

EPIZOOTIC HAEMATOPOIETIC NECROSIS

1. Scope

For the purpose of this chapter, epizootic haematopoietic necrosis is considered to be systemic clinical or subclinical infection of finfish with epizootic haematopoietic necrosis virus (EHNV).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

EHNV is a member of the genus *Ranavirus* in the family Iridoviridae with the type species Frog virus 3 (FV3) (7). Other species include Bohle virus (BIV), European catfish virus (ECV), European sheatfish virus (ESV) and Santee-Cooper ranavirus. Caution should be taken when speaking of ECV and ESV as two separate viruses because the scientific literature (17) indicates they are isolates of the same virus. There are many other tentative species in this genus. Ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (8, 10, 12, 18, 29, 38, 39). Ranaviruses have large (150–180 nm), icosahedral virions, a double-stranded DNA genome 150–170 kb, and replicate in both the nucleus and cytoplasm with cytoplasmic assembly (7). They possess common antigens that can be detected by several techniques.

Since the recognition of disease due to EHNV in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in farmed fish. These include catfish (*Ictalurus melas*) in France (ECV) (26), sheatfish (*Silurus glanis*) in Germany (ESV) (2, 3), turbot (*Scophthalmus maximus*) in Denmark (5) and others in Finland (4, 31).

EHNV and ECV are distinct viruses that can be differentiated using genomic analysis (1, 17, 23–25). This enables epidemiological separation of disease events in finfish in Australia (EHNV) and Europe (ECV), and differentiation of these from ranavirus occurrences in frogs (FV3 and BIV). However, many ranavirus isolates have not been characterised to this level.

2.1.2. Survival outside the host

EHNV is extremely resistant to drying and, in water, can survive for months (19). It can persist in frozen fish tissues for more than 2 years (19) and frozen fish carcasses for at least a year (33). For these reasons, it is presumed that EHNV would persist for months to years on a fish farm in water and sediment, as well as on plants and equipment.

2.1.3. Stability of the agent (effective inactivation methods)

EHNV is susceptible to 70% ethanol, 200 mg litre⁻¹ sodium hypochlorite or heating to 60°C for 15 minutes (19).

2.1.4. Life cycle

The route of infection is unknown but fish are susceptible experimentally following bath exposure. The virus infects a range of cell types including hepatocytes, haematopoietic cells and endothelial cells in many organs (27). Virus is shed into water from infected tissues and carcasses as they disintegrate.

2.2. Host factors

2.2.1. Susceptible host species

Natural EHNV infections are known from only two teleost species, redbfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) (19, 20–22), however, a number of other finfish species are susceptible to EHNV experimentally. Individuals of the following species have died after bath inoculation: Macquarie perch (*Macquaria australasica*), silver perch (*Bidyanus bidyanus*), mosquito fish (*Gambusia affinis*) and mountain galaxias (*Galaxias olidus*). Some species, for example goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*), are resistant.

2.2.2. Susceptible stages of the host

Susceptible stages of the host are all age classes of rainbow trout and redfin perch.

2.2.3. Species or subpopulation predilection (probability of detection)

Clinical signs are usually more obvious in fingerlings and juvenile fish than adults of both rainbow trout and redfin perch.

2.2.4. Target organs and infected tissue

Target organs and tissues infected with the virus are: liver, kidney, spleen and other parenchymal tissues. It is not known if EHNV can be detected in gonadal tissues, ovarian fluid or milt or whether these tissues are suitable for surveillance of broodstock.

2.2.5. Persistent infection with lifelong carriers

Rainbow trout: the high case fatality rate and low prevalence of EHNV infection in rainbow trout means that the recruitment rate of carriers is likely to be very low (<2%) (34). Persistent infection with very small numbers of infectious virions was detected in one clinically healthy rainbow trout fingerling 63 days after intraperitoneal inoculation (35), but the significance of this observation is unclear because of the artificial route of infection. EHNV has been detected in grower fish, but as histopathological lesions consistent with EHNV were also present there was active infection rather than a carrier state (36). Too few broodstock samples have been examined to be certain that broodstock are not infected (34). Anti-EHNV serum antibodies were not detected in 0+ fingerlings during or after an outbreak but were detected in a low proportion of 1+ to 2+ grower fish, hence, it is uncertain whether these were survivors of the outbreak (34, 36).

Redfin perch: this species is extremely susceptible to EHNV and it seems unlikely that it is a suitable reservoir host (35). However, there is some conflicting evidence. EHNV, or a related ranavirus, was isolated from 2 of 40 apparently healthy adult redfin perch during epizootics in juveniles in Victoria, Australia (20), but as the incubation period extends for up to 28 days (35), these fish may have been in the preclinical phase. Several ranavirus isolates have been obtained from redfin perch in Victoria at times when there was no obvious epizootic, and some apparently healthy redfin perch in Victoria had serum antibodies against EHNV or a related virus (Whittington & Hyatt, unpublished data).

Murray cod: this species may be a suitable carrier as infection without disease has occurred after bath inoculation (19).

Rainbow trout and Atlantic salmon: these species may be a suitable carrier as infection without disease has occurred after intraperitoneal or bath inoculation (19)

2.2.6. Vectors

Since EHNV is a resistant virus, it may be transferred on nets, boats and other equipment, or in fish used for bait by recreational fishers. Birds are potential mechanical vectors for EHNV, it being carried in the gut, on feathers, feet and the bill. Piscivorous birds feed on affected juvenile redfin perch and the gastrointestinal contents of these birds may contain EHNV (33). However, the virus is likely to be inactivated at typical avian body temperatures (40–44°C). Nevertheless, spread of EHNV by regurgitation of ingested material within a few hours of feeding is possible (33).

2.2.7. Known or suspected wild aquatic animal carriers

None known.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Rainbow trout: EHNV has spread between rainbow trout farms by transfer of infected fingerlings and probably transport water (21, 34, 36). It is assumed that consignments of fish contain a low proportion of individuals with progressive subclinical or clinical infection, rather than carrier fish. The low prevalence of infection in rainbow trout means that active infection can easily go unrecognised in a population and be spread by trading fish. There are no data on possible vertical transmission of EHNV on or within ova, and disinfection protocols for ova have not been evaluated. EHNV has not yet been isolated from ovarian tissues or from broodstock. Annual recurrence in farmed rainbow trout may be due to reinfection of successive batches of fish from wild redfin perch present in the same catchment.

Redfin perch: The occurrence of EHNV in redfin perch in widely separated river systems and impoundments, and its upstream progression, indicates that EHNV is spread by means other than water; mechanisms include

translocation of live fish or bait by recreational fishers. Redfin perch migrations in Australia are uncertain (see also Section 2.2.6 Vectors).

2.3.2. Prevalence

Rainbow trout: the disease is generally difficult to identify with very low mortality rates and EHNV may be present on a farm without causing suspicion. During outbreaks, EHNV has been detected by virus isolation in 60–80% of moribund or dead fish, but in only 0–4% of in-contact, clinically healthy fish. The 99% confidence limits for the prevalence of subclinical infection are 0–8% (34, 36). The virus could not be found at all in surviving cohorts after an outbreak. Anti-EHNV antibodies were detected in grower fish at low prevalence (0.7%, 95% confidence limits 0.02% to 3.7%).

