

COMMISSION

COMMISSION DECISION

of 13 June 2003

establishing criteria for zoning and official surveillance following suspicion or confirmation of the presence of infectious salmon anaemia (ISA)*(notified under document number C(2003) 1831)***(Text with EEA relevance)**

(2003/466/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 Regulation (EC) No 806/2003 ⁽²⁾, and in particular Article 15 thereof,

Having regard to Council Directive 93/53/EC of 24 June 1993 introducing minimum Community measures for the control of certain fish diseases ⁽³⁾, as last amended by Commission Decision 2001/288/EC ⁽⁴⁾, and in particular Article 5(2) and Article 6 thereof,

Whereas:

- (1) Directive 93/53/EEC establishes that sampling and laboratory testing for the presence of list I and list II diseases (which are referred to in Annex A to Directive 91/67/EEC) shall be carried out using the methods established in accordance with Article 15 of Directive 91/67/EEC.
- (2) The sampling plans and diagnostic methods for the detection and confirmation of the list II fish diseases, viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) are laid down by Commission Decision 2001/183/EC ⁽⁵⁾.
- (3) According to Article 5(2) and Article 6 of Directive 93/53/EEC, all farms situated in the same water catchment area or coastal area in which a farm suspected or confirmed infected with infectious salmon anaemia (ISA)

virus is situated, shall be placed under official surveillance. The criteria for zoning and official surveillance should be established.

- (4) In order to define the sampling plans and diagnostic methods for the detection and confirmation of ISA, and to establish the criteria for zoning and official surveillance following suspicion or confirmation of ISA, fish health and laboratory experts have been consulted. Furthermore, the guidelines for the diagnosis of ISA laid down in the current edition of the *Diagnostic manual for aquatic animal diseases* of the International Office for Epizooties (OIE) must be taken into account.
- (5) A sufficient period of time should be provided for the implementation of these new requirements.
- (6) The measures provided for in this Decision are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health,

HAS ADOPTED THIS DECISION:

Article 1

The sampling plans and diagnostic methods for the detection and confirmation of infectious salmon anaemia (ISA), as well as the criteria for zoning and official surveillance following suspicion or confirmation of ISA, are laid down in the Annex to this Decision.

Article 2

This Decision shall apply from 23 October 2003.

⁽¹⁾ OJ L 46, 19.2.1991, p. 1.

⁽²⁾ OJ L 122, 16.5.2003, p. 1.

⁽³⁾ OJ L 175, 19.7.1993, p. 23.

⁽⁴⁾ OJ L 99, 10.4.2001, p. 11.

⁽⁵⁾ OJ L 67, 9.3.2001, p. 65.

Article 3

This Decision is addressed to the Member States.

Done at Brussels, 13 June 2003.

For the Commission
David BYRNE
Member of the Commission

ANNEX

Sampling plans and diagnostic methods for the detection and confirmation of infectious salmon anaemia (ISA) and criteria for zoning and official surveillance following suspicion or confirmation of ISA

INTRODUCTION AND DEFINITIONS

This Annex:

- (a) provides for guidelines and minimum requirements on sampling plans and diagnostic methods for the detection and confirmation of the presence of ISA;
- (b) integrates the provisions and the definitions laid down in Directive 91/67/EEC and Directive 93/53/EEC;
- (c) sets out provisions aiming at the proper diagnosis, control and surveillance of ISA, in case of suspicion or confirmation of ISA;
- (d) is directed towards both the authorities responsible for the control of ISA and the laboratory personnel performing the tests with regard to this disease. Emphasis is put on the sampling procedures, principles and applications of laboratory tests and evaluation of their results, as well as on detailed laboratory techniques. However, when appropriate, the laboratories may apply modifications to the tests described in this Annex, or use different tests, provided that equal or superior sensitivity and specificity can be demonstrated. Furthermore, criteria for the establishment of zones and official surveillance following suspicion or confirmation of ISA are laid down.

For the purpose of this Annex, the following additional definitions shall apply.

'Water catchment area' means the entire catchment area from the sources of the waterways to the estuary, or part of a catchment area from the source of a waterway to a natural or artificial barrier preventing fish migrating from that barrier.

'Coastal area' means a part of the coast or seawater or an estuary with a precise geographical delimitation which consists of a homogenous hydrodynamic system or series of such systems.

Part I lays down the general principles and the criteria for the diagnosis and confirmation of ISA and criteria for zoning and official surveillance to be carried out following suspicion or confirmation of ISA.

Part II sets out the inspections and sampling to be carried out in order to detect the presence of ISA.

Part III lays down the methods to be used for virological examination.

Part IV outlines the procedure for the examination of samples by RT-PCR for the detection of ISA.

Part V describes the protocol to be used for the examination of kidney imprints by IFAT (indirect fluorescent antibody test) with regard to ISA.

