II

*(Acts whose publication is not obligatory)*

# COMMISSION

## COMMISSION DECISION

**of 5 February 1996**

**amending Decision 92/532/EEC laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases**

**(Text with EEA relevance)**

(96/240/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 91/67/EEC of 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products [1], as last amended by Directive 95/22/EC [2], and in particular Article 15 thereof,

Whereas the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases are laid down in Commission Decision 92/532/EEC [3];

Whereas since the time of adoption of this Decision, new practical and scientific developments have taken place which require the sampling plans and diagnostic methods to be updated;

Whereas such updating relates to sample size, the samples to be taken, the transport of samples and the method for isolating viruses possibly present in the sample;

Whereas the Scientific Veterinary Committee, established by Commission Decision 81/651/EEC [4], has been consulted;

Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

*Article 1*

The Annex to Decision 92/532/EEC is replaced by the Annex to this Decision.

*Article 2*

This Decision is addressed to the Member States.

Done at Brussels, 5 February 1996.

*For the Commission*

Franz FISCHLER

*Member of the Commission*

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[1] OJ No L 46, 19. 2. 1991, p. 1.
[2] OJ No L 243, 11. 10. 1995, p. 1.
[3] OJ No L 337, 21. 11. 1992, p. 18.
[4] OJ No L 233, 19. 8. 1981, p. 32.

*ANNEX*

PART I

## SAMPLING AND TESTING PROCEDURES FOR VHS AND IHN MONITORING

### I. Sampling

1. *Sampling time*

Farms are inspected clinically at least twice per year during the period October to June or whenever the water temperature is below 14 °C. Intervals between inspections must be at least four months. All production units (ponds, tanks, aquaria, netcages, etc.) are inspected for the presence of dead, weak or abnormally behaving fish. Particular attention has to be paid to the water outlet area (if feasible) where weak fish tend to accumulate because of the water current.

2. *Selection and collection of samples*

Thirty to 150 fish and/or ovarian fluid samples are collected for examination in connection with the inspections according to Table 1. If rainbow trout are present fish of that species should make up the whole sample. If rainbow trout are not present the sample has to contain fish of all other species present whenever these species are susceptible to VHS and/or IHN as listed in Annex A to Council Directive 91/67/EEC concerning the animal health conditions governing the placing on the market of aquaculture animals and products. The species have to be proportionally represented in the sample. During the first two years of the initial four-year control period which precedes achievement of approved status the sample size is 150 in order to ensure detection at a 95 % confidence level of virus carriers at a carrier prevalence of 2 %, except for salmonid farms without broodstock in coastal zones where the sample size is 30.

During the last two years of the control period the sample size can be reduced to 30 to ensure detection at a 95 % confidence level of virus at a prevalence of 10 %. During the subsequent years (maintenance of approved status) the sample size likewise can be reduced to 30.

In farms which have a documented history of at least four years of freedom from VHS and IHN (based on a regular official health inspection programme) the small sample size can be used during the whole initial four-year control.

If more than one water source is utilized for fish production, fish representing all water sources must be included in the 150 or 30 fish-sample. If weak, abnormally behaving or freshly dead (not decomposed) fish are present, these must primarily be included in the sample. If such fish are not present the sample must be composed of normally appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

3. *Preparation and shipment of samples from fish*

Before shipment or transfer to the laboratory pieces of the organs to be examined are removed from the fish with sterile scissors and forceps and transferred to 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. The tissue material to be examined is spleen, anterior kidney, and, in addition, either heart or encephalon. In some cases, ovarian fluid must be examined (Table 1).

Ovarian fluid or organ pieces from 10 fish (Table 1) may be collected in one 10 ml plastic tube containing 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 are placed in insulated containers (for instance thick-walled polystyrene boxes) together with sufficient ice or 'freeze blocks' to ensure chilling of the samples to between 0 and 5 °C 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.

The virological examination must be started as soon as possible and not later than 48 hours after the collection of the samples or in exceptional cases not later than 72 hours after the collection of the material to be examined is protected by transport medium and the temperature requirements during transportation can be fulfilled (1.1.3, paragraph 3).

Whole fish may be sent to the lab 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 above. Live fish can be shipped as well.

4. *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.

