II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DECISION

of 19 November 1992

laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases

(92/532/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic 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], and in particular Article 15 thereof,

Whereas, in accordance with Article 15 of Directive 91/67/EEC sampling plans and diagnostic methods to be applied for the detection and confirmation of diseases in aquaculture animals shall be established;

Whereas the Scientific Veterinary Committee, established by Commission Decision 81/651/EEC [2] 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 sampling plans and diagnostic methods for the detection and confirmation of infectious Haematopoietic Necrosis (IHN) and Viral haemorrhagic Septicaemia (VHS) are laid down in the Annex.

Article 2

This Decision is addressed to the Member States.

Done at Brussels, 19 November 1992.

For the Commission
Ray MAC SHARRY
Member of the Commission

[1] OJ No L 46, 19. 2. 1991, p. 1.
[2] 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 of 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 equally represented in the sample. During 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 %. During the subsequent years (maintenance of approved status) the sample size can be reduced to 30 to ensure detection at a 95 % confidence level of virus at a prevalence of 10 %.

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

If more than one water source is utilized for fish production, fish representing all water sources may 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 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 transportation 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, encephalon and, in some cases, ovarian fluid (Table 1).

Organ pieces from 5 to 10 fish (Table 1) may be collected in one tube and represent one pooled sample. The tissue in each sample should weigh a minimum of 1 gram (g) and such that the final dilution 1 : 10.

The tubes are placed in insulated containers (for instance thick-walled polystyrene boxes) together with sufficient ice of 'frost blocks' to ensure chilling of the samples to between 0 and 5 °C during transportation to the laboratory. Freezing must be avoided.

The virological examination must be started as soon as possible and not later than 48 hours after the collection of the samples. If the fish to be examined are less that 6 centimetres (cm) in length, the whole fish may be shipped to the laboratory in plastic bags chilled as mentioned above.

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, i.e. 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 at a 1 : 10 ratio in transport medium.

2. *Centrifugation of homogenate*

The homogenate is centrifuged in a refrigerated centrifuge at 2 - 5 °C at 2 000 to 4 000 x g for 15 minutes and the supernatant collected for examination.

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

If the sample was shipped as whole fish the homogenate following centrifugation has to be exposed to the antibiotics in the transport medium used for resuspension for either four hours at room temperature or over night at 4 °C.

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

Immediately after the centrifugation a volume of the supernatant is mixed with equal parts of a suitably diluted divalent antiserum to IPN virus (reference strains Sp and Ab) and incubated with this for a minimum of one hour at 15 °C or a maximum of 18 jours 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 is 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 duration of the virological examinations as well as the number of cases in which occurence 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*

BF-2 and either EPC or FHM cells are grown at 20 to 25 °C in 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, the medium is buffered with bicarbonate. The medium used for cultivation of cells in open units has to be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM) at a pH as close as possible to 7,6, a pH which is optimal for virus multiplication.

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 : 100 dilution thereof (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 seven 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.

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 occurence of CPE at about 40 × magnification. If obvious CPE is observed, virus identification procedures according to section IV have to be initiated immediately.

At the same time appropriate steps are taken to suspend the approved status of the production unit from where the virus positive sample originated as well as from any production units situated downstream.

Suspension of approved status has to be maintained until laboratory tests have proved the virus in question not to be VHSV or IHNV. A maximum of four weeks are allowed for identification of the virus, including the time needed for examination in reference laboratories.

5. *Subcultivation*

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

Aliquots of medium from all cultures/wells constituting the primary culture are pooled according to cell line following one freeze-thaw cycle of the cultures and 0,5 ml of the medium mixed with equal parts of antiserum to IPN as described in Section II.3. and the mixture incubated at 15 °C for 60 minutes. The mixture is then inoculated into homologous cell cultures at dilutions 1 : 1 and 1 : 100 as described in section III.2. The inoculation may be preceded by preincubation of the dilutions with divalent antiserum to IPN virus at appropriate dilution.

The inoculated cultures are then incubated for seven days at 15 °C with observation as in Section III.4.

IV. Virus identification

1. *Neutralization*

If evidence of CPE has been observed in a cell culture, medium is collected, cells removed by low speed centrifugation or membrane filtration (0.45 Um) and medium then diluted 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 (*)
— group specific antibody to infectious hematopoietic necrosis virus (IHNV)	1 : 50 (*)
— specific divalent antiserum to infectious pancreatic necrosis virus (IPNV) (reference strains Sp and Ab)	1 : 50 (*)
— medium	1 : 1

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

The reagents used have to be of reference quality with regard to titre and specificity.