Part VI includes the methodology for histology.

Part VII lists the acronyms and abbreviations used.

I. Criteria for diagnosis of ISA and for the establishment of zones, certain control measures and official surveillance**I.1. General principles for the diagnosis of ISA**

Reasonable grounds for fish to be suspected of being infected with ISAV are outlined in Part I.2 of this Annex. Member States shall ensure that, following the suspicion of fish on a farm being infected with ISAV, an official investigation to confirm or rule out the presence of the disease is carried out as quickly as possible, applying the inspections and clinical examinations, as well as the collection and selection of samples and the methods for laboratory examination as laid down in Part III-VI of this Annex. In order officially to confirm the presence of ISA, any one of the three sets of criteria laid down in Part I.3 of this Annex shall be fulfilled.

I.2. Suspicion of infection with ISA

I.2.1. The presence of ISA shall be suspected if at least one of the following criteria are met:

- (a) the presence of post-mortem findings consistent with ISA, with or without clinical signs of disease. Post-mortem findings and clinical signs of disease shall be in accordance with those laid down in the current edition of the OIE *Diagnostic manual for aquatic animal diseases*;
- (b) isolation and identification of ISAV in cell culture from a single sample from any fish on the farm as described in Part III;

- (c) reasonable evidence of the presence of ISAV from two independent laboratory tests such as RT-PCR (Part IV) and IFAT (Part V);
- (d) the transfer of live fish into a farm where there are reasonable grounds to suspect that ISA was present at the time of the fish transfer;
- (e) where an investigation reveals other substantial epidemiological links to ISA-suspected or confirmed farms.

I.2.2. Suspicion of ISA can be ruled out if continued investigations involving at least one clinical inspection per month for a period of six months reveal no further significant evidence of the presence of ISA.

I.3. Confirmation of ISA

The presence of ISA shall be considered as confirmed if the criteria in (a) or (b) or (c) are met:

- (a) clinical signs and post-mortem findings consistent with ISA in accordance with the current edition of the OIE *Diagnostic manual for aquatic animal diseases*, including dead, weak or abnormally behaving fish, signs of anaemia, other post-mortem findings and pathological changes, are observed, and ISAV is detected by one or more of the following methods:
 - (i) isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm as described in Part III,
 - (ii) detection of ISAV by means of RT-PCR by the methods described in Part IV,
 - (iii) detection of ISAV in tissues or tissue preparations by means of specific antibodies against ISAV (e.g. IFAT on kidney imprints as described in Part V);
- (b) isolation and identification of ISAV in two samples from one or more fish at the farm tested on separate occasions using the method described in Part III;
- (c) isolation and identification of ISAV from at least one sample from any fish on the farm using the method described in Part III with corroborating evidence of ISAV in tissue preparations from any fish on the farm using either RT-PCR (Part IV) or IFAT (Part V).

I.4. Criteria for the establishment and revocation of zones for control and official surveillance following suspicion and confirmation of ISA

I.4.1. For the purpose of establishing a risk-based official surveillance programme, Member States shall, in the vicinity of a farm officially suspected or confirmed infected with ISA, establish appropriate control and surveillance zones.

I.4.2. The zones to be established shall be defined based on a case-by-case analysis of the risks for further spread of the disease. In accordance with the epizootiological situation, the water catchment area or the coastal area of concern:

- shall be defined as a control zone, or
- may, in extensive water catchment or coastal areas, be divided into a control zone and a surveillance zone if the prevention of the spread of ISA is not compromised.

Furthermore, additional surveillance zones may, as necessary, be established outside the water catchment or coastal area.

I.4.3. The main factors to consider for the establishment of the zones above are those influencing the risks for the spread of the disease to farmed and wild fish, such as: the number, rate and distribution of mortalities of fish on the farm suspected or confirmed infected with ISAV; the cause of mortalities on the farm concerned; distance to and density of neighbouring farms; contact farms; species present at the farms; management applied in the affected and the neighbouring farms; the hydrodynamic conditions and other factors of epidemiological significance identified within the framework of the epizootic investigation carried out in accordance with Articles 5(2) and 8 of Directive 93/53/EEC.

I.4.4. The following minimum criteria shall apply for the establishment of zones.

I.4.4.1. A 'control zone' shall be established by the Member State in the close vicinity of a farm confirmed infected by ISAV as follows:

- in coastal areas: the area included in a circle with a radius of at least one tidal excursion or at least 5 km, centred on the confirmed ISAV-infected farm, or an equivalent area determined according to appropriate hydrodynamic or epidemiological data, or
- in inland areas: the entire water catchment area of the farm confirmed ISAV-infected; the Member State may, in extensive water catchment areas, limit the extension of the zone to parts of the water catchment area provided that the prevention of the spread of ISA is not compromised.