Redfin perch: the disease is recognised by epizootic mortality in fish of any age affecting a very large proportion of the population with dramatic population decline (20, 22, 33). Typically, fingerling and juvenile fish are affected in endemic areas, but in newly infected areas adults are also affected. When the disease is first recognised in an area there is a dramatic population decline (20, 22, 33).

2.3.3. Geographical distribution

Rainbow trout: infection with EHNV is known only from fish farms located in the Murrumbidgee and Shoalhaven river catchments in New South Wales, Australia. However, some farms within this region have remained free of the disease (36).

Redfin perch: EHN is endemic in south-eastern Australia, but there is a discontinuous distribution. The disease occurs in many small and large impoundments in Victoria and since 1986 has spread progressively upstream in the Murrumbidgee river catchment through New South Wales and the Australian Capital Territory. Similar spread has been observed in the Murray River in South Australia (33).

2.3.4. Mortality and morbidity

Rainbow trout: It appears that EHNV is poorly infective but has a high case fatality rate. EHNV may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate. EHNV has most often been reported in young fingerlings <125 mm fork length with daily mortality of less than 0.2% and total mortality of up to 4%. However, rainbow trout of all ages may be susceptible, although infection has not yet been seen in broodstock (34, 36). There is a low direct economic impact because of the low mortality rate. In keeping with the natural pattern of disease, rainbow trout were resistant to bath exposure in $10^{2.2}$ TCID₅₀ (50% tissue culture infective dose) ml⁻¹ (35), while only 1 of 7 became infected after bath inoculation for 1 hour in 10^3 TCID₅₀ ml⁻¹ (21).

Redfin perch: There is a very high rate of infection and mortality in natural outbreaks that, over time, leads to loss in wild fish populations (20, 22, 33). Experimental bath inoculation with as few as 0.08 TCID₅₀ ml⁻¹ was lethal, and doses too low to be detected by virus isolation in BF-2 cells were fatal by intraperitoneal inoculation (35).

2.3.5. Environmental factors

Rainbow trout: Natural outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. Water quality parameters are suboptimal, and intercurrent diseases, including skin diseases caused by protozoa and fungi, and systemic bacterial infection are common. Damage to skin may provide a route of entry for EHNV. Outbreaks have been seen on farms at water temperatures ranging from 11 to 20°C (34, 36). The incubation period after intraperitoneal inoculation was 3–10 days at 19–21°C compared with 14–32 days at 8–10°C (35).

Redfin perch: Natural epizootics of EHNV affecting juvenile and adult redfin perch occur mostly in summer (20, 22, 34). It has been assumed that the disease in juvenile fish is related to the annual appearance of large numbers of non-immune young fish and their subsequent exposure to the virus while schooling in shallow waters; adults are uncommonly involved in these outbreaks. It is possible that environmental temperature is the trigger for outbreaks as juvenile fish feed in warm shallow waters on planktonic fauna, whereas adults feed on benthic invertebrates and larger prey in deeper cooler water (35). Experimentally the incubation period ranged from 10 to 28 days at 12–18°C compared with 10–11 days at 19–21°C, and adult perch were refractory to infection at temperatures below 12°C (35).

2.4. Control and prevention

2.4.1. Vaccination

None available.

2.4.2. Chemotherapy

None available.

2.4.3. Immunostimulation

Not tested.

2.4.4. Resistance breeding

Not tested.

2.4.5. Restocking with resistant species

Not tested.

2.4.6. Blocking agents

Not tested.

2.4.7. Disinfection of eggs and larvae

Not tested.

2.4.8. General husbandry practices

Disease control in rainbow trout at the farm level relies on reducing the impact of infection by maintaining low stocking rates and adequate water quality. The mechanism of protection may be through maintenance of healthy integument.

3. Sampling**3.1. Selection of individual specimens**

A simple method for preparation of fish tissues for cell culture and enzyme-linked immunosorbent assay (ELISA) has been validated (37).

Bath large fish for 30 seconds in 70% ethanol; bath fingerlings for 5 seconds in 70% ethanol then rinse in sterile water. Dissect fish aseptically in a Class II biosafety cabinet.

Large fish (>60 mm fork length): remove 0.1 g liver, kidney, spleen (\pm other organs in specific situations) and place in sterile 1.5 ml tubes. Tubes suitable for use with pestles for grinding tissues (see below) are available, but standard 1.5 ml tubes may be suitable. In some situations liver, kidney and spleen may be pooled in a single tube (see Section 3.3).

Medium fish (30–60 mm fork length): scrape all viscera into the tube.

Small fish (<30 mm fork length): remove head and tail, place rest of fish into the tube.

3.2. Preservation of samples for submission

For cell culture and ELISA, freeze tubes containing tissues at -20°C to -80°C until needed.

For light microscopic examination, fix tissues in 10% neutral buffered formalin.

3.3. Pooling of samples

The effect of pooling tissues from multiple fish on the sensitivity of diagnostic tests has not been evaluated. However, tissues for virus isolation are commonly pooled in lots of 5 or 10 individual fish per test.

3.4. Best organs or tissues

Liver, anterior kidney, spleen.

3.5. Samples/tissues that are not appropriate

Inappropriate tissues include gonads, gonadal fluids, milt and ova, since there is no evidence of reproductive tract infection and broodstock are not known to participate in an infection cycle.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

There are no specific clinical signs. Fish are found dead. There may be clinical evidence of poor husbandry practices, such as overcrowding and suboptimal water quality manifesting as skin, fin and gill lesions (27).

4.1.2. Behavioural changes

Moribund fish may have loss of equilibrium, flared opercula and may be dark in colour (27).

4.2. Clinical methods

4.2.1. Gross pathology

There may be no gross lesions or nonspecific lesions on the skin, fins and gill. A small proportion of fish may have enlargement of kidney, liver or spleen. There may be focal white to yellow lesions in the liver corresponding to areas of necrosis (27).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Acute focal, multifocal or locally extensive coagulative or liquefactive necrosis of liver, haematopoietic kidney and spleen are commonly seen in routine haematoxylin and eosin (H&E)-stained sections of formalin-fixed material. A small number of basophilic intracytoplasmic inclusion bodies may be seen, particularly in areas immediately surrounding necrotic areas in the liver and kidney. Necrotic lesions may also be seen in heart, pancreas, gastrointestinal tract, gill and pseudobranch (27).

4.2.4. Wet mounts

Not applicable.

4.2.5. Smears

Not tested.

