

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

Infection with infectious haematopoietic necrosis virus means infection with the pathogenic agent Salmonid Novirhabdovirus (also known as infectious haematopoietic necrosis virus [IHNV]) of the Genus *Novirhabdovirus* and Family *Rhabdoviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The fish rhabdovirus, IHNV, has a bullet-shaped virion containing a non-segmented, negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides that encodes six proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-virion protein (NV), and a polymerase (L). The presence of the unique NV gene and sequence similarity with certain other fish rhabdoviruses, such as viral haemorrhagic septicaemia virus, has resulted in the creation of the *Novirhabdovirus* genus of the family *Rhabdoviridae*, with IHNV as the type species. The type strain of IHNV is the Western Regional Aquaculture Center (WRAC) strain available from the American Type Culture Collection (ATCC VR-1392). The GenBank accession number of the genomic sequence of the WRAC strain is L40883 (Morzunov et al., 1995; Winton & Einer-Jensen, 2002).

Sequence analysis has been used to compare IHNV isolates from North America, Europe and Asia (Emmenegger et al., 2000; Enzmann et al., 2005; Enzmann et al., 2010; Johansson et al., 2009; Kim et al., 2007; Kolodziejek et al., 2008; Kurath et al., 2003; Nishizawa et al., 2006; Troyer & Kurath, 2003). Within the historical natural range of the virus in western North America, most isolates of IHNV from Pacific salmon form two genogroups that are related to geographical location and not to year of isolation or host species. The isolates within these two genogroups show a relatively low level of nucleotide diversity, suggesting evolutionary stasis or an older host-pathogen relationship. Conversely, isolates of IHNV from farmed rainbow trout in the USA form a third genogroup with more genetic diversity and an evolutionary pattern indicative of ongoing adaptation to a new host or rearing conditions. Isolates from farmed rainbow trout in Europe and Asia appear to have originated from North America, but show further, independent, divergence within their new geographical range (Enzmann et al., 2010; Kim et al., 2007; Nishizawa et al., 2006).

On the basis of antigenic studies using neutralising polyclonal rabbit antisera, IHNV isolates form a single serogroup (Engelking et al., 1991), while mouse monoclonal antibodies have revealed a number of neutralising epitopes on the glycoprotein (Huang et al., 1994; Ristow & Arnzen de Avila, 1991; Winton et al., 1988), as well as the existence of a non-neutralising group epitope borne by the nucleoprotein (Ristow & Arnzen, 1989). However, there appears to be little or no correlation between genotypes and serotypes (Johansson et al., 2009). Variations in the virulence and host preference of IHNV strains have been recorded during both natural cases of disease and in experimental infections (Garver et al., 2006; LaPatra et al., 1993a).

2.1.2. Survival outside the host

IHNV is heat, acid and ether labile. The virus will survive in fresh water for at least 1 month at cooler temperatures, especially if organic material is present.

2.1.3. Stability of the agent (effective inactivation methods)

IHNV is readily inactivated by common disinfectants and drying (Wolf, 1988).

2.1.4. Life cycle

Reservoirs of IHNV are clinically infected fish and covert carriers among cultured, feral or wild fish. Virus is shed via urine, sexual fluids and from external mucus, whereas kidney, spleen and other internal organs are the sites in which virus is most abundant during the course of overt infection (Bootland & Leong, 1999; Wolf, 1988).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*Oncorhynchus keta*), coho salmon (*Oncorhynchus kisutch*), cutthroat trout (*Oncorhynchus clarkii*), lake trout (*Salvelinus namaycush*), masu salmon (*Oncorhynchus masou*), marble trout (*Salmo marmoratus*), pike (*Esox lucius*), rainbow trout and sockeye salmon (*Oncorhynchus nerka*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence of susceptibility according to Chapter 1.5. of the *Aquatic Code* include: Pacific herring (*Clupea pallasii*), shiner perch (*Cymatogaster aggregate*), tube-snout (*Aulorhynchus flavidus*), burbot (*Lota lota*) and white sturgeon (*Acipenser transmontanus*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: all varieties and subspecies of common carp (*Cyprinus carpio*) and American yellow perch (*Perca flavescens*).