I.4.4.2. A 'temporary control zone' shall be established in case of suspicion of the presence of ISA, based on the same criteria as outlined for the control zone.

I.4.4.3. A 'surveillance zone' shall be established as necessary by the Member State outside the control zone in areas where less intensive surveillance is deemed sufficient and shall correspond to:

- in coastal areas: an area surrounding the control zone of overlapping tidal excursion zones, an area surrounding the control zone and included in a circle of radius 10 km from the centre of the control zone or an equivalent area determined according to appropriate hydrodynamic or epidemiological data, or
- in inland areas: if needed, as an extended area outside the established control zone.

I.5. *Fallowing and revocation of zones established*

I.5.1. The competent authority of the Member State shall ensure that all farms within the control zone are subject to an appropriate period of fallowing after being emptied of fish and disinfected as necessary. The duration of the fallow period in farms confirmed to be infected with ISA shall be no less than six months. The length of the fallowing period for other farms in control zones shall be determined by the competent authority on a case-by-case risk evaluation. When all farms in the control zone are emptied, at least six weeks of synchronised fallowing shall apply.

Furthermore, the competent authority may decide upon fallowing of farms in established surveillance zones.

I.5.2. Established control zones may not be revoked and restocked until all farms situated in the zones have been emptied of fish, disinfected as necessary and fallowed in accordance with I.5.1. When restocking of the zones is carried out, the control zones shall be converted into surveillance zones as laid down in 1.4.4.3.

I.5.3. Established temporary control zones may not be revoked until the suspicion of ISA has been ruled out in accordance with Part I.2.2. In case of confirmation of ISA in accordance with Part I.3, the temporary control zone shall be converted into a control zone.

I.5.4. Established surveillance zones may not be revoked until two years after the revocation of the control zone.

I.6. *Official surveillance following suspicion or confirmation of ISA*

I.6.1. With reference to Articles 5(2) and 6 of Directive 93/53/EEC and in order to establish the distribution and the evolution of the disease following a suspicion or confirmation of ISA on a farm, a risk-based official surveillance programme must be carried out by the competent authority, or by qualified fish health services in consultation with and under the control of the competent authority, on all farms situated in the zones established.

I.6.2. For the purpose of the application of such an official surveillance programme, the competent authority must, if necessary by an inspection on the spot, identify all farms in the established zones and make an official census of the species, categories and numbers of fish kept at the farms, including mortality figures.

- I.6.3. Following the initial official census, farms within temporary established control zones that are keeping Atlantic salmon (*Salmo salar*), or any other species referred to in the most recent edition of the OIE *Aquatic animal health code* as being susceptible to or a potential carrier of ISA, shall report upon mortalities every 14 days to the competent authority. Increased mortality shall be reported per day and cage. The competent authority shall investigate any significantly increased mortality on a farm.

If suspicion is confirmed, all farms in the established control zone shall report weekly on mortality, on a per cage and day basis, to the competent authority.

Farms in surveillance zones shall report mortalities to the competent authority every 14 days.

Furthermore, inspections shall be carried out regularly throughout the year in the established zones and with a frequency as outlined in Table 1. However, when climatic conditions make such inspections impossible during part of the year, Member States may lay down other inspection frequencies in the contingency plan.

Table 1

Official surveillance programme

Farm location	Minimum number of inspections per year	Minimum number of inspections per year after the control zone is revoked
Control zone	12	
Surveillance zone	6	6
Temporary control zone	6	

The surveillance programme shall be carried until the zones are revoked.

- I.6.4. The inspections, as well as selection, collection, preparation and shipment of samples shall be carried out as defined in Parts II.1 to II.4. The examination of the samples shall be in accordance with Parts III to VI.

II. Inspection and sampling

II.1. Inspection, selection and collection of samples at a farm where the presence of ISA is suspected

- II.1.1. At the regular inspections carried out within the framework of the official surveillance programme outlined in Part I.6, and in farms that are suspected of being infected with ISA, all farm facilities (cages, tanks or ponds) shall be inspected for the presence of dead, weak or abnormally behaving fish. Where possible, recent mortalities (not decomposed) and weak or abnormally behaving fish shall be examined for clinical signs or post-mortem findings of ISA as described in the current edition of the OIE *Diagnostic manual for aquatic animal diseases*.
- II.1.2. If recent clinical signs consistent with ISA are observed, or an inspector or veterinarian has any other reason to suspect that fish may be infected, a minimum of 10 fish shall be sampled. The sample shall be made up of recent mortalities and weak or abnormally behaving fish where possible. If there are insufficient numbers of clinically affected fish then numbers in the sample shall be supplemented with healthy fish selected from the cages, tanks or ponds showing the highest number of mortalities or fish showing clinical signs of disease.
- II.1.3. If recent mortalities or weak or abnormally behaving fish are observed, but the clinical signs and post-mortem findings are not consistent with ISA, sampling is not obligatory, although such samples as may be required to carry out a differential diagnosis may be taken at the inspector's or veterinarian's discretion.