## II. Preparation of samples for virological examination

1. *Homogenization of organs*

In the laboratory the tissue in the tubes must be completely homogenized (either by stomacher, blender or mortar and pestle) and subsequently suspended in the original transport medium. If a sample consisted of whole fish less than 6 cm long, these are minced with sterile scissors after removal of the body behind the gut opening, homogenized as described above and suspended in transport medium, The final ratio between tissue material and transport medium must be adjusted to 1: 10.

2. *Centrifugation of homogenate*

The homogenate is centrifuged in a refrigerated centrifuge at 2 to 5 °C at 2 000 to 4 000 × 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 h after the sampling it may be thawed and 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 1/2 000 in a 50 % plaque neutralization 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 VHS or IHN.

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

## III. Virological examination

1. *Cell cultures and media*

Either 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 as close as possible to 7,6.

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.

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: 1 000, respectively, (in order to prevent homologous interference). At least two cell lines have to be inoculated (see III.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 utilized. Use of cell culture trays is recommended, but other units of similar or bigger growth area are acceptable as well.

3. *Incubation of cell cultures*

Inoculated cell cultures are incubated at 15 °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 ot ensure cell susceptibility to virus infection.

Titration every six months of frozen stocks of VHSV and IHNV is performed to verify the susceptibility of the cell cultures to infection.

4. *Microscopy*

Inoculated cell cultures are inspected daily for the occurrence of CPE at about 40 × magnification. If obvious CPE is observed, virus identificaiton procedures according to Section IV have to be initiated immediately.

5. *Subcultivation*

If no CPE has developed after the primary incubation for 7 to 10 days, subcultivation is performed to fresh cell cultures utilizing 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 Section I.III.2. The inoculation may be preceded by preincubation of the dilutions with the antiserum to IPN virus at appropriate dilution as described in Section I.II.2.

The inoculated cultures are then incubated for 7 to 10 days at 15 °C with observation as in Section III.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 sub-cultivated 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.

## IV. **Virus identification**

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: neutralization, immunofluorescence (IF), enzyme linked immunosorbent assay (Elisa).

If the tests has not allowed safe identification of the virus within one week, the virus must be transferred to a national reference laboratory for fish diseases or to the Community Reference Laboratory for fish diseases for immediate identification.

The use of immunoreagents for virus identification must be of reference quality and approved, with regard to titre and specificity, by the national reference laboratory for fish diseases.

2. *Neutralization*

Remove cells from collected medium by centrifugation (2 000 to 4 000 × g) or membrane filtration (0,45 µm) and dilute the medium 1: 100 and 1: 10 000 in cell culture medium.

Aliquots of the dilutions are mixed and incubated for 60 minutes at 15 °C with equal parts of the following reagents separately:

| | |
|---|---|
| — group specific antibody to VHSV (Egtved virus) | 1:50 (1) |
| — group specific antibody to infectious hematopoietic necrosis virus (IHNV) | 1:50 (1) |
| — specific pool of antisera to the indigenous serotypes of infectious pancreatic necrosis virus (IPNV) (reference strains Sp, Ab or VR 299) | 1:50 (1) |
| — medium | 1:1 |

From each virus-serum mixture at least two cell cultures are inoculated with 50 µl each and then incubated at 15 °C. Development of CPE is checked as described in Section III.4.

Other neutralization tests of proven efficiency may be used alternatively.

(1) Or as specified by the reference laboratory with regard to the possible cytotoxicity of the antisera.

3. *Immunofluorescence (IF)*

For each virus isolate to be identified, at least eight coverglasses or equivalent are seeded with EPC cells at a density leading to about 60 to 90 % confluence after 24 hours of cultivation. EPC cells are chosen for this purpose because of their strong adherence to glass surfaces.

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 of 1: 10, and four cultures at a ratio of 1:1 000.

Between 20 and 30 hours post inoculation, the cultures are rinsed twice in Eagle's MEM without serum, fixed in acetone and then stained by means of a two-layer IFAT. The first reagent layer consists or poly- or monoclonal antibodies of reference quality. The second reagent layer is a FITC-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.

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

Some Egtved virus strains react strongly with antiserum to reference strain F1 in IFAT although not reacting in neutralization tests.