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

If the test has not allowed safe identification of the virus within one week, one of the following steps has to be taken :

(a) transfer of the virus to a national fish virus reference laboratory for immediate identification ;

(b) application of immunofluorescence (IFAT) (Section IV.2.), enzyme-linked immunosorbent assay (ELISA) (Section IV.3.) or other virus identification techniques with reagents of reference quality.

2. *Immunofluorescence (IFAT)*

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 % confluency 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 to 1 : 10, and four cultures at a ratio of 1 : 100.

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 of approved antiserum (as in Section IV.1.). The second reagent layer is an 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. Dilutions of primary antiserum and FITC conjugated anti-species immunoglobulin will depend on the microscope set-up chosen or available.

Some Egtved virus strains react strongly with antiserum to reference strain F 1 in IFAT although not reacting in neutralization tests.

3. *Enzyme linked immunosorbent assay (ELISA)*

Wells in microtiter plates (for instance Nunc-immunoplates, Maxisorp, Nunc, Denmark) are coated overnight with recommended dilutions of Protein-A purified immunoglobulin fractions of the antisera mentioned in Section IV.1.

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 with 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, TMB 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	Examination of organs from number of fish	Examination of ovarian fluid from number of fish
Continental zones:			
(a) farms with broodstock	2	120 (1st series) 150 (2nd series)	30 (1st series) 0
(b) farms with broodstock only	2	0	150 (1st or 2nd series)
(c) farms without broodstock	2	150 (1st & 2nd series)	0
Coastal zones:			
(a) farms without broodstock	2	30 (1st & 2nd series)	0
(b) farms with broodstock	2	120 (1st series) 150 (2nd series)	30 (1st series) 0

Maximum number of fish per pool: 5

TABLE 1 B

Maintenance of status

	No of clinical inspections per year	Examination of organs from number of fish	Examination of ovarian fluid from number of fish
Continental zones:			
(a) farms with broodstock	2	15 (1st or 2nd series)	15 (1st or 2nd series)
(b) farms with broodstock only	2	0	30 (1st or 2nd series)
(c) farms without broodstock	2	30 (1st or 2nd series)	0
Coastal zones:			
(a) farms without broodstock	1	30 (1) (1st or 2nd series)	0
(b) farms with broodstock	2	0	30 (1st or 2nd series)

Maximum number of fish per pool : 10

(1) The samples have to be collected during the months two to six 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. Rapid 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 in some cases may 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.

A. Conventional virus isolation with subsequent serological virus identification

A.I.1. *Selection of samples*

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

A.I.2. *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 transportation 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 ml can be recommended but other antibiotics of proven efficiency can be used as well. The organs to be examined are spleen, anterior kidney, and encephalon.

Organ pieces from 5 to 10 fish may be collected in one tube and represent one pooled sample. The tissue in each sample should weigh a minimum of 1g and such that the final dilution is about 1 : 10.

The tubes are placed in insulated containers (for instance thick-walled polystyrene boxes) together with sufficient ice or 'frost blocks' to ensure chilling of the samples to between 0 and 5 °C during transportation to the laboratory. Freezing must be avoided.

The virological examination must be started as soon as possible and not later than 48 hours after the collection of the samples. If the fish to be examined are less than 6 cm in length, the whole fish may be shipped to the laboratory in plastic bags chilled as mentioned above.

A.I.3. *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.

A.II.1. *Homogenization of organs*

In the laboratory the tissue in the tubes is completely homogenized (stomacher, blender or mortar and pestle) and subsequently the homogenate is suspended in the original transport medium. If a sample consisted of whole fish, i.e. 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 1 : 10 in transport medium.

A.II.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 for examination.

If shipment of the sample has been made in transport medium (i.e. with exposure to antibiotics) the supernatant needs no further treatment with antibiotics.

If the sample was shipped as whole fish, the homogenate following centrifugation has to be exposed to the antibiotics in the transport medium used for resuspension for either four hours at room temperature or over night at 4 °C.

The antibiotic treatment aims at controlling bacterial contamination in the samples and makes filtration through membrane filters unnecessary. Immediately after the centrifugation a volume of the supernatant is mixed with equal parts of a suitably diluted divalent antiserum to IPN virus (reference strains Sp and Ab) 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 up to 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.