II.1.4. Where fish from the wild are suspected of being infected with ISA, Member States shall ensure that appropriate samples are taken and examined by the appropriate clinical and laboratory methods as laid down in Parts II to VI in order to rule out or confirm the presence of ISA and to estimate if the occurrence of the disease presents a significant threat to farmed fish.

II.2. *Preparation of samples from fish*

II.2.1. Samples for histological examination shall only be taken from freshly killed fish exhibiting clinical signs or post-mortem findings consistent with the presence of disease. Any external or internal lesions shall be sampled and in any case samples of liver, mid-kidney, heart and spleen shall be removed from individual fish using a scalpel and transferred to 8 to 10 % (vol/vol) buffered formol saline. The ratio of fixative to tissue must be at least 20:1 to ensure satisfactory preservation of the tissues.

II.2.2. Tissues for virological examination shall be taken from all of the fish sampled. Duplicate samples shall be taken for corroborative purposes. Pieces of the liver, anterior kidney, heart and spleen shall be removed from the fish using a sterile instrument and transferred to plastic tubes containing 9 ml transport solution, i.e. cell culture medium with antibiotics. A combination of 12,5 µg ml⁻¹ fungizone, 200 IU ml⁻¹ polymixin B, and 200 µg ml⁻¹ kanamycin is suitable but other combinations of proven efficiency may be used. Tissues from up to five fish may be collected in one tube containing transport solution and represent one pooled sample. The weight of tissue in one sample shall be 1,0 ± 0,5 g.

II.2.3. Kidney imprints shall be taken for IFAT examination from freshly killed fish only, i.e. within two hours of death. A piece of mid-kidney shall be removed from the fish using sterile instruments. The tissue shall be blotted on absorbent paper to remove excess blood then repeatedly pressed against a poly-L-lysine-coated glass slide. The individual impressions shall be adjacent to each other, but not overlapping, to give a continuous single layer of cells. Blood and tissue fluid are not relevant material for this test. Leaving the kidney sample to 'drain' on the absorbent paper shall be avoided as this can lead to blood clotting causing large amounts of serum proteins to be deposited on the test slide. The imprints shall be air dried then kept cool and dry if they are not to be fixed immediately. Fixation of imprints shall be carried out within 72 hours of the fish being sampled. Alternatively, imprints may be frozen following air drying and stored for up to one month at - 20 °C prior to fixation.

II.2.4. Fish showing signs of anaemia may be stunned and heparinised blood samples taken immediately for haematological examination, such as measurement of haematocrit.

II.2.5. Tissue for RT-PCR analysis shall be taken from all of the fish sampled. A piece of anterior or mid-kidney shall be removed from the fish using a sterile instrument and transferred to a microfuge tube containing 1 ml RNA preservative solution of proven efficacy. Tissue from up to five fish may be collected in one tube of preservative solution and represent one pooled sample. The weight of tissue in one sample shall be approximately 0,5 g. When the fish are too small to obtain a sample of the required weight, pieces of kidney, heart, spleen, liver or pyloric caeca may be taken, in that order of preference, to make up 0,5 g.

II.3. *Shipment of samples from fish*

II.3.1. Blood samples and tubes containing fish tissues for virological examination or RT-PCR analysis shall be placed in insulated containers (for instance thick-walled polystyrene boxes) together with sufficient ice or 'freeze blocks' to ensure chilling of the samples during transportation to the laboratory. Freezing must be avoided and ice shall still be present in the transport box at receipt or one or more of the 'freeze blocks' must still be partly or completely frozen. In exceptional circumstances RT-PCR samples and samples for virological examination may be snap-frozen and transported to the laboratory at - 20 °C or below.

II.3.2. Slides for IFAT shall be shipped in slide holders with sufficient desiccant to keep the imprints dry and chilled as above.

II.3.3. If fish tissues are transported in fixative for histological examination they shall be shipped in leak-proof tubes in impact-resistant containers, such as thick-walled polystyrene boxes.

- II.3.4. Unless samples have been frozen, the virological examination must be started as soon as possible and not later than 72 hours after the collection of the samples. The sample for corroborative analysis shall be stored at -20°C or below on arrival at the laboratory.
- II.3.5. Whole fish may be transported to the laboratory if the temperature requirements during transportation, as described in II.3.1, can be fulfilled. Whole fish shall be wrapped in absorbent paper and shipped in a plastic bag, chilled as mentioned above.
- II.3.6. Live fish may also be shipped but only under the supervision of the official service.
- II.3.7. For RT-PCR analysis of tissues preserved in *RNAlater*, RNA extraction must be carried out within a certain time for samples stored at different temperatures. These times are given below:
- | | |
|-------------------------|--------------|
| — 37°C | one day |
| — 25°C | one week |
| — 4°C | one month |
| — -20°C | indefinitely |
- II.3.8. All packaging and labelling must be performed in accordance with present national and international transport regulations as appropriate.