4.2.6. Electron microscopy/cytopathology

Affected tissues (e.g. kidney liver and spleen) contain cells exhibiting necrosis. Cells contain conspicuous cytoplasmic inclusions that are rarefied areas of the cytoplasm in which the viruses are assembled. Within the cytoplasm, aggregates (paracrystalline arrays) of large (175 nm ± 6 nm) nonenveloped icosahedral viruses are apparent; single viruses are also present. Complete viruses (containing electron-dense cores) bud/egress from the infected cells through the plasma membrane. The nuclei of infected cells are frequently located peripherally and are distorted in shape.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

Light microscopy: routine methods can be used for tissue fixation in 10% buffered neutral formalin, paraffin embedding, preparation of 10 µm sections and staining with H&E to demonstrate tissue necrosis and basophilic intracytoplasmic inclusion bodies. These inclusion bodies are indicative but not confirmatory for EHN. Formalin-fixed paraffin-embedded sections can also be stained using an immunoperoxidase method (see below) to identify EHN antigen associated with necrotic lesions.

Electron microscopy: Ultrathin routine sectioning methods can be used for preparation of tissues and cell cultures (11) to demonstrate tissue necrosis, presence of viruses and virus inclusion bodies. Tissues and cells fixed with an alternative fixation and embedding regime can be used for antigen detection (15).

Negative contrast electron microscopy: supernatants from dounce homogenised tissues (10% [w/v]) and cell cultures can be used to detect viruses. Ranaviruses have a definitive appearance. They vary in diameter (150–180 nm) and have a limiting cell-derived (plasma membrane) envelope that surrounds a capsid of skewed symmetry. Underlying the capsid is a *de novo* membrane that itself surrounds a core containing the double-stranded (ds) DNA and minor proteins. These preparations can also be used to confirm ranavirus antigenicity (11).

4.3.1.1.1. Wet mounts

Not applicable.

4.3.1.1.2. Smears

Not applicable.

4.3.1.1.3. Fixed sections

See Section 4.3.1.1 on microscopic methods.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Preparation of fish tissues for virus isolation and ELISA

A simple method for preparation of fish tissues for cell culture and ELISA has been validated (37) (see sampling Section 3.1).

- i) Freeze tubes containing tissues at -80°C until needed.
- ii) Add 0.5 ml of homogenising medium (minimal essential medium Eagle, with Earle's salts with glutamine) [MEM] with 200 International Units [IU] ml^{-1} penicillin, 200 $\mu\text{g ml}^{-1}$ streptomycin and 4 $\mu\text{g ml}^{-1}$ amphotericin B) to each tube. Grind tissue to a fine mulch with a sterile fitted pestle.
- iii) Add another 0.5 ml of homogenising medium to each tube and mix with a pestle.
- iv) Add three sterile glass beads to each tube (3 mm diameter) and close the lid of the tube.
- v) Vortex the suspension vigorously for 20–30 seconds and place at 4°C for 2 hours.
- vi) Vortex the suspension again as above and centrifuge for 10 minutes at 2500 *g* in a benchtop microcentrifuge.
- vii) Transfer the supernatant, now called clarified tissue homogenate, to a fresh sterile tube. Homogenates may be frozen at -80°C until required for virus isolation and ELISA.

Cell culture/artificial media

Cell culture is the gold-standard test but is costly and time consuming. EHNV grows well in many fish cell lines including BF-2 (bluegill fry ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (*epithelioma papulosum cyprini* [13]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (9), but BF-2 are preferred by the reference laboratory where an incubation temperature of 22°C both before and after inoculation with virus is used. The procedure for BF-2 cells is provided below. A procedure for CHSE-214 cells is provided under immunoperoxidase staining below (Section 4.3.1.2.2).

The identity of viruses in cell culture is determined by immunostaining, ELISA, immuno-electron microscopy, polymerase chain reaction (PCR) or other methods.

Samples: tissue homogenates.

Cell culture technical procedure: cells are cultured (in flasks, tubes or multi-well plates) with growth medium (MEM + 10% foetal calf serum [FCS] with 100 IU ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin and 2 $\mu\text{g ml}^{-1}$ amphotericin B). The cells are incubated until almost confluent at 22°C , which can take up to 4 days depending on the seeding rate. Medium is changed to a maintenance medium (MEM with 2% FCS and 100 IU ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin and 2 $\mu\text{g ml}^{-1}$ amphotericin B) on the day of inoculation. A 1/10 dilution using homogenising medium is made of single or pooled homogenates. Each culture is inoculated with 100 μl of sample per ml of culture medium. This represents a 1/100 dilution of a 0.1 mg ml^{-1} tissue homogenate. One culture is inoculated with undiluted homogenate, and two with 1/10 homogenate. No adsorption step is used. As an alternative,

two to three cultures can be inoculated directly with 10 µl undiluted homogenate per ml of culture medium. Note that a high rate of cell toxicity or contamination often accompanies the use of a large undiluted inoculum. The cultures are incubated at 22°C in an incubator for 6 days. Cultures are read at day 3 and day 6. Cultures are passed at least once to detect samples with low levels of virus. On day 6, the primary cultures (P1) are frozen overnight at –20°C, thawed, gently mixed and then the culture supernatant is inoculated onto fresh cells as before (P2), i.e. 100 µl P1 supernatant per ml culture medium. Remaining P1 supernatants are transferred to sterile 5 ml tubes and placed at 4°C for testing by ELISA or PCR or another means to confirm the cause of cytopathic effect (CPE) as EHN. P2 is incubated as above, and a third pass is conducted if necessary.

Interpretation of results

CPE is well developed and consists of focal lysis surrounded by rounded granular cells. This change extends rapidly to involve the entire monolayer, which detaches and disintegrates.

4.3.1.2.2. Antibody-based antigen detection methods

It should be noted that antibodies used in all related methods (immunoperoxidase, antigen-capture ELISA and immunoelectron microscopy) cross-react with all known ranaviruses (17).

4.3.1.2.2.1. Detection of EHN using immunoperoxidase staining of infected cell cultures

Principle of the test: EHN replicates within cultured cells. The addition of a mild detergent permeabilises the cells allowing an affinity purified rabbit antibody to bind to intracellular viral proteins. EHN is detected by a biotinylated anti-species antibody and a streptavidin–peroxidase conjugate. The addition of a substrate results in ‘brick-red’ staining in areas labelled with antibodies.

Samples: tissue homogenates.

Operating characteristics: when performed as described in this protocol, the staining is conspicuous and specific. However, the test has not been validated with respect to sensitivity or reproducibility.

Preparation of cells: the procedure described below is for CHSE-214 cells. Other recommended cell lines can also be used.