2.2.3. Susceptible stages of the host

Infection with IHNV occurs among several species of salmonids with fry being the most highly susceptible stage. Older fish are typically more resistant to clinical disease, but among individuals, there is a high degree of variation in susceptibility to infection with IHNV. As with viral haemorrhagic septicaemia virus, good fish health condition seems to decrease susceptibility to overt infection with IHNV, while co-infections with bacterial diseases (e.g. bacterial coldwater disease), handling and other stressors can cause subclinical infections to become overt. Fish become increasingly resistant to infection with age until spawning, when they once again become highly susceptible and may shed large amounts of virus in sexual products. Survivors of infection with IHNV demonstrate a strong protective immunity with the synthesis of circulating antibodies to the virus (LaPatra et al., 1993b).

2.2.4. Species or subpopulation predilection (probability of detection)

IHNV shows a strong phylogeographic signature (Enzmann et al., 2010; Kurath et al., 2003; Nishizawa et al., 2006) that reflects the host species from which the virus is most commonly isolated in various geographical areas (e.g. sockeye salmon in the Northeast Pacific – U genogroup; Chinook salmon in California, USA – L genogroup; and rainbow trout in Europe, Asia and Idaho, USA – E, J and M genogroups, respectively).

2.2.5. Target organs and infected tissue

Virus entry is thought to occur through the gills and at bases of fins while kidney, spleen and other internal organs are the sites in which virus is most abundant during the course of overt infection (Bootland & Leong, 1999; Wolf, 1988).

2.2.6. Persistent infection with lifelong carriers

Historically, the geographic range of infection with IHNV was limited to western North America, but the disease has spread to Europe and Asia via the importation of infected fish and eggs. Once IHNV is introduced into a farmed stock, the disease may become established among susceptible species of wild fish in the watershed. The length that individual fish are infected with IHNV varies with temperature; however, unlike infectious pancreatic necrosis virus (IPNV) or channel catfish virus (CCV), a true, life-long IHNV carrier state appears to be a rare event.

2.2.7. Vectors

Horizontal transmission of IHNV is typically by direct exposure, but invertebrate vectors have been proposed to play a role in some cases (Bootland & Leong, 1999).

Mayfly (*Callibaetis* sp.) (Shors & Winston, 2003) and salmon lice (*Lepeophtheirus salmonis*) (Jakob et al., 2011) are potential vectors for IHNV.

2.2.8. Known or suspected wild aquatic animal carriers

IHNV is endemic among many populations of free-ranging salmonids. A marine reservoir has been proposed, but not confirmed.

2.3. Disease pattern

Infection with IHNV often leads to mortality due to the impairment of osmotic balance, and occurs within a clinical context of oedema and haemorrhage. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissues, and cells of the kidney underlies the clinical signs.

2.3.1. Transmission mechanisms

The transmission of IHNV between fish is primarily horizontal and high levels of virus are shed from infected juvenile fish, however, cases of vertical or egg-associated transmission have been recorded. Although egg-associated transmission is significantly reduced by the now common practice of surface disinfection of eggs with an iodophor solution, it is the only mechanism accounting for the occurrence of infection with IHNV in new geographical locations among alevins originating from eggs that were incubated and hatched in virus-free water (Winton, 1991).

2.3.2. Prevalence

Infection with IHNV is endemic and widely prevalent among populations of free-ranging salmonids throughout much of its historical range along the west coast of North America. The virus has also become established with a high prevalence of infection in major trout growing regions of North America, Europe and Asia where IHNV was introduced through the movement of infected fish or eggs.

2.3.3. Geographical distribution

Infection with IHNV has been detected in North America, Asia and Europe, but not in the Southern Hemisphere. Countries reporting confirmed or suspect cases of infection with IHNV to the OIE include: Austria, Belgium, Canada, China (People's Rep. of), Croatia, Czech Republic, France, Germany, Iran, Italy, Japan, Korea (Rep. of), Netherlands, Poland, Russia, Slovenia, Spain, Switzerland and United States of America. Infections and overt disease have been reported among fish reared in both fresh and sea water.