II.4. *Collection of supplementary diagnostic material*

With the agreement of the diagnostic laboratory, other fish tissues may be collected and prepared for supplementary examination.

III. **Virological examination**

III.1. *Preparation of samples*

- III.1.1. Where practical difficulties arise which make it impossible to inoculate cells within 72 hours after collection of the tissue samples, it is acceptable to freeze the tissue at -80°C for up to 28 days. The tissue must be frozen and thawed only once before examination.
- III.1.2. Each sample (tissue pool in transport solution) shall be completely homogenised using a stomacher, blender or mortar and pestle, centrifuged at $2\,000$ to $4\,000 \times g$ for 15 minutes at 0 to 6°C , and the supernatant shall be filtered ($0,45\ \mu\text{m}$) and incubated with an equal volume of a suitably diluted pool of antisera to the indigenous serotypes of IPNV. The titre of the antiserum must be at least 1:2000 in a 50 % plaque neutralisation test. The mixture shall be incubated for one hour at 15°C . This represents the inoculum.

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 ISAV.

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

III.2. *Inoculation of cell cultures*

- III.2.1. SHK-1 cells (pass 80 or lower) or TO cells shall be grown in L-15 medium containing 5 % foetal bovine serum, 2 % (v/v) 200 mM L-glutamine, and 0,08 % (v/v) 50 mM 2-mercaptoethanol in 12- or 24-well plates. Other cell lines of proven effectiveness and sensitivity in isolating ISAV may be used, taking into consideration strain variability and the ability of different strains to replicate in different cell lines. Antiserum-treated organ suspension shall be inoculated into young actively growing cell cultures to give a final dilution of tissue material to culture medium of 1:1 000. For each organ suspension 40 μl of inoculum shall be added to one well containing 2 ml of culture medium. To minimise the risk of cross-contamination it is recommended that separate 12- or 24-well plates shall be used for samples from different fish farm sites.

III.2.2. One plate shall be left uninoculated to serve as a negative control. A separate plate shall be inoculated with a reference isolate of ISAV as a positive control, as follows. One hundred μl of a stock preparation of ISAV (minimum titre 10^7 TCID₅₀ ml⁻¹) shall be inoculated into the first well and mixed well. A volume of this material shall be transferred from the first well to the second well to make a 1:10 dilution and mixed well. This shall be repeated across the plate to make six 10-fold dilutions. Stock ISAV may be stored at -80°C for at least two years but once thawed must be used within three days. Note: care must be taken to prevent cross-contamination of test plates with positive control material. To avoid this risk positive controls shall be set up and handled separately from test plates.

III.2.3. Samples shall be incubated at $14 \pm 2^\circ\text{C}$ for up to 15 days.

III.3. *Microscopy*

Using a microscope, cell cultures shall be examined for CPE twice, between five to seven and 12 to 14 days following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately (III.6). If no CPE is observed by day 14, a haemadsorption test (III.4) shall be performed.

III.4. *Haemadsorption*

Replication of ISAV in cell cultures does not always result in a CPE. Therefore, every well shall be subject to a haemadsorption test as described below, or alternatively, every well shall be subject to an IF test as described in III.6.1.

III.4.1. Cell culture medium shall be removed from each well, including those of positive and negative controls, and placed in labelled sterile tubes. Five hundred μl of a 0,2 % (v/v) suspension of washed rabbit or horse red blood cells, or a 0,05 % (v/v) suspension of washed rainbow trout or Atlantic salmon red blood cells, shall be added to each well and incubated at room temperature for 45 minutes. The red blood cells shall be removed and each well shall be washed twice with L-15 medium. Each well shall be examined using a microscope.

III.4.2. The presence of clusters of red blood cells attaching to the surface of SHK-1 or TO cells shall be indicative of presumptive infection with an orthomyxovirus. If a haemadsorption test is positive a virus identification test shall be performed immediately (III.6).

III.5. *Subcultivation or passage*

III.5.1. Subcultivation shall be carried out between days 13 to 15. Two hundred and twenty-five μl of culture supernatant shall be added to wells containing fresh actively growing SHK-1 cells in 12-well plates and incubated at $14 \pm 2^\circ\text{C}$ for up to 18 days. Using a microscope, cell cultures shall be examined for CPE twice, between days five to seven and 14 to 18 following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately (III.6). If no CPE is observed by days 14 to 18, a haemadsorption test shall be performed (III.4).

