplementary material for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.3.

II.1.4. Preparation of samples for cell culture examination

The preparation of samples for cell culture examination for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.4.

II.1.5. Virological examination on cell culture

The virological examination for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.5.

II.1.6. Virus identification

The virus identification for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.6.

II.2. Virus detection by RT-qPCR

II.2.1. Selection of samples

The selection of samples for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.1.2.

II.2.2. Preparation and shipment of samples from fish

The preparation and shipment for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.2.

II.2.3. Collection of supplementary diagnostic material

The collection of supplementary diagnostic material for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.3.

II.2.4. Sample preparation for RT-qPCR

The sample preparation for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.6.4.1.

II.2.5. RT-qPCR

The virus detection by RT-qPCR shall follow the methods and procedures laid down in points I.6.3.1, I.6.3.2 and I.6.4.3.

II.3 Confirmation of first case of infection with VHSV or IHNV in previously non-infected areas

The confirmation of the first case of infection with VHSV or IHNV in Member States, zones or compartments previously not infected must be based:

- on conventional virus isolation in cell culture with subsequent immunochemical or molecular identification;
- or on genome detection and confirmation by sequencing of the amplification (RT-PCR) product.

II.3.1. Sequencing of VHSV

To confirm the identification of the sample testing positive for the detection of VHSV RNA, at least the protocol given in paragraph 1.6.4. shall be used. All samples suspected for being positive for VHSV shall be confirmed by sequencing (PCR amplification primers described in 1.6.4 can be used). To perform sequencing, PCR products shall be cleaned, and sequencing reaction should be performed using sequence specific primers (one primer per reaction). Sequencing reaction shall be done using commercially available sequencing kits (e.g BigDye Terminator) according to manufacturer's instructions. Alternatively a sequencing provider can be used to sequence PCR products.

The sequencing results shall be analysed using BLAST tool against the NCBI (National Centre for Biotechnology Information) database to confirm VHSV.

Other RT-PCR methods of proven similar efficiency followed by sequencing and BLAST analyses may be used instead

II.3.2. Sequencing of IHNV

To confirm the identification of the sample testing positive for the detection of IHNV RNA, at least the protocol given in paragraph 1.6.5 shall be used. All samples suspected for being positive for IHNV shall be confirmed by sequencing (PCR amplification primers described in 1.6.5 can be used). To perform sequencing, PCR products shall be cleaned, and sequencing reaction should be performed using sequence specific primers (one primer per reaction). Sequencing reaction shall be done using commercially available sequencing kits (e.g BigDye Terminator) according to manufacturer's instructions. Alternatively a sequencing provider can be used to sequence PCR products.

. The sequencing results shall be analysed using BLAST tool against the NCBI (National Centre for Biotechnology Information) database to confirm IHNV.

Other RT-PCR methods of proven similar efficiency followed by sequencing and BLAST analyses may be used instead

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.

At least two VHSV isolates and one isolate of IHNV shall be used. The isolates shall represent the major group of viruses within the European Union, namely for VHSV one pathogenic isolate from rainbow trout in freshwater and one marine isolate, and for IHNV one rainbow trout pathogenic strain from the European Union. Well-defined isolates from the Member States shall be used. Batches of virus shall be propagated in cell culture flasks on BF-2 or RTG-2 cells for VHSV and on EPC or FHM cells for IHNV. 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 supernatant at 2000 x g for 15 minutes, filter sterilised through 0,45 µm membrane filter and aliquoted in labelled cryotubes. The virus shall be kept at -80 °C. Once thawed the tube shall not be frozen again and reused.

Three replicate vials with each virus shall be 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, each virus isolate shall be thawed and titrated on the cell line or sublineage to be examined

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.

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.

IV. References

CUENCA, A., VENDRAMIN, N., & OLESEN, N. J. (2020). Analytical validation of one-step realtime RT-PCR for detection of infectious hematopoietic necrosis virus (IHNV). Bulletin of The European Association of Fish Pathologists, 40(6), 261-272.

EMMENEGGER E.J., MEYERS T.R., BURTON T.O & KURATH G. (2000). Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. Dis. Aquat. Org., 40, 163–176

FIJAN, N., SULIMANOVIC, D., BEARZOTTI, M., MUZINIC, D., ZWILLENBERG, L.O., CHILMONCZYK, S.,VAUTHEROT, J.F., DE KINKELIN, P., 1983. Some properties of the Epithelioma papulosum cyprinid (EPC) cell line from carp Cyprinus carpio. Ann. Inst. Pasteur Virol. 134, 207–220

FREGENEDA-GRANDES J.M. & OLESEN N.J. (2007). Detection of rainbow trout antibodies against viral haemorrhagic septicaemia virus (VHSV) by neutralisation test is highly dependent on the virus isolate used. Dis. Aquat. Org., 74, 151–158.

GRAVELL M, MALSBERGER RG. A permanent cell line from the fathead minnow (Pimephales promelas). Ann. N.Y. Acad. Sci. 126: 555-565, 1965. PubMed: 5220177

HOFERER M, AKIMKIN V, SKRYPSKI J, SCHÜTZE H, STING R. Improvement of a diagnostic procedure in surveillance of the listed fish diseases IHN and VHS. J Fish Dis. 2019 Apr;42(4):559-572. doi: 10.1111/jfd.12968. Epub 2019 Feb 19. PMID: 30779211.

JONSTRUP S.P., KAHNS S., SKALL H.F., BOUTRUP T.S. & OLESEN N.J. (2013). Development and validation of a novel Taqman-based real-time RT-PCR assay suitable for demonstrating freedom from viral haemorrhagic septicaemia virus. J. Fish Dis., 36 (1), 9–23.

KIM, H.J., CUENCA, A., OLESEN, N.J. Validation of a novel one-step reverse transcription polymerase chain reaction method for detecting viral haemorrhagic septicaemia virus (2018) *Aquaculture*, 492, pp. 170-183. http://www.journals.elsevier.com/aquaculture/ doi: 10.1016/j.aquaculture.2018.03.047

LORENZEN N., OLESEN N.J. & VESTERGÅRD JØRGENSEN P.E. (1988). Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. Dis. Aquat. Org., 4, 35–42.

MORTENSEN HF, HEUER OE, LORENZEN N, OTTE L, OLESEN NJ (1999) Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea, Kattegat, Skagerrak and the North Sea. Virus Res 63: 95–106

PURCELL MK, THOMPSON RL, GARVER KA, HAWLEY LM, BATTS WN, SPRAGUE L, SAMPSON C AND WINTON JR (2013). Universal reversetranscriptase real-time PCR for infectious hematopoietic necrosis virus (IHNV). Diseases of Aquatic Organisms 106, 103–115

WOLF K (1988) Fish viruses and fish viral diseases. Cornell University Press, Ithaca, NY

WOLF K, QUIMBY MC (1962) Established eurytherrnic line of fish cells in vitro Science 135:1065-1066