- i) CHSE-214, 24-well plates are seeded the day before use with 250,000 cells/well (or 4 million cells in 40 ml of growth medium per plate) in 1.5 ml of growth medium (Earle’s MEM with non-essential amino acids [EMEM], 10% FCS, 10 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid [HEPES], 2 mM glutamine, 100 IU penicillin and 100 µg streptomycin) and incubated in 5% CO₂ at 22°C overnight. (NOTE: cultures must be nearly confluent and have healthy dividing cells prior to use.)
- ii) Discard the medium, inoculate each well with 150 µl of a 10% suspension of ground tissue (e.g. liver, kidney or spleen), incubate for 1 hour (22°C) then add 1.5 ml of fresh maintenance medium (as for growth medium except 2% FCS) and return to the incubator (22°C).
- iii) Observe cultures for CPE. If no CPE occurs by day 10, pass the cultures on to fresh CHSE cells by collecting the cells and medium and adding 150 µl to the cells of the fresh plate; note that cells are not freeze–thawed. There is no need to discard the existing medium, just return the new plate to the incubator (22°C). Again, observe daily for CPE.
- iv) Fix cells (add 50 µl for 96-well plate cultures with 200 µl culture medium/well or 400 µl (for 24-well plate cultures with 1.6 ml culture medium/well) of a 20% formalin solution to each well), without discarding the culture medium when CPE is first observed. After incubation (22°C) for 1 hour at room temperature (RT), the medium/formalin mixture is discarded and the wells are rinsed twice with PBS-A (phosphate buffered saline, Ca⁺⁺ and Mg⁺⁺ free) to remove the formalin. More PBS-A is added if the plates are to be stored at 4°C.

Protocol

- i) Dilute primary anti-EHN antibody and normal serum to working strength as described below (fixation protocol for immunocytochemistry) for the relevant agent in 1% skim milk (SM) solution (PBS-A [SM]) to the volume required for the test.
- ii) Remove PBS-A from wells (with fixed cell cultures) and wash wells twice with 0.05% (v/v) PBS/Tween 20 (PBST). Add 50 µl of primary antibody solutions to each well in a 96-well plate well or 200 µl in a 24-well plate well. Incubate on a plate shaker at 100–200 rpm at RT (22–24°C) for 15–30 minutes or without shaking at 37°C for 1 hour.

- iii) Dilute biotinylated anti-species serum (secondary antibody) in 0.1% SM solution as described in the fixation protocol (below) for the relevant agent to the volume required for the test.
- iv) Remove primary antibody solution and wash wells three times with PBST. Add secondary antibody to all wells. Incubate on a plate shaker at 100–200 rpm at RT for 15–30 minutes or without shaking at 37°C for 1 hour.
- v) Dilute streptavidin–peroxidase conjugate in 0.1% SM solution for the relevant agent to the volume required for the test.
- vi) Remove secondary antibody from wells and wash wells three times with PBST. Add conjugate to each well. Incubate on a plate shaker at 100–200 rpm at RT for 15–30 minutes or without shaking at 37°C for 1 hour.
- vii) Prepare stock substrate of 3-amino-9-ethylcarbazole (AEC) solution: dissolve one AEC tablet (20 mg) in 2.5 ml of dimethyl formamide.
- viii) Remove conjugate from wells. Wash (three times) with PBST.
- ix) Dilute dissolved AEC stock in 47.5 ml of acetate buffer (4.1 ml anhydrous sodium acetate in 1 litre of deionised water; the pH is adjusted to 5.0 with glacial acetic acid). Just before use, add 25 µl 30% hydrogen peroxide to AEC solution then add to each well. Incubate at RT for 20 minutes.
- x) Remove substrate solution and wash wells twice with deionised water to stop reaction.
- xi) To visualise all cells counterstain with Mayer's haematoxylin (50 µl/well or 200 µl/well) for 1 minute and rinse with deionised water.
- xii) Add 50 µl Scott's tap water and rinse with deionised water and air dry.

Interpretation of the results

Positive reaction: granular-like, focal, brick-red staining of cells indicates presence of virus identified by the diagnostic antibody.

Negative reaction: no red staining apparent – all cells should be stained pale blue due to counterstain.

Background staining: nongranular, nonfocal, more generalised, pale, pinkish staining may occur throughout the culture. This background staining could be caused by any number of reasons, e.g. nonspecific antibody reaction with nonviral components, inefficient washing, and expiration of other reagents.

Reagents for immunocytochemistry tests

20% Formaldehyde (PBS-A) saline

Formalin (36–38% formaldehyde)	54 ml
Distilled water	36 ml
10 × PBS-A	10 ml

10 × PBS-A

To make up 1 litre of 10 × PBS-A use:

NaCl	80.0 g
Na ₂ HPO ₄	11.5 g
KCl	2.0 g
KH ₂ PO ₄	2.0 g
Distilled water	1.0 litre

NOTE: some salts are supplied with extra water groups. If using these reagents adjust the masses to ensure the appropriate mass of salt is added, e.g. for Na₂HPO₄·2H₂O add 15 g instead of 11.5 g (156 mw/120 mw × 11.5 g = 14.95 g) to remove the effect of the water molecules.

4.3.1.2.2.2 Detection of EHNV using antigen-capture ELISA

Antigen-capture ELISA has been validated to detect EHNV in cell cultures and directly in fish tissue homogenates. The analytical sensitivity is 10³ to 10⁴ TCID₅₀ ml⁻¹. Specificity approaches 100% and sensitivity for direct detection in fish tissues is 60% relative to the gold standard of virus isolation in BF-2 cells (16, 37 and unpublished data). ELISA is useful for both diagnosis and certification. Neutralisation tests cannot be used to identify EHNV because neutralising antibodies are not produced following immunisation of mammals or fish. Mouse monoclonal antibodies produced against

EHNV are directed against major capsid protein (MCP) epitopes and are non-neutralising (unpublished data). Rabbit-anti-EHNV antibodies have been developed for use in antigen-capture ELISA, immunoperoxidase staining and immunoelectron microscopy (14, 16, 27). Reagents and protocols are available from the reference laboratory.

Samples: tissue homogenate samples prepared using a validated protocol (see below), and cell cultures.

Principle of the test: EHNV particles are captured from the sample by an affinity purified rabbit antibody that is coated to the plate. EHNV is detected by a second antibody and a peroxidase-labelled conjugate using the chromogen ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid). The enzyme is inactivated after 20 minutes and the resulting optical density (OD) is compared with standards.

Operating characteristics: the protocol is based on published procedures (16, 30, 32, 37). When performed as described in this protocol, the operating characteristics of the test are as given in Table 4.1. The precision of the assay is <10% coefficient of variation, measured as variation in the OD of the controls between plates and over time, when the recommended normalisation procedure is followed.

Table 4.1. EHNV ELISA operating characteristics compared with the gold standard of cell culture in BF-2 cells

Sample	Positive-negative cut-off**	Sensitivity %	Specificity %
Tissues of fish*	OD 0.5	60	>99
Tissue culture supernatants with cytopathic effect (BF2 cells)	OD 0.3	>99	>99

* redefin perch and rainbow trout only. Higher background OD occurs with golden perch. There are no data for other species.

** these cut-offs are determined by the OIE Reference Laboratory for EHNV and will vary with the batch of control antigen. Values above are for batch 86/8774-4-5-01.

