CHAPTER 2.1.15.

INFECTIOUS PANCREATIC NECROSIS

SUMMARY

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of young fish of salmonid species held under intensive rearing conditions (14, 32, 33). The disease most characteristically occurs in rainbow trout (Oncorhynchus mykiss), brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), Atlantic salmon (Salmo salar), and several Pacific salmon species (Oncorhynchus spp.). Susceptibility generally decreases with age, with resistance to clinical disease in salmonid fish usually being reached at about 300 degree-days (value obtained by multiplying the age in days by the average temperature in degrees centigrade during the lifespan) (11), except for Atlantic salmon smolts, which can be affected after transfer from fresh water to seawater (29). The first sign of an outbreak in salmonid fry is frequently a sudden and usually progressive increase in daily mortality, particularly in the faster growing individuals. Clinical signs include darkening pigmentation, a pronounced distended abdomen and a corkscrewing/spiral swimming motion. Cumulative mortalities may vary from less than 10% to more than 90% depending on the combination of several factors, such as virus strain (11) and quantity (24), host and environment (10). For further details see reviews by Hill (14), Reno (27) and Wolf (32).

The disease is transmitted both horizontally via the water route and vertically via the egg (3, 4, 12). Surface disinfection of eggs is not entirely effective in preventing vertical transmission (8).

The disease has a wide geographical distribution, occurring in most major salmonid-farming countries of North and South America, Europe and Asia. Oceania is free of the disease.

IPN virus (IPNV), or viruses showing serological relatedness to IPNV, have been reported to cause diseases in some farmed marine fish species, such as yellowtail (Seriola quinqueradiata) (21), turbot (Scophthalmus maximus) (7, 20, 22), dab (Limanda limanda) (25), halibut (Hippoglossus hippoglossus) (20, 28) and Atlantic cod (Gadus morhua). Subclinical covert infections have been detected in a wide range of estuarine and freshwater fish species, such as loach (Misgurnus anguillicaudatus) (8), pike (Esox lucius) (2) and numerous other species in the families Anguillidae, Atherinidae, Bothidae, Carangidae, Cobitidae, Cichlidae, Clupeidae, Cobitidae, Cyprinidae, Esocidae, Moronidae, Paralichthyidae, Percidae, Poeciliidae, Sciaenidae, Soleidae and Thymallidae (27).

The causative agent, IPNV, is a bi-segmented double-stranded RNA virus belonging to the family Birnaviridae (see review by Dobos & Roberts [10]). Isolates display wide antigenic diversity (11, 16, 19, 23) and fall into two serogroups that do not cross-react in serum neutralisation tests (3, 25, 31), with the majority of strains belonging to serogroup A, which comprises at least nine serotypes (11). Isolates also show marked differences in degrees of virulence (11, 19, 21).

Control methods currently rely on the implementation of control policies and of hygiene practices in salmonid husbandry, through the avoidance of the introduction of fertilised eggs originating from IPNV-carrier broodstock, and the use of a protected water supply (e.g. spring or borehole pond) where the ingress of fish, particularly possible virus carriers, is prevented. In outbreaks, a reduction in the population density ('thinning out') may help to reduce the overall mortality. No treatment or entirely effective vaccine is available at present.
The screening procedure for infectious pancreatic necrosis (IPN) is based on IPN virus (IPNV) isolation tests in cell culture (1) followed by immunological identification, either by serum neutralisation (16) or enzyme-linked immunosorbent assay (ELISA) (9), of virus isolated. Diagnosis of clinical cases is normally based on histology (18) and/or immunological demonstration of IPNV antigen in infected tissues (13), confirmed by isolation and immunological identification of IPNV in tissue culture, as for screening.

Due to insufficient knowledge of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine screening method for assessing the viral status of fish populations. However, the validation of some serological techniques for certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for health screening purposes.

Fish material suitable for virological examination is:

- Asymptomatic fish (apparently healthy fish): liver, kidney and spleen (any size fish) and/ or ovarian fluid from broodfish at spawning time.
- Clinically affected fish: whole alevin (body length ≤4 cm), entire viscera including kidney (4 cm ≤body length ≤6 cm) or, for larger size fish, liver, kidney and spleen.

Sampling procedures: see Chapter I.1 Section B.