III.5.2. If cytotoxicity occurs within the first seven days of incubation, subcultivation shall be performed at that stage, and the cells must be incubated for 14 to 18 days and subcultivated again with a further 14 to 18 days incubation. If cytotoxicity occurs after seven days, subcultivation shall be performed once and the cells shall be incubated to achieve the total of 28 to 36 days incubation from the primary inoculation.

III.5.3. If bacterial contamination occurs in the primary culture, the test must be set up again using the tissue homogenate stored at -80°C . Prior to inoculation the tissue homogenate is centrifuged at $4\,000 \times g$ for 30 minutes at 0 to 6°C and the supernatant is filtered at $0,22 \mu\text{m}$. If bacterial contamination occurs during the subcultivation step the supernatant shall be filtered at $0,22 \mu\text{m}$, inoculated onto fresh cells and incubated for a further 14 to 18 days.

III.6. Virus identification tests

If evidence of CPE is observed at any stage, or if a haemadsorption test is positive, virus identification shall be carried out. The methods of choice for identification of ISAV are IF (III.6.1) and RT-PCR (Part IV). If it is considered that other viruses may be present it is recommended that supplementary virus identification tests are carried out. If these tests have not allowed definitive identification of the virus within one week, the supernatant must be forwarded to a national reference laboratory or to the EU reference laboratory for fish diseases for immediate identification.

III.6.1. IF

- III.6.1.1. SHK-1 cells (pass 80 or lower) or TO cells shall be grown in L-15 medium containing 5 % foetal bovine serum, 2 % (v/v) 200 mM L-glutamine and 0,08 % (v/v) 50 mM 2-mercaptoethanol in 24- or 96-well plates and used at greater than 50 % confluence. Other cell lines or growth medium of proven efficacy may also be used. Two hundred and twenty five μ l of putative virus-infected culture supernatant shall be added to each of two wells, mixed and 225 μ l transferred to two further wells, i.e. a 1:5 dilution. Two additional wells shall be left uninoculated to act as controls. Samples from each fish farm site shall be handled on separate plates, as shall the virus control. A virus control shall be established using a reference isolate of ISAV.
- III.6.1.2. Plates shall be incubated at 14 ± 2 °C and examined microscopically for up to seven days. When early CPE is observed, or if no CPE is observed within seven days, the next step shall be fixation. Wells shall hereby be washed with PBS and fixed by incubation with 80 % acetone for 20 minutes at room temperature. Plates shall be air-dried and stained immediately or stored at 0 to 6 °C for no more than 24 hours prior to staining.
- III.6.1.3. Replicate wells shall be stained with monoclonal antibody 3H6F8 to ISAV, or other monoclonal antibody of proven effectiveness and specificity, diluted in PBS and incubated at 37 ± 4 °C for 30 minutes. Monoclonal antibody shall be removed and plates washed three times with 0,05 % Tween 20 in PBS. Anti-mouse IgG FITC conjugate diluted in PBS shall be added to each well and incubated at 37 ± 4 °C for 30 minutes. Note: the dilutions of different batches of monoclonal antibody and FITC conjugate shall be optimised in each laboratory. Antibody shall be removed and plates shall be washed three times with 0,05 % Tween 20 in PBS.
- III.6.1.4. Wells shall be examined immediately using an inverted microscope set up for fluorescence microscopy with a suitable filter for excitation of FITC. A test shall be considered positive if fluorescent cells are observed. For a test to be valid the positive controls must score positive and the negative controls must score negative.

IV. Examination of samples by RT-PCR

IV.1. *This section describes the procedures required for PCR amplification of part of segment 8 of the ISAV genome that may be carried out on fish tissue or ISAV in culture*

IV.1.1. RNA extraction

- (a) RNAlater is removed from each sample. 1 ml of DEPC-treated dH₂O shall be added to each tube and tubes shall be centrifuged at 13 000 rpm for five minutes at 0 to 6 °C.
- (b) The supernatant shall be removed from each sample, and 800 μ l TRIzol (Invitrogen), or alternative reagent proven to be of equal or greater efficacy, shall be added to each sample and a control tube containing suitable control material (400 μ l dH₂O or kidney homogenate from specified-pathogen-free fish). If necessary, tissues shall be disrupted by repeated pipetting. Tubes shall be incubated at room temperature for five minutes. 160 μ l chloroform shall be added to each tube and tubes shall be shaken vigorously for three minutes, then centrifuged at 13 000 rpm for 15 minutes at 0 to 6 °C.
- (c) The aqueous upper layer shall be removed to a labelled 1,5 ml microfuge tube containing 500 μ l isopropanol and tubes shall be incubated for 10 minutes at room temperature, then centrifuged at 6 500 rpm for 15 minutes at 0 to 6 °C.