Test components and preparation of reagents

- i) Flat bottom microtitre plates are required.
- ii) Affinity purified rabbit anti-EHNV immunoglobulin and sheep anti-EHNV antiserum reagents are supplied in freeze-dried form. Reconstitute using 1 ml of purified water and allow the vial to stand at RT for 2 minutes. Mix the vial very gently. These reagents are stable when stored at –20°C for at least 4 years. For routine use in ELISA, it is recommended that working stocks of both antibodies be prepared as a 1/10 dilution in tris saline glycerol merthiolate TSGM (formula at end of this section). These are stable at –20°C for at least 5 years and do not solidify at this temperature.
- iii) The peroxidise labelled anti-sheep immunoglobulin conjugate (commercial reagent, KPL #14-23-06; 0.5 mg) is supplied as a freeze-dried powder. This reagent has displayed remarkable consistency in activity between different lots over a period of 15 years. The product should be reconstituted in sterile 50% glycerol water, dispensed in 150 µl aliquots and stored at –20°C as undiluted stock. A working stock is prepared by adding 900 µl of TSGM to 100 µl of undiluted stock. The working stock is also stored at –20°C and is stable for at least 1 year. New batches of this conjugate should be titrated against an older batch using standard protocols.
- iv) EHNV control antigen, heat-inactivated, is supplied as freeze-dried powder. Reconstitute in 1 ml sterile water and store in small aliquots at –20°C. Prepare dilutions using PBSTG (PBS + Tween + gelatin) on the same day the test is performed. Control EHNV antigen dilutions (A, B, D and F) cover the range of the signal response of the assay and enable a normalisation procedure to be undertaken.

Equipment

An automatic plate washer is recommended although plates can be washed by hand. The assay is sensitive to plate washing conditions. If the OD of the controls is unexpectedly low, and the conjugate and other reagents are within date, the plate washer should be adjusted so that washing pressure during filling of wells and aspiration of wells is minimised.

An automatic plate reader is recommended although plates can be read by eye.

Precision calibrated pipettes (e.g. Gilson) should be used to prepare dilutions of all reagents and to load reagents into microtitre plate wells.

Protocol

- i) Coat a 96-well ELISA plate (100 µl/well) with affinity purified rabbit-anti-EHNV diluted 1/12,800 in borate coating buffer. Incubate overnight at 4°C.
- ii) Wash plate five times with wash buffer (Milli-Q (MQ) purified water plus 0.05% Tween 20). Note that distilled and deionised water can also be used in this and all other steps.
- iii) Prepare a blocking solution: warm the solutions in a microwave oven or water bath to dissolve the gelatin, then cool to RT.
- iv) Block remaining binding sites using blocking solution (100 µl/well) (1% [w/v] gelatin in PBSTG diluent [PBS, 0.05% [v/v] Tween 20, 0.1% [w/v] gelatin]). Incubate at RT for 30 minutes.
- v) Wash plate five times as above.
- vi) Work in a Class II biological safety cabinet. Dilute the control antigen (see below) in PBSTG and add to the lower right-hand corner of the plate. Add tissue homogenate samples or culture supernatant samples and control antigens at 100 µl/well. All samples and controls are added to duplicate wells. Incubate for 90 minutes at RT.

The control antigens are dilutions of a heat killed cell culture supernatant of EHNV 86/8774. The controls are expected to give the following OD, although there will be some variation from laboratory to laboratory and ±10% variation should therefore be allowed:

Control	Dilution in PBS*	OD (405 nm)*
A	1/5	>2.0
B	1/40	1.90
D	1/200	0.68
F	1/3000	0.16

* These dilutions and OD values are determined by the OIE Reference Laboratory for EHNV and will vary with the batch of control antigen. The values above are for batch 86/8774-4-5-01. The positive-negative cut-off for clarified tissue homogenate samples from redfin perch and rainbow trout in this ELISA is approximated by the OD value of control D on each plate.

- vii) Wash the plate by hand to avoid contamination of the plate washer. Work in a Class II cabinet. Aspirate wells using a multichannel pipette. Rinse the plate twice.
- viii) Wash the plate five times on the plate washer, as above.
- ix) Add the second antibody sheep-anti-EHNV diluted 1/32,000 in PBSTG (100 µl/well). Incubate for 90 minutes at RT.
- x) Wash the plate five times on the plate washer.
- xi) Add the conjugate diluted 1/1500 in PBSTG (100 µl/well). Incubate for 90 minutes at RT.
- xii) Wash the plate five times on the plate washer.
- xiii) Add ABTS substrate (22 ml ABTS + 10 µl H₂O₂) (100 µl/well) and place the plate on a plate shaker. Time this step from the moment substrate is added to the first wells of plate 1. Incubate for 20 minutes.
- xiv) Immediately add ABTS stop solution (50 µl/well), shake the plate briefly and read OD at 405 nm. Calculate mean ELISA OD of duplicate wells. Calculate the coefficient of variation of the duplicates: samples with CV >15% should be retested if the mean OD lies near the positive-negative cut-off.

Normalisation of data and decision limit quality control

If it is desired to normalise data from plate to plate and over time, or to undertake decision limit quality control, the following procedure can be followed. Run control antigens in ELISA on at least five occasions over a period of 3 weeks (a total of 20 separate ELISA plates). Calculate the mean OD for each control antigen. Then, for each plate subsequently used, calculate a plate correction factor (PCF) as follows:

PCF = (mean OD control A/actual OD + mean OD control B/actual OD + mean OD control D/actual OD + mean OD control F/actual OD)/4. Multiply the actual mean OD of each sample by the PCF for that plate and report these values.

PCF is allowed to vary between 0.8 and 1.2, which approximates to a coefficient of variation of 10%. Values outside this range suggest that a plate needs to be retested. Plots of PCF over time provide a ready means for monitoring the stability of reagents, procedural variations and operator errors. This QC method has been validated for antigen capture ELISA.

Buffers and other reagents

Borate coating buffer

Boric acid	6.18 g
Disodium tetraborate (Na ₂ B ₄ O ₇ ·10H ₂ O)	9.54 g
NaCl	4.38 g
MQ water to	1 litre
Autoclave	

10 × phosphate buffered saline

NaCl	80.00 g
KCl	2.00 g
Na ₂ HPO ₄	11.50 g
KH ₂ PO ₄	2.00 g
MQ water to	900 ml
Adjust pH to 7.2 with HCl or NaOH; make up to 1 litre	
Autoclave	

For working strength dilute 1/10 and recheck pH.

For storage of powder in jars, make up twice the above quantity of powder; store; to make up add 1.8 litres MQW, pH, make up to 2 litres.

ABTS

Citrate phosphate buffer	
Citric acid	21.00 g
Na ₂ HPO ₄	14.00 g
MQ water to 800 ml; adjust pH to 4.2; make up to 1 litre	
ABTS	0.55 g
Citrate phosphate buffer to	1 litre
Dispense in 22-ml aliquots and freeze.	
Immediately prior to use add 10 µl H ₂ O ₂ per 22-ml aliquot.	

ABTS stop solution (0.01% NaN₃ in 0.1 M citric acid)

Citric acid	10.5 g
MQW to	500 ml
Add 50 mg sodium azide or 1 ml of 5% solution.	