2.3.4. Mortality and morbidity

Depending on the species of fish, rearing conditions, temperature, and, to some extent, the virus strain, outbreaks of infection with IHNV may range from explosive to chronic. Losses in acute outbreaks will exceed several per cent of the population per day and cumulative mortality may reach 90–95% or more (Bootland & Leong, 1999). In chronic cases, losses are protracted and fish in various stages of disease can be observed in the pond.

2.3.5. Environmental factors

The most important environmental factor affecting the progress of infection with IHNV is water temperature. Experimental trials have demonstrated infection with IHNV can produce mortality from 3°C to 18°C (Bootland & Leong, 1999); however clinical disease typically occurs between 8°C and 15°C under natural conditions.

2.4. Control and prevention

Control methods for infection with IHNV currently rely on avoidance of exposure to the virus through the implementation of strict control policies and sound hygiene practices (Winton, 1991). The thorough disinfection of fertilised eggs, the use of virus-free water supplies for incubation and rearing, and the operation of facilities under established biosecurity measures are all critical for preventing infection with IHNV at a fish production site.

2.4.1. Vaccination

Experimental vaccines to protect salmonids against infection with IHNV have been the subject of research for more than 40 years with some showing promise in both laboratory and field trials when delivered by immersion or injection (Kurath, 2008; Winton, 1991; Winton, 1997). Both autogenous, killed vaccines and a DNA vaccine have been licensed for commercial use in Atlantic salmon net-pen aquaculture on the west coast of North America where such vaccines can be delivered economically by injection. However, vaccines against infection with IHNV have not yet been licensed in other countries where the application of vaccines to millions of smaller fish will require additional research on novel mass delivery methods.

2.4.2. Chemotherapy

Although chemotherapeutic approaches for control of infection with IHNV have been studied, they have not found commercial use in aquaculture against the disease (Winton, 1991).

2.4.3. Immunostimulation

Immunostimulants are an active area of research, but have not found commercial use in aquaculture against infection with IHNV.

2.4.4. Resistance breeding

Experimental trials of triploid or inter-species hybrids have shown promise (Barroso et al., 2008; Winton, 1991) and the genetic basis of resistance to IHNV has been an active area of recent research (Miller et al., 2004; Purcell et al., 2010).

2.4.5. Restocking with resistant species

Within endemic areas, the use of less susceptible species has been used to reduce the impact of infection with IHNV in aquaculture.

2.4.6. Blocking agents

Natural compounds have been identified from aquatic microbes that have antiviral activity; however, these have not found commercial use in aquaculture against infection with IHNV (Winton, 1991).

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs is a highly effective method to block egg-associated transmission of IHNV in aquaculture settings (Bovo et al., 2005).

The method is widely practiced in areas where the virus is endemic.

2.4.8. General husbandry practices

In addition to disinfection of eggs, use of a virus-free water supply has been shown to be a critical factor in the management of infection with IHNV within endemic areas. Several approaches include use of wells or springs that are free of fish or other sources of IHNV and disinfection of surface water sources using UV light or ozone (Winton, 1991).

3. Sampling

3.1. Selection of individual specimens

Clinical inspections are best carried out during a period whenever the water temperature is below 14°C. All production units (ponds, tanks, net-cages, etc.) must be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention must be paid to the water outlet area where weak fish tend to accumulate.

In farms with salmonids, if rainbow trout are present, only fish of that species are selected for sampling. If rainbow trout are not present, the sample has to be obtained from fish of all other species present that are susceptible to infection with IHNV, as listed in Section 2.2.1. Susceptible species should be sampled proportionally, or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown risk status).