- (d) The supernatant shall be removed and 1 ml 75 % ethanol added to the RNA pellet. Tubes shall be then centrifuged at 6 500 rpm for five minutes at 0 to 6 °C.
- (e) The supernatant shall be removed and the tubes left open for approximately three minutes to let the remaining ethanol evaporate. 15 µl of DEPC-treated dH₂O shall be added to resuspend the pellet, vortexing briefly if necessary.
- (f) A spectrophotometer shall be used to calculate RNA concentration and purity of samples. Optical densities are measured at 260 and 280 nm.
- (g) RNA that is to be used immediately (the same day), can be temporarily stored at 0 to 6 °C. RNA not used at once shall be stored at – 80 °C.

IV.1.2. RT

- (a) Two µg RNA shall be diluted in DEPC-treated dH₂O in 1,5 ml microfuge tubes. Where the RNA concentration of a sample is too low to allow use of 2 µg in the RT reaction, the maximum possible amount of RNA shall be used. Diluted RNA shall be incubated at 55 to 60 °C for 10 minutes.
- (b) Tubes containing RNA shall then be placed on ice and RT reagents added to give final concentrations of 1× buffer, 1mM dNTPs, 100 ng random hexamers, 20 U RNase inhibitor and 200 U MMLV-RT in a total volume of 20 µl.
- (c) Tubes shall be incubated at 37 °C for one hour.
- (d) cDNA shall be stored at 0 to 6 °C until required and shall be used in PCR as soon as possible.

IV.1.3. PCR

- (a) Five µl cDNA shall be added to 45 µl PCR mix to give final concentrations of 1× buffer, 1,5 mM MgCl₂, 0,2 mM each dNTP, 25 pmol each primer and 1U Taq polymerase. Primers are ISA+ (5'-GGC-TAT-CTA-CCA-TGA-ACG-AAT-C-3') (forward primer) and ISA- (5'-GCC-AAG-TGT-AAG-TAG-CAC-TCC-3') (reverse primer). Negative controls for extraction, RT and PCR steps shall be included.
- (b) Tubes shall be placed in a thermocycler programmed at 94 °C for five minutes followed by 35 cycles of 94 °C for one minute, 55 °C for one minute and 72 °C for one minute with a final incubation at 72 °C for five minutes.
- (c) Results of the PCR shall be assessed following electrophoresis using a 2 % agarose gel stained with ethidium bromide and including size markers alongside the samples and the negative controls from RT and PCR steps. A single PCR product of 15 5bp shall be considered indicative of the presence of ISAV RNA. Samples that contain one additional product, of 310bp, shall also be considered to contain ISAV RNA. Samples that yield multiple PCR products, including at least one of approximately 155 bp, may contain ISAV RNA. These can be investigated further using DNA probes or nucleotide sequencing.

IV.1.4. PCR confirmation of isolation of ISAV in tissue culture

If full CPE has occurred during virological examination of tissue samples in SHK-1 cells, 400 µl of supernatant shall be removed from the well and placed in a sterile 1,5 ml tube. RNA shall be extracted from this sample as in III.1 and RT-PCR shall be carried out. If cultures without full CPE are used, the supernatant shall be removed, cells shall be scraped from the surface of the well or flask and placed in a sterile 1,5 ml tube for RNA extraction and RT-PCR.

IV.1.5. DNA probe confirmation of PCR products

- (a) The specificity of a 155 bp PCR product can be assessed by probing with an oligonucleotide that hybridises to a region of the PCR product, internal to the primers. PCR products shall be subjected to electrophoresis in a 1 % agarose gel alongside size markers and a positive control and the negative controls from the RT and PCR steps.

- (b) DNA shall be Southern blotted onto a membrane and the labelled oligonucleotide (5'-CGGGAGTTGATCAGACATGCACTGA AGGTG-3') shall be incubated with the membrane after appropriate pre-hybridisation steps.
- (c) Unbound and non-specifically bound probes shall be washed from the membrane and bound probes visualised.
- (d) Probes binding to a fragment of 155 bp (and 310 bp if present) shall be evidence for specificity of the PCR and indicates that ISAV RNA was present in the sample.

IV.1.6. Nucleotide sequencing of PCR products

The specificity of the PCR can be assessed by examination of the nucleotide sequence of the 155 bp PCR product.

- (a) PCR product shall be purified from agarose gel or solution.
- (b) The fragment shall be sequenced using the same primers as were used in the PCR or vector primers if cloned into a vector prior to sequencing.
- (c) The nucleotide sequence shall be compared with those for ISAV segment 8 available on the EMBL nucleotide sequence database (accession numbers Y10404, AJ012285, AJ242016).
- (d) Presence of sequence corresponding to that of ISAV segment 8 is evidence that the sample contained ISAV RNA.