KPL Conjugate #14-23-06¹

TSGM cryoprotectant

10 × Tris/saline, pH 7.4	50 ml
Glycerol	250 ml
Sterile purified water to	500 ml
Autoclave	
Add 10% Merthiolate	1 ml
Store in dark bottle at 4°C.	

1 Reagent Supplier: Bio-Mediq DPC Australia, P.O. Box 106, Doncaster, Victoria 3108, Australia; Tel.: (+61-3) 9840 2767; Fax: (+61-3) 9840 2767. Visit: www.kpl.com for links to worldwide network distributors. Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

10 × Tris/saline (250 mM Tris, 1.5 M NaCl)

Tris	15.14 g
NaCl	43.83 g
Sterile purified water	500 ml
pH adjust to	7.4

4.3.1.2.2.3. Immunoelectron microscopy

Gold-labelling of sections containing tissues or cell cultures

Principle of the test: cell cultures, tissues and/or tissue homogenates can be used for examination by electron microscopy. Conventional electron microscopy (examination of ultra-thin sections) will generate data on virus structure and morphogenesis. Negative contrast electron microscopy will produce images that can be used to examine the particulate structure of the virus. The use of ranavirus-specific antibodies and conjugated gold with these preparations permits both ultrastructure and antigenicity to be examined (15). These collective data enable classification to the genus Ranavirus.

Cell cultures and tissues

- i) Fix tissues or cell cultures as described in ref. 15. Briefly, 2.5% (v/v) buffered glutaraldehyde (cacodylate or phosphate) is used to fix cells for 40 minutes. Following primary fixation the cells are rinsed in the same buffer (3 × 20 minutes), post-fixed in 1% (w/v) buffered osmium tetroxide (1 hour), washed (3 × 5 minutes) in double-distilled/reverse osmosis (RO) water, dehydrated through graded alcohol (70–100%) and infiltrated and embedded in an epoxy resin (e.g. Spurr's or epon). For gold labelling of ultra-thin resin sections, attention must be given to fixation and embedding regimes. For example, cells should be fixed in 0.25% (v/v) glutaraldehyde with 2–4% paraformaldehyde. No secondary fixation is used and the cells are infiltrated and embedded in an acrylic resin such as LR White.
- ii) Following fixation and embedding, cut and transfer ultrathin sections onto filmed nickel grids.
- iii) Cut sections from the appropriate blocks.
- iv) Block in 2% (w/v) skim milk powder in PBS-A (10 minutes).
- v) Block free aldehydes with 0.1 M glycine in PBS-A (20 minutes).
- vi) Wash in PBS-A (3 × 1 minutes). This is an optional step used only if there is an excess of free aldehydes (a high background may be indicative of this).
- vii) If protein A-gold is not being used then block in normal species serum – this serum should be homologous to that complexed to gold. Recommended dilution is approximately 1/40 (10 minutes).
- viii) Incubate in primary antibody. If incubation details are unknown then perform initial reactions with 1/100 to 1/2700 dilutions (with three-fold dilutions). Dilute antibodies in 1% (v/v) cold water fish gelatin in PBS-A, (60 minutes, RT).
- ix) Rinse in 1% (v/v) coldwater fish gelatin in PBS-A, (6 × 3 minutes).
- x) Incubate in gold-labelled secondary antibody or protein A-gold or protein G-gold. Suggested dilution 1/40 in a PBS-A containing 1% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Tween 20 and 0.1% (v/v) Triton X, 60 minutes, RT.
- xi) Rinse in PBS-A (6 × 3 minutes, RT).
- xii) Post-fix in 2.5% (v/v) glutaraldehyde in PBS-A (5 minutes, RT).
- xiii) Rinse in water (RO) (3 × 3 minutes, RT).
- xiv) Dry on filter paper (type not critical).
- xv) Stain in uranyl acetate and lead acetate.

Interpretation of results

Viruses within the cytoplasm of infected cells will be specifically gold-labelled. Viruses will be located singularly, within assembly bodies (inclusion bodies) and within paracrystalline arrays.

Gold-labelling of virus particles (viruses adsorbed to grids)

- i) Dounce homogenise 10% (w/v) liver, kidney or spleen and clarify (5 minutes, 2500 g).

- ii) Adsorb the supernatant (from homogenate or cell cultures) to grid substrate.
- iii) Use carbon-coated 200 mesh gold grids.
- iv) Fix the sample with 0.1% (v/v) glutaraldehyde and 1% (v/v) Nonidet P40 (NP40) in PBS (2 minutes).
- v) Wash in PBS (3 × 3 minutes).
- vi) Block with 5% (v/v) cold water fish gelatin (Sigma) in PBS (10 minutes) followed with incubation buffer (PBS/0.1% cold water fish gelatin).
- vii) Incubate with antibody (affinity purified rabbit anti-EHNV, Lot No. M708; supplied by the OIE Reference Laboratory; suggested dilution 1/500) for 1 hour, at RT.
- viii) Wash grids (6 × 3 minutes) in incubation buffer.
- ix) Incubate with 10 nm protein A-gold (for dilution refer to suppliers recommendation) for 1 hour, at RT.
- x) Wash (6 × 3 minutes).
- xi) Fix with 2.5% glutaraldehyde (5 minutes).
- xii) Wash with distilled water (3 × 3 minutes) and stain with 2% phosphotungstic acid (pH 6.8) for 1 minute.

Interpretation of results

The inclusion of NP40 will permit antibodies and protein A-gold to penetrate the outer membrane and react with the underlying capsid. Labelling should be specific for the virus. Non-EHNV affinity purified rabbit serum (1/500) should be included as a negative control.

4.3.1.2.2.4. Immunohistochemistry (immunoperoxidase stain)

Samples: formalin-fixed paraffin-embedded tissue sections.

Technical procedure

The following protocol is intended for the qualitative demonstration of EHNV antigens in formalin-fixed paraffin-embedded tissue sections (27). It assumes that antigens may have become cross linked and therefore includes a protease digestion step that may be omitted if unfixed samples are examined. A commercial kit (DAKO[®] LSAB K0679) with peroxidase-labelled streptavidin and a mixture of biotinylated anti-rabbit/anti-mouse/anti-goat immunoglobulins as link antibodies is used for staining. Other commercially supplied reagents are also used. For convenience these are also supplied by DAKO². The primary affinity purified rabbit anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the OIE Reference Laboratory.

- i) Cut 5 µm sections and mount on SuperFrost[®] Plus G/Edge slides (Menzel-Glaser, HD Scientific Cat. No. HD 041300 72P3). Mark around the section with a diamond pencil to limit the spread of reagents.
- ii) Deparaffinise the section:

Preheat slides in a 60°C incubator for 30 minutes.

Place slides in a xylene bath and incubate for 5 minutes. Repeat once. Note that xylene replacements can be used without deleterious effects.

Tap off excess liquid and place slides in absolute ethanol for 3 minutes. Repeat once.

Tap off excess liquid and place slides in 95% ethanol for 3 minutes. Repeat once.