If more than one water source is used for fish production, fish from all water sources must be included in the sample. If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish are selected. If such fish are not present, the fish selected must include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

3.2. Preservation of samples for submission

Before shipment or transfer to the laboratory, parts of the organs to be examined must be removed from the fish with sterile dissection instruments and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. Addition of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used.

3.3. Pooling of samples

Ovarian fluid or organ pieces from a maximum of ten fish may be collected in one sterile tube containing at least 4 ml transport medium and this represents one pooled sample. The tissue in each sample should weigh a minimum of 0.5 g. The tubes should be placed in insulated containers (for instance, thick-walled polystyrene boxes) together with sufficient ice or 'freezer blocks' to ensure chilling of the samples during transportation to the laboratory. Freezing must be avoided. The temperature of a sample during transit should never exceed 10°C and ice should still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen. Virological examination must be started as soon as possible and not later than 48 hours after collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after collection of the material, provided that the material to be examined is protected by transport medium and that the temperature requirements during transportation are fulfilled.

Whole fish may be sent to the laboratory if the temperature requirements during transportation can be fulfilled. Whole fish may be wrapped in paper with absorptive capacity and must be shipped in a plastic bag, chilled as mentioned above. Live fish can also be shipped. All packaging and labelling must be performed in accordance with present national and international transport regulations, as appropriate.

3.4. Best organs or tissues

The optimal tissue material to be examined is spleen, anterior kidney, and either heart or encephalon. In some cases, ovarian fluid and milt must be examined.

In case of small fry, whole fish less than 4 cm long can be minced with sterile scissors or a scalpel after removal of the body behind the gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected. If a sample consisted of whole fish less than 4 cm long, these should be minced with sterile scissors or a scalpel, after removal of the body behind the gut opening. If a sample consisted of whole fish with a body length between 4 cm and 6 cm, the viscera, including kidney, should be collected. If a sample consisted of whole fish more than 6 cm long, tissue specimens should be collected as described above. The tissue specimens should be minced with sterile scissors or a scalpel, homogenised and suspended in transport medium.

3.5. Samples/tissues that are not suitable

IHNV is very sensitive to degradation, therefore sampling tissues with high enzymatic activities or large numbers of contaminating bacteria such as the intestine or skin should be avoided when possible. Muscle tissue is also less useful as it typically contains a lower virus load.

4. Diagnostic methods

The "Gold Standard" for detection of IHNV is the isolation of the virus in cell culture followed by its immunological or molecular identification. While the other diagnostic methods listed below can be used for confirmation of the identity of virus isolated in cell culture or for confirmation of overt infections in fish, they are not approved for use as primary surveillance methods for obtaining or maintaining approved infection with IHNV-free status.

Due to substantial variation in the strength and duration 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 diagnostic method for assessing the viral status of fish populations. In the future, validation of serological techniques for diagnosis of fish virus infections could render the use of fish serology more widely acceptable for diagnostic purposes. However, when present, a positive

serological response is considered presumptive evidence of past exposure to infection with IHNV (Jorgensen et al., 1991).

4.1. Field diagnostic methods

4.1.1. Clinical signs

The disease is typically characterised by gross signs that include lethargy interspersed with bouts of frenzied, abnormal activity, darkening of the skin, pale gills, ascites, distended abdomen, exophthalmia, and petechial haemorrhages internally and externally.

4.1.2. Behavioural changes

During outbreaks, fish are typically lethargic with bouts of frenzied, abnormal activity, such as spiral swimming and flashing. A trailing faecal cast is observed in some species. Spinal deformities are present among some of the surviving fish (Bootland & Leong, 1999).

4.2. Clinical methods

4.2.1. Gross pathology

Affected fish exhibit darkening of the skin, pale gills, ascites, distended abdomen, exophthalmia, and petechial haemorrhages internally and externally. Internally, fish appear anaemic and lack food in the gut. The liver, kidney and spleen are pale. Ascitic fluid is present and petechiae are observed in the organs of the body cavity.