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.

4. *Enzyme linked immunosorbent assay (Elisa)*

Wells in microtitre plates (for instance Nunc-immunoplates, Maxisorp, Nunc, Denmark) are coated overnight with recommended dilutions of protein-A purified immunoglobulin fractions of antibodies of reference quality.

After rinsing of wells with PBS-Tween-20 buffer, the virus to be identified is added to the wells in two or four-fold dilution steps and allowed to react with the coating antibody for 60 minutes at 37 °C. Following rinsing which PBS-Tween-20 buffer, biotinylated antibodies of a specificity corresponding to that of the coating antibodies are added and allowed to react for 60 minutes at 20 °C. Following another rinse as above, HRP conjugated streptavidin is added and allowed to react for one hour at 20 °C. After a last rinse, bound enzyme is visualized using appropriate Elisa substrates (OPD or others).

The above biotin-avidin based Elisa version is given as an example. Other Elisa versions of proven efficiency may be used instead.

*Table 1 A*

**Achievement of status**

| | No of clinical inspections per year: Years 1 and 2 | Examinations of organs from number of growing fish: Years 1 and 2 | Examination of ovarian fluid from number of broodstock fish: Years 1 and 2 |
|---|---|---|---|
| *Continental zones* | | | |
| (a) Farms with broodstock | 2 | 120 (first inspection (*))<br>150 (second inspection) | 30 (first inspection)<br>0 |
| (b) Farms with broodstock only | 2 | 0 | 150 (first or second inspection) |
| (c) Farms without broodstock | 2 | 150 (first and second inspections) | 0 |
| *Coastal zones* | | | |
| (a) Salmonid farms without broodstock | 2 | 30 (first and second inspections) | 0 |
| (b) Non-salmonid farms without broodstock | 2 | 150 (first and second inspections) | 0 |
| (c) Farms with broodstock | 2 | 120 (first inspection)<br>150 (second inspection) | 30 (first inspection)<br>0 |

| | No of clinical inspections per year: Years 3 and 4 | Examination of organs from number of fish: Years 3 and 4 | Examination of overian fluid from number of fish: Years 3 and 4 |
|---|---|---|---|
| *Continental zones* | | | |
| (a) Farms with broodstock | 2 | 20 (first or second inspection) | 10 (first or second inspection) |
| (b) Farms with broodstock only | 2 | 0 | 30 (first or second inspection) |
| (c) Farms without broodstock | 2 | 30 (first or second inspection) | 0 |
| *Coastal zones* | | | |
| (a) Farms without broodstock | 2 | 30 (first or second inspection) | 0 |
| (b) Farms with broodstock | 2 | 20 (first or second inspection) | 10 (first or second inspection) |

Maximum number of fish per pool: 10

(*) Clinical inspections.

*Table 1 B*

**Maintenance of status**

| | No of clinical inspections per year | Examination of organs from number of growing fish (1) | Examination of ovarian fluid from number of broodstock fish (1) |
|---|---|---|---|
| *Continental zones* | | | |
| (a) Farms with broodstock | 2 | 20 (first or second inspection) | 10 (first or second inspection) (2) |
| (b) Farms with broodstock only | 2 | 0 | 30 (first or second inspection) (2) |
| (c) Farms without broodstock | 2 | 30 (first or second inspection) | 0 |
| *Coastal zones* | | | |
| (a) Farms without broodstock | 1 | 30 (3) | 0 |
| (b) Farms with broodstock | 2 | 20 (first or second inspection) | 10 (first or second inspection) (2) |

Maximum number of fish per pool: 10

(1) Samples only have to be collected by rotation in 50 % of the fish farms in the zone each year.

(2) In exceptional circumstances, if it is impossible to obtain ovarian fluid, organs may be sampled instead.

(3) The samples have to be collected not before three weeks after transfer of fish from fresh to salt-water.

PART II

## DIAGNOSTIC PROCEDURES FOR THE CONFIRMATION OF IHN AND VHS IN CASE OF SUSPECTED OUTBREAKS

Diagnosis of IHN and VHS can be achieved by one of the following techniques:

— A. conventional virus isolation with subsequent serological virus identification,

— B. virus isolation with simultaneous serological virus identification,

— C. other diagnostic techniques (IFAT, Elisa).