Tap off excess liquid and place slides in distilled or deionised water for 30 seconds.
- iii) Expose antigens using a protease treatment. Flood slide with proteinase K (5–7 µg ml⁻¹) and incubate for 20 minutes (ready-to-use solution, DakoCytomation Cat. No. S3020). Rinse slide by immersing three times in water. Place in a PBST bath for 5 minutes (PBS pH 7.2, 0.05% [v/v] Tween 20). Tap off the excess wash solution and carefully wipe around the section.

2 Dako Cytomation California Inc., 6392 via Real, Carpinteria, CA 93013, USA, Tel.: (+1-805) 566 6655, Fax: (+1-805) 566 6688; Dako Cytomation Pty Ltd, Unit 4, 13 Lord Street, Botany, NSW 2019, Australia, Fax: (+61-2) 9316 4773; Visit www.dakocytomation.com for links to other countries.

- iv) Perform the immunostaining reaction using the Universal DAKO LSAB[®]+ Kit, Peroxidase (DakoCytomation Cat No. K0679). Ensuring the tissue section is completely covered, add the following reagents to the slide. Avoid drying out.
- v) 3% hydrogen peroxide: cover the section and incubate for 5 minutes. Rinse gently with PBST and place in a fresh wash bath.
- vi) Primary antibody (affinity purified rabbit anti-EHNV 1:/1500 Lot No. M708) and negative control reagent (non-immune rabbit serum at a dilution of 1/1500) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides.
- vii) Link: cover the section and incubate for 15 minutes. Rinse slides.
- viii) Streptavidin peroxidase: cover the section and incubate for 15 minutes. Rinse slides.
- ix) Substrate–chromogen solution: cover the section and incubate for 5 minutes. Rinse slides gently with distilled water.
- x) Counterstain by placing slides in a bath of DAKO[®] Mayer's Haematoxylin for 1 minute (Lillie's Modification, Cat. No. S3309). Rinse gently with distilled water. Immerse 10 times into a water bath. Place in distilled or deionised water for 2 minutes.
- xi) Mount and cover-slip samples with an aqueous-based mounting medium (DAKO[®] Faramount Aqueous Mounting Medium Cat. No. S3025).

Interpretation of results

EHNV antigen appears as a brown stain in the areas surrounding degenerate and necrotic areas in parenchymal areas. There should be no staining with negative control rabbit serum on the same section.

Availability of test and reagents: antibody reagents and test protocols are available from the OIE Reference Laboratory.

4.3.1.2.3. Molecular techniques

Identification of ranavirus at genus and species level is possible using two PCR methods based on the MCP gene. In the first method, two PCR assays using MCP primers are used with restriction analysis to detect and rapidly differentiate EHNV from the European (ECV), North American (FV3) and other Australian ranaviruses (BIV) (25). This can be completed in less than 24 hours at relatively low cost. In the second method, a single MCP PCR assay is used to generate a 580 bp product, which is then sequenced to identify the type of ranavirus.

Samples: virus from cell culture or direct analysis of tissue homogenate.

4.3.1.2.3.1. PCR and restriction endonuclease analysis (REA): technical procedure

Amplified product from PCR assay MCP-1 digested with PflM I enables differentiation of Australian iridoviruses (EHNV and BIV) from non-Australian iridoviruses (FV3, Americas; and ECV, Europe). Amplified product from PCR assay MCP-2 digested with Hinc II, Acc I and Fnu4H I (individually) enables differentiation of EHNV and BIV (Australia) from each other and from FV3 (Americas) and ECV (Europe).

Preparation of reagents

EHNV-purified DNA and BIV-purified DNA PCR control reagents are supplied by the reference laboratory in freeze-dried form. Reconstitute using 0.5 ml of Tris-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and allow the vial to stand at RT for 2 minutes. Mix the vial very gently. For routine use, as a PCR control, it is recommended that working stocks be prepared as a 1/10 dilution in TE buffer (pH 8.0). Aliquots of 250 µl should be stored at –20°C. Each aliquot is sufficient for at least 50 reactions (1 to 5 µl added to cocktail) and has a minimum shelf life of 6 months from date of diluting.

Primers M151 and M152 (MCP-1, 321 bp), M153 and M154 (MCP-2, 625 bp) are supplied in working strength and should be stored at –20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 4.2.

Table 4.2. MCP-1 and MCP-2 primer sequences

PCR assay	Primer	Sequence	Product size	Gene location
MCP-1	M151	AAC-CCG-GCT-TTC- GGG-CAG-CA	321 bp	266–586
	M152	CGG-GGC-GGG-GTT- GAT-GAG-AT		
MCP-2	M153	ATG-ACC-GTC-GCC- CTC-ATC-AC	625 bp	842–1466
	M154	CCA-TCG-AGC-CGT- TCA-TGA-TG		

PCR cocktail

Amplification reactions in a final volume of 50 µl (including 5 µl DNA sample) contain 2.5 µl of each working primer, 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 10 × PCR buffer (66.6 mM Tris/HCl, 16.6 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 1.65 mg ml⁻¹ BSA, 10 mM beta-mercaptoethanol) and 2 U Taq polymerase. Instructions on preparation of 10 × PCR buffer are included in Table 4.3.

Table 4.3. 10 × PCR buffer preparation

Ingredients	Amount	Final concentration in 50 µl PCR mix
Tris	4.050 g	66.6 mM
Ammonium sulphate	1.100 g	16.6 mM
BSA (albumin bovine fraction V fatty acid free)	0.825 g	1.65 mg ml ⁻¹
Magnesium chloride	1.25 ml	2.5 mM
TE buffer (sterile)	50 ml	

NOTE: alternative commercial buffers may also be used.

Two negative controls are included, one comprising PCR cocktail only and the second containing 5 µl TE buffer.

The MCP-1 and MCP-2 reactions have the following profile: 1 cycle of denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; a final extension of 72°C for 5 minutes, and cooling to 4°C.

NOTE: the annealing temperature may be increased to 60 or 62°C to reduce nonspecific amplification when the assay is used to test fish tissues.

PCR results are assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. EHNV PCR control DNA (1/10 working stock) should give a result similar in intensity to the 10–3 band in both cases.

Restriction endonuclease analysis (REA)

PCR amplicons are subjected to REA with the enzymes described in Table 4.4. All endonucleases should be used according to the manufacturers' instructions. REA reactions are prepared by adding 1–4 µl of PCR product, 2 U of the appropriate restriction endonuclease, 1.6 µl of buffer (supplied with each restriction endonuclease), 1.6 µl of 100 µg ml⁻¹ BSA (for PfiM I and Hinc II) and made up to a final volume of 16 µl with sterile purified water. Restriction digests are incubated for 2–4 hours at the recommended temperatures and assessed by agarose gel electrophoresis in 3% gels. The predicted band sizes after restriction are given in Table 4.4.