1. Standard Screening Method for IPN

1.1. Isolation of IPNV in cell culture

Cell line to be used: BF-2 or CHSE-214 or RTG-2

a) Inoculation of cell monolayers

i) Make an additional tenfold dilution of the 1/10 organ homogenate supernatants and transfer an appropriate volume of each of the two dilutions on to 24-hour cell monolayers. Inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 0.5–1 hour at 10–15°C and, without withdrawing the inoculate, add the cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1ml/ well for 24-well cell culture plates) and incubate at 15°C.

b) Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at x40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid).

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the homogenate, IPNV identification procedures must be undertaken immediately (see Section 1.2. below).

iv) If no CPE occurs after 7 days of incubation (except in positive control cell cultures), subcultivation of the inoculated cell cultures must be performed.
c) **Subcultivation procedures**

i) Subject cell culture monolayers to one freeze–thaw cycle. Pool aliquots of the supernatants from all cell monolayers inoculated with dilutions of organ homogenates.

ii) Dilute 1:20 and 1:100 and inoculate cell monolayers as described above in Section 1.1.a.

iii) Incubate at 5°C and monitor as described in above Section 1.1.b.

iv) If no CPE occurs, the test may be declared negative.

v) If CPE occurs, the IPNV identification procedures must be undertaken immediately.

1.2. Identification of IPNV isolated in cell culture

a) **Neutralisation test**

i) Dilute the virus-containing medium from $10^{-2}$ to $10^{-4}$.

ii) Mix aliquots of each dilution with equal volumes of a neutralising antibody (NAb) solution against the indigenous serotypes of IPNV known in the region. If IPNV is not known to be indigenous to the region, mix aliquots of each dilution with equal volumes of a pooled NAb solution against all IPNV serotypes (g). Similarly, treat aliquots of each virus dilution with cell culture medium. In parallel, a neutralisation test must be performed against homologous IPNV (positive neutralisation test).

(The NAb solution must have a 50% plaque reduction titre of at least 2000.)

iii) In parallel, other neutralisation tests must be performed against:

- a homologous virus strain (positive neutralisation test)
- a heterologous virus strain (negative neutralisation test).

iv) Incubate all the mixtures at 5°C for 1 hour.

v) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution).

vi) Incubate at 5°C.

vii) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase-contrast preferable) or after discarding the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

viii) The tested virus is identified as IPNV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the IPNV-specific antibody, whereas CPE is evident in all other cell cultures.

ix) If CPE develops in the presence of antisera against the indigenous serotypes of IPNV, the neutralisation test must be repeated using pooled antibody against the non-indigenous serotypes of IPNV.

b) **Indirect fluorescent antibody test**

i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of IPNV in a similar way, in order to obtain a virus titre of about 5000-10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 5°C for 24 hours.
v) Remove the cell culture medium, rinse once with 0.01M phosphate buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at -20°C) for cover-slips or a mixture of acetone 30%/ethanol 70%, also at -20°C, for plastic wells.

vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to IPNV in 0.01M PBS, pH 7.2, containing 0.05% Tween 80 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin (Ig) used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover-slips using glycerol saline at pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with x10 eye pieces and x20–40 objective lens having high numerical aperture. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of purified Ig specific for IPNV, in 0.02 M carbonate buffer, pH 9.5 (200 µl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of IPNV, monoclonal antibodies (MAbs) specific for certain domains of the capsid protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse twice with 0.01M PBS.

iv) Block with bovine serum albumin (BSA) (15% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl/well).

v) Rinse four times with PBS containing 0.05% Tween 20 (PBST).

vi) Add an equal volume of PBST to the virus suspension to be identified.

vii) Dispense 20 µl/well of two- or four-step dilutions of the suspension of virus to be identified and of the non-infected cell culture harvest (negative control), and the IPNV control virus (positive control); allow to react with the coated antibody to IPNV for 30 minutes at 37°C.

viii) Rinse once with PBST followed by three washes, allowing to soak for 3 minutes between washes.

ix) Add to the wells, horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to IPNV; or polyclonal IgG to IPNV or MAb to capsid protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.

x) Incubate for 30 minutes at 37°C.

xi) Rinse and wash with PBST as in step viii above.

xii) Add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and repeat steps x and xi.
xi) Add 200 µl of the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.

xii) Alternatively, add substrate and chromogen to those wells containing the peroxidase-conjugated antibody and proceed as above.