A.III.1. *Cell cultures and media*

BF-2 and either EPC or FHM cells are grown at 20 to 25 °C in 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, medium is buffered with bicarbonate. The medium used for cultivation of cells in open units has to be buffered with Tris-Hcl (23 mM) and bicarbonate (6 mM) at a pH as close as possible to 7,6, a pH which is optimal for virus multiplication.

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

A.III.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 : 100 dilution thereof (in order to prevent homologous interference). At least two cell lines have to be inoculated (see A.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 1 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.

A.III.3. *Incubation of cell cultures*

Inoculated cell cultures are incubated at 15 °C for seven 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.

Titration every six months of frozen stocks of VHS and IHN virus is performed to verify the susceptibility of the cell cultures to infection.

A.III.4. *Microscopy*

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

At the same time appropriate steps are taken to suspend the approved status of the production unit from where the virus positive sample originated as well as from any production units situated downstream.

Suspension of approved status has to be maintained until laboratory tests have proved the virus in question not to be VHS or IHN. A maximum of four weeks are allowed for identification of the virus, including the time needed for examination in reference laboratories.

A.III.5. *Subcultivation*

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

Aliquots of medium from all cultures/wells constituting the primary culture are pooled according to cell line following one freeze-thaw cycle of the cultures and 0,5 ml of the medium mixed with equal parts of antiserum to IPN as described in Section A.II.2 and the mixture incubated at 15 °C for 60 minutes. The mixture is then inoculated into cell cultures at dilutions 1 : 1 and 1 : 100 as described in Section A.III.2. The inoculation may be preceded by preincubation of the dilutions with divalent antiserum to IPN virus at appropriate dilution.

The inoculated cultures are then incubated for seven days at 15 °C with observation as in section A.III.4.

A.IV.1. *Neutralization*

If evidence of CPE has been observed in a cell culture, medium is collected, cells removed by low speed centrifugation or membrane filtration (0,45 Uμ) and medium then diluted 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 VHS virus (Egtved virus)	1 : 50 (*)
— group specific antibody to infectious hematopoietic necrosis virus (IHN virus)	1 : 50 (*)
— specific divalent antiserum to infectious pancreatic necrosis virus (IPN virus) (reference strains Sp and Ab)	1 : 50 (*)
— medium	1 : 1

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

The reagents used have to be of reference quality with regard to titre and specificity.

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 A.III.4.

If the test has not allowed safe identification of the virus within one week, one of the following steps has to be taken :

a. Transfer of the virus to a national fish virus reference laboratory for immediate identification.

b. Application of IFAT (section A.IV.2.), ELISA (section A.IV.3.) or other virus identification techniques with reagents of reference quality.

A.IV.2. *Immunofluorescence (IFAT)*

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 % confluency 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 into 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 : 100.

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 of approved antiserum (as in section IV.1.). The second reagent layer is an 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 UV light. Use 10 × or 12 × eyepieces and × 40 objective lens with numerical apertures > 07 and > 1,3 respectively. Dilutions of primary antiserum and FITC conjugated anti-species immunoglobulin will depend on the microscope set-up chosen or available.

Some Egtved virus strains react strongly with antiserum to reference strain F 1 in IFAT although not reacting in neutralization tests.

A.IV.3. *Enzyme linked immunosorbent assay (ELISA)*

Wells in microtiter plates (for instance Nunc- immunoplates, Maxisorp, Nunc, Denmark) are coated over night with recommended dilutions of Protein-A purified immunoglobulin fractions of the antisera mentioned in section A.IV.1.

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 with 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 rise 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, TMB or others).

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

B. Virus isolation with simultaneous serological virus identification

B.I.1. *Selection of samples*

As A.I.1-2.

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

As A.I.3.

B.II.1. *Homogenization of organs*

As A.II.1.

B.II.2. *Centrifugation of homogenate*

As A.II.2.

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 A.IV.1.

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

As A.III.1.

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

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

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

As A.III.3.

B.III.4. *Microscopy*

Inoculated cell cultures are inspected daily for the occurence 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 A.IV. have to be performed.

B.III.5. *Subcultivation*

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

C. Rapid diagnostic techniques (IFAT, ELISA)

Supernatant prepared as described under A.II.2. is submitted to IFAT or ELISA according to A.IV.2. or A.IV.3., 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.