V. Examination of kidney imprints by IFAT

V.1. *The following protocol has been established for examination of kidney imprints by IFAT*

V.2. *Preparation and staining of imprints*

V.2.1. Slides shall be fixed in acetone or methanol/acetone (1:1) for three minutes and air-dried. Before staining each slide shall be examined and appropriate regions of the slide shall be circumscribed with ImmEdge™ pen, or similar, and allowed to air-dry. The slides shall be then placed in blocking solution (6 % skimmed milk in PBS containing 0,2 % Tween 20) and incubated with gentle agitation for 30 minutes at room temperature. Each slide shall be drained and placed horizontally in a slide box containing wet tissue paper to maintain a humid atmosphere.

V.2.2. Each imprint shall be covered with a solution of monoclonal antibody 3H6F8 to ISAV (or other antibody of proven specificity and efficacy) and the slide box shall be closed and incubated with agitation for 60 minutes at room temperature. The antibody shall normally be diluted 1:10 to 1:100 in 1 % skimmed milk but the actual dilution needs to be determined for each batch. Slides shall be washed three times for two minutes in PBS containing 0,1 % Tween 20. Each imprint shall be covered with a solution containing FITC goat anti mouse conjugate diluted 1:1 000 in 1 % skimmed milk and incubated in a humid environment for 60 minutes at room temperature. Slides shall be washed three times for two minutes in PBS containing 0,1 % Tween 20. Each slide shall be covered with CITIFLUOR™ solution (500 ul CITIFLUOR™ mixed with 1,5 ml 0,1 % (v/v) Tween 20 in PBS) or other suitable mounting medium for 10 minutes. Slides shall be washed three times in PBS containing 0,1 % Tween 20. If a counter-stain is required, each imprint may be covered with propidium iodide (0,01 mg/ml) in PBS containing 0,1 % Tween 20 and incubated for three minutes at room temperature. Slides shall be washed three times for two minutes in PBS containing 0,1 % Tween 20. Slides shall be drained and mounted in CITIFLUOR™ or other suitable mounting medium. Slides shall be stored in the dark at 4 °C prior to microscopic examination.

V.3. *Examination using fluorescent microscopy*

Each slide shall be examined on a microscope suitable for epi-fluorescent illumination, using a suitable filter that will excite FITC, causing it to emit characteristic green fluorescent. All fields within the regions defined by the ImmEdge™ pen shall be examined under $\times 10$ and $\times 20$ objectives and suspicious areas (those showing a green fluorescence) shall be further examined under a $\times 40$ objective and phase/fluorescent illumination to ensure that the fluorescent staining is cell-associated. The stage coordinates for the suspicious regions shall be recorded for later confirmation of the nature of the fluorescence by a second examiner. Following examination by the primary reader slides that are positive or suspicious shall be re-examined by a second reader and the results confirmed.

V.4. *Controls*

V.4.1. Three types of control must be included with each batch of slides stained for IFAT:

- kidney imprint from uninfected Atlantic salmon (negative control),
- uninfected SHK-1 cell culture or other susceptible cell culture (negative control),
- ISAV-infected SHK-1 cell culture or other susceptible cell culture (positive control).

V.4.2. If available, a kidney imprint from an ISAV-infected Atlantic salmon is recommended as an additional positive control.

V.4.3. If a positive result is obtained with any negative controls the test is considered invalid for all slides in that batch. If all slides in a batch, including positive controls, are negative the test is considered invalid for all slides in that batch. In cases where failure of controls invalidates a batch of slides, those slides shall be destroyed and a re-test shall be carried out using the duplicate imprints.

V.5. *Examination of other tissues*

This technique can be applied to other fish tissues such as liver, spleen and heart providing a reasonable quantity of endothelial cells, leucocytes or lymphocytes can be deposited on the slide. The staining procedure remains the same for each tissue, although for some tissues it may be preferable to omit the propidium iodide staining relying on phase illumination to identify the cell types present in the imprint.

VI. **HISTOLOGY**

Paraffin-embedded sections shall be cut at 5 µm and stained using haematoxylin and eosin. Histological changes associated with ISA are described in the current edition of the OIE *Diagnostic manual for aquatic animal diseases*.

VII. **Acronyms and abbreviations**

cDNA	Complementary Deoxyribonucleic acid
CPE	Cytopathic effect
DEPC	Diethylpyrocarbonate
dNTP	Deoxynucleotide triphosphate
FITC	Fluorescein isothiocyanate
IF	Immunofluorescence
IFAT	Indirect fluorescent antibody test
OIE	<i>Office international des epizooties</i>
IPN(V)	Infectious pancreatic necrosis (virus)
ISA(V)	Infectious salmon anaemia (virus)
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RT-(PCR)	Reverse transcriptase (polymerase chain reaction)
SHK-1	Salmon head kidney cells
TCID ₅₀	Tissue culture infective dose at the 50 % end point