4.2.2. Clinical chemistry

The blood of affected fry shows reduced haematocrit, leukopenia, degeneration of leucocytes and thrombocytes, and large amounts of cellular debris. As with other haemorrhagic viraemias of fish, blood chemistry is altered in severe cases (Bootland & Leong, 1999).

4.2.3. Microscopic pathology

Histopathological findings reveal degenerative necrosis in haematopoietic tissues, kidney, spleen, liver, pancreas, and digestive tract. Necrosis of eosinophilic granular cells in the intestinal wall is pathognomonic of infection with IHNV (Bootland & Leong, 1999).

4.2.4. Wet mounts

Wet mounts have limited diagnostic value.

4.2.5. Tissue imprints and smears

Necrobiotic bodies and foamy macrophages, indicative of a clinical manifestation of infection with IHNV, can be best observed using tissue imprints obtained from the kidney and spleen rather than smears.

4.2.6. Electron microscopy/cytopathology

Electron microscopy of virus-infected cells reveals bullet-shaped virions of approximately 150–190 nm in length and 65–75 nm in width (Wolf, 1988). The virions are visible at the cell surface or within vacuoles or intracellular spaces after budding through cellular membranes. The virion possesses an outer envelope containing host lipids and the viral glycoprotein spikes that react with immunogold staining to decorate the virion surface.

4.3. Agent detection and identification methods

The traditional procedure for detection of IHNV is based on virus isolation in cell culture. Confirmatory identification may be achieved by use of immunological (neutralisation, indirect fluorescent antibody test or enzyme-linked immunosorbent assay), or molecular (polymerase chain reaction, DNA probe or sequencing) methods (Arakawa et al., 1990; Arnzen et al., 1991; Deering et al., 1991; Dixon & Hill, 1984; Jorgensen et al., 1991; LaPatra et al., 1989; Purcell et al., 2006; Winton & Einer-Jensen, 2002).

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Wet mounts are not appropriate for detection or identification of IHNV.

4.3.1.1.2. Smears

Smears are not appropriate for detection or identification of IHNV.

4.3.1.1.3. Fixed sections

Immunohistochemistry and *in-situ* hybridisation (ISH) methods have been used in research applications, but are not appropriate for detection or identification of IHNV in a diagnostic setting.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Cell lines to be used: EPC or FHM.

Detection of virus through the development of viral cytopathic effect (CPE) in cell culture would be followed by virus identification through either antibody-based tests or nucleic acid-based tests. Any antibody-based tests would require the use of antibodies validated for their sensitivity and specificity.

4.3.1.2.1.1. Virus extraction

In the laboratory the tissue in the tubes must be completely homogenised (either by stomacher, blender mortar and pestle with sterile sand or any other suitable and validated homogeniser) and subsequently suspended in the original transport medium. The final ratio between tissue material and transport medium must be adjusted in the laboratory to 1:10.

The homogenate is centrifuged in a refrigerated centrifuge at 2°C–5°C at 2000–4000 *g* for 15 minutes and the supernatant collected and treated for either four hours at 15°C or overnight at 4°C with antibiotics (e.g. 1 mg ml⁻¹ gentamicin may be useful at this stage). If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted. The antibiotic treatment aims at controlling bacterial contamination in the samples and makes filtration through membrane filters unnecessary.

Where practical difficulties arise (e.g. incubator breakdown, problems with cell cultures, etc.), which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples, it is acceptable to freeze the supernatant at –80°C and carry out virological examination within 14 days. If the collected supernatant is stored at –80°C within 48 hours after the sampling it may be reused only once for virological examination.

Optional treatment of homogenate to inactivate competing virus: treatment of inocula with antiserum to IPNV (which in some parts of the world occurs in 50% of fish samples) aims at preventing CPE due to IPNV from confounding the ability to detect IHNV in cell culture. When samples come from production units, which are considered free from IPN, treatment of inocula with antiserum to IPNV should be omitted. Prior to the inoculation of the cells, the supernatant is mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV 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 1/2000 in a 50% plaque neutralisation test.