(The procedure described above is just one example of an ELISA. Other procedures that use smaller volumes [50–100 µl] of reagents and/ or that use alkaline phosphatase as the enzyme are also acceptable.)

2. Diagnostic Methods for Clinically Diseased Fish

2.1. Direct detection in fish tissues

a) Indirect fluorescent antibody test

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store the kidney pieces (as indicated in Chapter I.1. Section B.3.1) together with the other organs required for virus isolation in case this becomes necessary later.

iv) Allow the imprint to air-dry for 20 minutes.

v) Fix with acetone or ethanol/acetone and dry as indicated in Section 1.2.b. steps v–vii.

vi) Rehydrate the above preparations (see Section 1.2.b. step ix) and block with 5% skim milk or 1% BSA, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST.

viii) Treat the imprints with the solution of antibody to IPNV and rinse as indicated in Section 1.2.b.

ix) Block and rinse as described previously in steps vi and vii.

x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated in Section 1.2.b. steps xii–xv.

If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture as described in Section 1.1.

b) Enzyme-linked immunosorbent assay

i) Microplate processing

As in Section 12.c. of this chapter up to step iv (inclusive).
ii) 
Sampling procedures

See the following sections in Chapter I.1:
B.1. for the selection of fish specimens
B.2. for the selection of materials sampled.

iii) 
Processing of organ samples

See the following sections in Chapter I.1:
B.3.1. for transportation
B.3.2. for virus extraction and obtaining organ homogenates.

iv) 
Enzyme-linked immunosorbent assay procedure

- Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.
- Add a volume of 100 mM phenyl methyl sulphonide fluoride (PMSF) to the homogenate to a final concentration of 2 mM PMSF, and mix gently.
- Complete the other steps of the procedure described in Section 1.2.c.

If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture as described in Section 11.

c) 
Co-agglutination test

IPNV may be detected by a rapid co-agglutination test method (30). The test comprises two test solutions - an anti-IPNV solution, consisting of Staphylococcus aureus cells (Phadebact coating kit, Boule Diagnostics AB, Sweden) coated with rabbit-anti-IPNV antibody, according to the manufacturers instructions, and a negative control solution of S. aureus cells coated with normal rabbit serum - and cardboard slides, a stomacher plastic bags and PBS.

The procedure for the test is as follows:

i) 
Use 0.5–1g tissue material, preferably kidney, but if small fry are to be examined, all of the visceral organs or whole fry except head and tail may be used. Homogenise the tissue sample with 0.5–1 ml of PBS.

ii) 
Centrifuge the homogenate at 2000 g for 10 minutes, remove the supernatant and dilute in an equal volume of PBS.

iii) 
Add one drop of the anti-IPNV solution and one drop of the negative control solution separately to a cardboard slide. Add one drop of the diluted homogenate (see step ii) separately to the drop containing anti-IPNV and to the drop containing negative control solution. Use a bacterial loop or syringe point to mix the contents of the drops.

iv) 
A positive reaction is shown as agglutination in the sample where anti-IPNV antibody is present, but not in the negative control solution.

d) 
Immunohistochemistry

i) 
**Fixation:** The tissues for examination have to be fixed in aldehyde fixatives (10% formaldehyde, 3% paraformaldehyde, 2.5% glutaraldehyde) for 48 hours or in absolute alcohol or 96% alcohol. Tissues for freeze sections are frozen in Freon gas or in liquid nitrogen as fast as possible. Such tissues must be kept at -70°C prior to and after sectioning.

ii) 
**Pretreatment of tissue samples:** After fixation, the sample is processed through a histokinette according to normal histological procedures and embedded in liquid paraffin. Freeze sections are thawed, dried by a fan without heat and fixed in acetone, acetone/ethanol, ethanol or other similar solvents.

iii) 
**Histological sections are put on slides coated with poly-L-lysine. (Poly-L-lysine glass is prepared in the following manner: 0.5% poly-L-lysine is dissolved in distilled water to reach a user concentration of 0.05%. The slide is left in poly-L-lysine solution for 30 minutes, and then rinsed for 5 minutes in running tap water. The slides are air dried over night. Note: Poly-L-lysine-coated slides are also commercially available.)**

iv) 
The sections are then deparaffinised and hydrated in the following way:

- Heat the chamber for 25 minutes at 56–58°C. The temperature must not exceed 60°C. Sections are then placed in the following xylene/ethanol baths in a fume-hood:
• 5 minutes in xylene,
• 5 minutes in xylene,
• 5 minutes in absolute alcohol,
• 5 minutes in absolute alcohol,
• 5 minutes in 96% alcohol,
• 5 minutes in 70% alcohol,
• 5 minutes in running tap water.

v) Blocking of endogenous peroxidase activity can be done by two different methods:

• \( \text{H}_2\text{O}_2/\text{methanol (30\% H}_2\text{O}_2 \text{diluted in 3\% methanol)} \) in staining jars. The sections are kept in this solution for 20 minutes and then washed in PBS.

• Phenylhydrazine diluted in PBS to a final concentration 0.05%. The PBS must be preheated to 37°C before phenylhydrazine is added. Mix well. The sections are covered with this solution and incubated, in a humid chamber, at 37°C for 40 minutes and then washed in PBS.

vi) Pretreatment of sections to unmask antigens: As antigens may be masked by methylene bridges from the formalin fixation, it is necessary to break the cross-bindings. This can be done by pretreatment in a microwave oven, or by trypsin treatment or by boiling under pressure/autoclaving.

vii) Immunohistochemical procedure

After deparaffinisation and unmasking procedures are completed, the sections are overlaid with 5% (w/v) BSA for 20 minutes. The solution is then blotted off the slides (without washing) and the specific polyclonal or MAbs against IPNV are incubated at concentrations/dilutions that will give an optimal signal to noise ratio. After washing for 5 minutes in Tris-buffered saline (TBS, pH 7.4), the secondary antibody, biotinylated anti-rabbit, diluted from 1/300 to 1/500, or anti-mouse immunoglobulin, is incubated for 30 minutes. After washing in TBS, streptavidin alkaline phosphatase (Boehringer Mannheim GmbH D 89-1 E 1) diluted 1/1000 (30 minutes incubation), avidin/ biotin/ alkaline phosphatase (Dakopatts, D 365) or avidin/ biotin/ peroxidase (ABC-PO, Vector PK-4001) (the two latter complexes diluted according to the manufacturer’s instructions) should be incubated for 30–45 minutes at room temperature. After washing, the specimens are incubated with either Fast Red in the case of alkaline phosphatase or with AEC (3-amino-9-ethylcarbazole, 0.27 g/litre, Sigma A-5754) in the case of peroxidase. Fast Red TR salt (Sigma F 150) (1g/litre) should be dissolved in TBS, and naphthol AS-MX phosphate (0.2 g/litre), N,N-dimethylformamide (20 ml/litre) (Sigma D-4254) and 1mM levamisole are then added. Levamisole should be added as an inhibitor of endogenous alkaline phosphatase. The sections are incubated for 20 minutes. AEC is dissolved in N,N-dimethylformamide (67 ml/litre) in 0.1 M acetate buffer, pH 5.2, and 0.03% \( \text{H}_2\text{O}_2 \) is added. The sections should be incubated for 15 minutes. The sections are then washed in running tap water for 10 minutes and counterstained with Mayer’s haematoxylin for 2 minutes. Finally, the sections are washed in tap water for another 2 minutes and cover-slips are applied with an aqueous medium (Aquamount, BDH- Laboratory Supplies, UK, 36086). All incubations, unless otherwise stated, are performed at room temperature in a humidified chamber.

Performance controls should include application of a non-immune serum (normal rabbit serum) at the same dilution as the immune serum or of heterologous MAbs (of the same isotype) at almost identical protein concentrations. Tissue sections from non-infected fish (same species as subject to analysis) should be incubated with immune and non-immune serum-specific or heterologous MAbs. Estimation of end-point dilution value (the highest dilution giving a positive reaction that was discernible from background) must be performed for antibody solutions using a 60-minute incubation at 37°C to validate the method.

2.2. Virus isolation in cell culture

See Section 11

REFERENCES


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NB: There is an OIE Reference Laboratory for Infectious pancreatic necrosis (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).