The first diagnosis of IHN and VHS in farms in approved zones, must not be based on method C alone. Either method A or B must also be used.

The tissue material meant for virological examination may in some cases have to be accompanied by supplementary material for bacteriological, parasitological, histological or other examination to allow for a differential diagnosis. Such material is collected according to procedures outlined by OIE.

### II.A. Conventional virus isolation with subsequent serological virus identification

II.A.I.1. *Selection of samples*

At least 10 fish showing typical signs of IHN or VHS must be selected for examination.

II.A.I.2. *Preparation and shipment of samples from fish*

As I.I.3.

II.A.I.3. *Collection of supplementary diagnostic material*

As I.I.4.

II.A.II. *Preparation of samples for virological examination*

As I.II.

II.A.III. *Virological examination*

As I.III, except that either BF-2 or RTG-2 and either EPC or FHM cells may be used for inoculation with tissue material.

II.A.IV. *Virus identification*

As I.IV.

### II.B. Virus isolation with simultaneous serological virus identification

II.B.I.1. *Selection of samples*

As II.A.I.1.

II.B.I.2. *Preparation and shipment of samples from fish*

As I.I.3.

II.B.I.3. *Collection of supplementary diagnostic material*

As I.I.4.

II.B.II.1. *Homogenization of organs*

As I.II.1.

II.B.II.2. *Centrifugation of homogenate*

As I.II.2.

II.B.II.3. *Treatment of supernatant with diagnostic antisera*

The antibiotic and anti-IPN treated organ suspension is diluted 1: 10 and 1: 10 000 in cell culture medium and aliquots mixed and incubated for 60 minutes at 15 °C with equal parts of the reagents listed in Section I.IV.2.

II.B.III.1. *Cell cultures and media*

Either 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 as close as possible to 7,6.

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.

II.B.III.2. *Inoculation of cell cultures*

From each virus-serum mixture (prepared according to II.B.II.3) at least two cell cultures per cell line are inoculated with 50 µl each.

II.B.III.3. *Incubation of cell cultures*

As I.III.3.

II.B.III.4. *Microscopy*

Inoculated cell cultures are inspected daily for the occurrence of CPE at about 40 × magnification. If CPE is prevented by one of the antisera used, the virus can be considered to be identified accordingly.

If CPE is not prevented by any of the antisera, virus identification procedures according to I.IV have to be performed.

II.B.III.5. *Subcultivation*

If no CPE has occurred after seven days, subcultivation has to be performed from cultures inoculated with supernatant plus medium (II.B.II.3) according to I.III.5.

### C. Other diagnostic techniques

Supernatant prepared as described under II.A.II.2 can be submitted to IFAT or Elisa according to II.A.IV.3 or II.A.IV.4 respectively. These rapid techniques have to be supplemented with a virological examination according to either A or B within 48 hours after sampling, if:

(a) a negative result is obtained;

or

(b) a positive result is obtained with material representing the first case of IHN or VHS in approved zones.

Tissue material may be subjected to other diagnostic techniques such as IF on frozen sections or immunohistochemistry on formalin fixed tissue material. These techniques must always be accompanied by inoculation of non-fixed tissue material on cell cultures.

ABBREVIATIONS

| | |
|---|---|
| BF-2 | bluegill fibroblast (cell line) |
| CPE | cytopathic effect |
| Elisa | enzyme-linked immunosorbent assay |
| EPC | *Epithelioma papulosum cyprini* (cell line) |
| FHM | fathead minnow (cell line) |
| FITC | fluorescein isthiocyanate |
| HRP | horse radish peroxidase |
| IF | immunofluorescense |
| IFAT | indirect fluorescent antibody test |
| IHN(V) | infectious hematopoietic necrosis (virus) |
| IPN(V) | infectious pancreatic necrosis (virus) |
| MEM | minimum essential medium |
| OPD | Ortho phenylene diamine |
| PBS | phosphate buffered saline |
| RTG-2 | rainbow trout gonad (cell line) |
| Tris-HCl | tris (hydroxymethyl) aminomethane — HCl |
| VHS(V) | viral haemorrhagic septicaemia (virus) |