4.3.1.2.1.2. Inoculation of cell monolayers

EPC or FHM cells are grown at 25°C in suitable medium, e.g. Eagle's MEM (or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris/HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7.6 ± 0.2. Cell cultures to be used for inoculation with tissue material should be young (4–48 hours old) and actively growing (not confluent) at inoculation.

Antibiotic-treated organ suspension is inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and, in addition, a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively, (in order to prevent homologous interference). 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 cm² cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are acceptable as well.

4.3.1.2.1.3. Incubation of cell cultures

Inoculated cell cultures are incubated at 15°C for 7–10 days. If the colour of the cell culture medium changes from red to yellow, indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances has to be performed to maintain cell susceptibility to virus infection.

At least every 6 months or if decreased cell susceptibility is suspected, titration of frozen stocks of IHNV is performed to verify the susceptibility of the cell cultures to infection.

4.3.1.2.1.4. Microscopy

Inoculated cell cultures must be inspected regularly (at least three times a week) for the occurrence of CPE at 40–150 × magnification. The use of a phase-contrast microscope is recommended. If obvious CPE is observed, virus identification procedures have to be initiated immediately.

4.3.1.2.1.5. Subcultivation

If no CPE has developed after the primary incubation for 7–10 days, subcultivation is performed to fresh cell cultures utilising a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to the cell line 7–10 days after inoculation. The pools are then inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:10 and 1:100, respectively, of the supernatant) as described in Section 4.3.1.2.1.2. above.

Alternatively, aliquots of 10% of the medium constituting the primary culture are inoculated directly into a well with fresh cell culture (well-to-well subcultivation). In case of salmonid samples, the inoculation may be preceded by preincubation of the dilutions with the antiserum to IPNV at an appropriate dilution as described above.

The inoculated cultures are then incubated for 7–10 days at 15°C with observation as in Section 4.3.1.2.1.4. If toxic CPE occurs within the first three days of incubation, subcultivation may be performed at that stage, but the cells must then be incubated for seven days and subcultivated again with a further seven days incubation. When toxic CPE develops after 3 days, the cells may be passed once and incubated to achieve the total of 14 days from the primary inoculation. There should be no evidence of toxicity in the final seven days of incubation.

If bacterial contamination occurs, despite treatment with antibiotics, subcultivation must be preceded by centrifugation at 2000–4000 *g* for 15–30 minutes at 2–5°C, and/or filtration of the supernatant through a 0.45 µm filter (low protein-binding membrane). In addition to this, subcultivation procedures are the same as for toxic CPE.

If no CPE occurs the test may be declared negative.

4.3.1.2.2. Antibody-based antigen detection methods

4.3.1.2.2.1. Neutralisation test (identification in cell culture)

- i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge an aliquot at 2000 *g* for 15 minutes at 4°C, or filter through a 0.45 µm (or 450 nm) pore membrane to remove cell debris.
- ii) Dilute virus-containing medium from 10²–10⁴.
- iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of an IHNV antibody solution. The neutralising antibody (Nab) solution must have a 50% plaque reduction titre of at least 2000. Likewise, treat a set of aliquots of each virus dilution with cell culture medium to provide a non-neutralised control.

- iv) In parallel, a neutralisation test must be performed against a homologous IHN virus strain (positive neutralisation test) to confirm the reactivity of the antiserum.
- v) Incubate all the mixtures at 15°C for 1 hour.
- vi) Transfer aliquots of each of the above mixtures on to 24-hour-old monolayers overlaid with cell culture medium containing 10% FBS (inoculate two wells per dilution) and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.
- vii) Check the cell cultures for the onset of CPE and read the results for each suspect IHN virus sample as soon as it CPE occurs in non-neutralised controls. Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.
- viii) The tested virus is identified as IHN virus when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the IHN virus-specific antibody, whereas CPE is evident in all other cell cultures.

Other neutralisation tests of proven efficiency may be used alternatively.

4.3.1.2.2.2. Indirect fluorescent antibody test (IFAT)

Antibody-based antigen detection methods such as IFAT, ELISA and various immunohistochemical procedures for the detection of IHN virus have been developed over the years. These techniques can provide detection and identification relatively quickly compared with virus isolation in cell culture. However, various parameters such as antibody sensitivity and specificity and sample preparation can influence the results; a negative result should be viewed with caution. These techniques should not be used in attempts to detect carrier fish.

4.3.1.2.2.2.1. Indirect fluorescent antibody test in cell cultures

- 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 FBS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of black 96-well plates for immunofluorescence is recommended.
- 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 IHN virus in a similar way, in order to obtain a virus titre of about 5,000–10,000 plaque-forming units (PFU) per ml in the cell culture medium.
- iv) Incubate at 15°C for 24 hours.
- v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with a cold mixture of acetone 30%/ethanol 70% (v/v) (stored at –20°C).
- 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 IHN virus antibody or serum in 0.01 M 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 (e.g. by adding a piece of wet cotton to the humid chamber). 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 FITC- or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibody to the

immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These conjugated 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, for example, glycerol saline, pH 8.5 prior to microscopic observation.
- xv) Examine under incident UV light using a microscope with $\times 10$ eye pieces and $\times 20$ – 40 objective lens having numerical aperture >0.65 and >1.3 , respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.3.1.2.2.2.2. *Indirect fluorescent antibody test on imprints*

- 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 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.
- vi) Rehydrate the above preparations and block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C .
- vii) Rinse four times with PBST.
- viii) Treat the imprints with the solution of antibody to IHNV and rinse.
- ix) Block and rinse.
- x) Reveal the reaction with suitable fluorescein isothiocyanate (FITC)-conjugated specific antibody, rinse and observe.
- xi) If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture, as described above.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of proven efficiency may be used alternatively.

4.3.1.2.2.3. *Enzyme-linked immunosorbent assay (ELISA)*

- i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for IHNV, in 0.01 M PBS, pH 7.2 (200 μl /well).
- ii) Incubate overnight at 4°C .
- iii) Rinse four times with 0.01 M PBS containing 0.05% Tween-20 (PBST).
- iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 μl /well).
- v) Rinse four times with PBST.
- vi) Add 2% Triton X-100 to the virus suspension to be identified.
- vii) Dispense 100 μl /well of two- or four-step dilutions of the virus to be identified and of IHNV control virus, and a heterologous virus control (e.g. viral haemorrhagic septicaemia virus). Allow the samples to react with the coated antibody to IHNV for 1 hour at 20°C .
- viii) Rinse four times with PBST.
- ix) Add to the wells either biotinylated polyclonal IHNV antiserum or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.
- x) Incubate for 1 hour at 37°C .
- xi) Rinse four times with PBST.
- xii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C .
- xiii) Rinse four times with PBST. Add the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.

- xiv) Interpretations of the results is according to the optical absorbencies achieved by negative and positive controls and must follow the guidelines for each test, e.g. absorbency at 450 nm of positive control must be minimum 5–10 × A450 of negative control.

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

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Polymerase chain reaction

4.3.1.2.3.1.1. Viral RNA preparation

Total RNA from infected cells is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially-available RNA isolation kit used according to the manufacturer's instructions. While all of these methods work well for drained cell monolayers or cell pellets, RNA binding to affinity columns can be affected by salts present in tissue culture media and phase-separation methods should be used for extraction of RNA from cell culture fluids.

4.3.1.2.3.1.2. Reverse-transcription (RT) and standard PCR protocol

- i) Prepare a master mix for the number of samples to be analysed. Work under a hood and wear gloves.
- ii) The master mix for one 50 µl reverse-transcription PCR is prepared as follows: 23.75 µl ribonuclease-free (DEPC-treated) or molecular biology grade water; 5 µl 10 × buffer; 5 µl 25 mM MgCl₂; 5 µl 2 mM dNTP; 2.5 µl (20 pmoles µl⁻¹) Upstream Primer 5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3'; 2.5 µl (20 pmoles µl⁻¹) Downstream Primer 5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'; 0.5 µl *Taq* polymerase (5 U µl⁻¹); 0.5 µl AMV reverse transcriptase (9 U µl⁻¹); 0.25 µl RNasin (39 U µl⁻¹).
- iii) Centrifuge the tubes briefly (10 seconds) to make sure the contents are at the bottom.
- iv) Place the tubes in the thermal cycler and start the following cycles – 1 cycle: 50°C for 30 minutes; 1 cycle: 95°C for 2 minutes; 30 cycles: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; 1 cycle: 72°C for 7 minutes and soak at 4°C.
- v) Visualise the 693 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

NOTE: These PCR primers target a central region of the IHNV G gene (Emmenegger et al., 2000). While other primer sets can be used for amplification of portions of the N or G genes of IHNV (Winton & Einer-Jensen, 2002), the primer sequences listed above have been shown to be conserved among a broad range of IHNV isolates and are not present in the G gene of the related fish rhabdoviruses, viral haemorrhagic septicaemia virus or hirame rhabdovirus. Additionally, the new primers produce an amplicon that can be used as a template for sequence analysis of the 'mid-G' region of the IHNV genome for epidemiological purposes (Emmenegger et al., 2000; Kurath et al., 2003).

4.3.1.2.3.2. Other amplification-based assays

Other methods to detect IHNV based on amplification of target sequences of genomic or messenger RNA have been developed that use a loop-mediated isothermal amplification (LAMP) method (Gunimaladevi et al., 2005) or a highly sensitive quantitative reverse-transcriptase PCR assay (Overturf et al., 2001). However, these assays have not yet undergone sufficient laboratory validation using a panel of isolates representing the various IHNV genotypes to make them suitable for listing as a confirmatory method.

4.3.1.2.3.3. Sequencing

Sequence analysis of PCR amplicons has become much more rapid and less costly in recent years and is a good method for confirmation of IHNV (Winton & Einer-Jensen, 2002). In addition, sequence analysis provides one of the best approaches for identification of genetic strains and for epidemiological tracing of virus movement (Emmenegger et al., 2000; Kim et al., 2007; Kurath et al., 2003; Nishizawa et al., 2006).

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of infection with IHNV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Gametes	Fry	Juveniles	Adults		
Gross signs	d	c	c	d	b	d
Virus isolation	a	a	a	a	a	c
Direct LM	d	c	d	d	b	c
Histopathology	d	c	d	d	b	c
Transmission EM	d	d	d	d	b	c
Antibody-based assays	d	c	c	c	a	b
PCR assays	c	c	c	c	a	a
Sequencing	d	d	d	d	c	a

LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infectious haematopoietic necrosis

The method for targeted surveillance to declare freedom from infection with IHNV is isolation of virus in cell culture. For this purpose, the most susceptible stages of the most susceptible species should be examined. Reproductive fluids and tissues collected from adult fish of a susceptible species at spawning should be included in at least one of the sampling periods each year.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case is defined as the presence of typical, gross clinical signs of the disease in a population of susceptible fish, OR a typical internal histopathological presentation among susceptible species, OR detection of antibodies against IHNV in a susceptible species, OR typical cytopathic effect in cell culture without identification of the agent, OR a single positive result from one of the diagnostic assays ranked as 'a' or 'b' in Table 5.1.

7.2. Definition of confirmed case

A confirmed case is defined as a suspect case that has EITHER: 1) produced typical cytopathic effect in cell culture with subsequent identification of the agent by one of the antibody-based or molecular tests listed in Table 5.1., OR: 2) a second positive result from a different diagnostic assay ranked as 'a' or 'b' in the last column of Table 5.1.

8. References

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NB: There are OIE Reference Laboratories for infectious haematopoietic necrosis (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: <http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>). Please contact the OIE Reference Laboratories for any further information on infectious haematopoietic necrosis.

NB: FIRST ADOPTED IN 1995 AS INFECTIOUS HAEMATOPOIETIC NECROSIS;
MOST RECENT UPDATES ADOPTED IN 2019.