Table 4.4. Restriction endonuclease analysis of ranavirus MCP amplicons

PCR Assay	Restriction enzyme	Predicted band sizes after restriction (bp)	Pattern applies to
MCP-1 (321bp)	<i>Pf</i> MI	321	EHN, BIV
		131, 190	FV3, WV
MCP-2 (625bp)	<i>Hinc</i> II	100, 138, 387	EHN
		100, 525	BIV, FV3
		100, 240, 285	WV
	<i>Acc</i> I	238, 387	EHN
		625	BIV, ESV, ECV, WV
		164, 461	FV3, GV
	<i>Fnu</i> 4H I	33, 38, 44, 239, 271	EHN
		3, 33, 38, 44, 108, 399	BIV
		3, 38, 44, 108, 432	FV3, GV
		3, 9, 38, 44, 108, 151, 272	ESV, ECV
		3, 44, 71, 108, 399	WV

Aliquot into 500 µl volumes and store at –20°C. For a working solution, add 3.5 µl of beta-mercaptoethanol per 500 µl 10 × buffer. Any remaining buffer should be discarded after preparing the PCR cocktail.

The sensitivity of PCR in diagnostic applications directly on fish tissues is being evaluated.

Detailed protocols to enable completion of the test, worksheets and purified control EHN DNA are available from the OIE Reference Laboratory.

4.3.1.2.3.2. Alternative PCR and sequencing for viral identification

In this assay two primers, a reverse primer (5'-AAA-GAC-CCG-TTT-TGC-AGC-AAA-C-3') and a forward primer (5'-CGC-AGT-CAA-GGC-CTT-GAT-GT-3'), are used for amplification of the target MCP sequence (580 base pairs [bp]) of EHN DNA by PCR. This PCR procedure can be used for the specific detection of ranaviruses from redbfin perch, rainbow trout, sheatfish, catfish, guppy fish (*Poecilia reticulata*), doctor fish (*Labroides dimidatus*) and a range of amphibian ranaviruses (17). Nucleic acid (1 µl) is added to Taq polymerase buffer containing 0.1 µM of each primer, 2.5 U Taq polymerase (Promega) and 2.5 mM MgCl₂. The mixture is incubated in an automatic thermal cycler programmed for 35 cycles at 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and finally held at 72°C for 15 minutes. Amplified DNA (580 bp) is analysed by agarose gel electrophoresis, excised and sequenced using a range of standard technologies). Each viral species is identified by its unique DNA sequence available from GenBank. Samples can be submitted to the OIE reference laboratory for specific identification.

4.3.1.2.4. Agent purification

Purification of EHN has been described (16, 30) and a protocol is available from the reference laboratory.

4.3.2. Serological methods

Neutralising antibodies have not been detected in fish or mammals exposed to EHN. Indirect ELISA for detection of antibodies induced following exposure to EHN has been described for rainbow trout and redbfin perch (34, 35). The sensitivity and specificity of these assays in relation to a gold standard test are not known and interpretation of results is currently difficult. Protocols and specific anti-immunoglobulin reagents required to conduct these tests are available from the reference laboratory.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of EHNV 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; d = the method is presently not recommended for this purpose; and NA = not applicable. 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 (see Chapter 1.1.2 of this *Aquatic Manual*), 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
	Ova/milt	Fry/fingerlings	Juveniles	Adults		
Gross signs	na	d	d	d	d	d
Histopathology	na	d	d	d	b	d
Immunoperoxidase stain	na	c	c	c	c	c
Transmission EM	na	d	d	d	c	b
Immuno-EM	na	d	d	d	c	b
Cell culture	na	a	a	a	a	b
Antigen-capture ELISA	na	a	a	a	a	a
Antibody-capture ELISA	na	d	d	c	c	d
PCR-REA	na	d	a	d	c	a
PCR sequence analysis	na	d	d	d	c	a

EM = electron microscopy; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; REA = restriction endonuclease analysis; na = not applicable.

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

Statistically valid sampling practices need to be used and the correct organs/samples need to be collected;

Standardised tests of specified sensitivity and specificity should be used. This restricts certification testing to cell culture, the gold standard test, and antigen-capture ELISA.

The chances of detecting EHNV infection in apparently healthy rainbow trout is extremely low, even where disease is active in the same population, because the prevalence of infection is low and there is a high case fatality rate. For practical purposes, EHNV can only be detected in fish that are clinically affected or that have died with the infection. From a random sample of live rainbow trout it would be possible to misclassify a farm as being free of EHNV even during an outbreak of the disease because the prevalence of infection is generally very low. Consequently, the examination of 'routine' mortalities is recommended (36).

During a low-grade outbreak of disease in rainbow trout, the prevalence of EHNV among mortalities may be 60–80% and the contribution of EHNV to 'background' mortality is high enough to enable detection of the virus in the absence

of overt disease in the population. For EHN detection and certification purposes the population of interest is 'the population of mortalities' and sampling rates can be selected to detect at least one EHN-infected individual at a given level of confidence given a certain prevalence of infection and test sensitivity (6, 28). During an outbreak of EHN the virus was detected in at least 2% of dead fish (36). For this reason, assume a prevalence of 2% for sampling of EHN for certification purposes. The antigen-capture ELISA used to screen tissue homogenates for EHN has a sensitivity of at least 60% compared with cell culture (37). The sample size required from a very large population of 'routine' mortalities (36) to provide 95% confidence in detecting at least one infected individual using a test of 60% sensitivity is approximately 250. In practice, 'routine' mortalities should be collected daily and stored in plastic bags in groups of 20 at -20°C until a sample of 250 has been gathered. Where possible, young age classes should be selected to simplify dissections and tissue processing. Individual clarified homogenates that are positive in antigen-capture ELISA are then subjected to cell culture to confirm the presence of EHN. This is an economical approach as it greatly reduces the number of cell cultures required. Alternatively, cell culture could be used and samples from five fish pooled to reduce costs.

Serology might also play a useful role in surveys to identify infected trout populations. Assuming 1% prevalence of seropositive grower fish on an endemically infected farm, a sample of 300 fish would be required to be 95% certain of detecting at least one infected individual (6). Further research is required to confirm the validity of this approach.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Finfish, apparently healthy, moribund or dead in which parenchymal tissues contain histological evidence of focal, multifocal or locally extensive liquefactive or coagulative necrosis with or without intracytoplasmic basophilic inclusion bodies.

7.2. Definition of confirmed case

Finfish, apparently healthy, moribund or dead in which parenchymal tissues contain histological evidence of focal, multifocal or locally extensive liquefactive or coagulative necrosis with or without intracytoplasmic basophilic inclusion bodies and/or in which EHN is demonstrated by the following means:

1. Characteristic CPE in cell culture and cell culture is positive for EHN in immunoperoxidase test or antigen-capture ELISA or PCR,

or

2. Tissues positive in antigen-capture ELISA or immunoperoxidase stain or immunoelectron microscopy or PCR

And for both 1 and 2,

3. Sequence consistent with EHN is demonstrated by PCR-REA or PCR-sequencing.

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NB: There is an OIE Reference Laboratory for epizootic haematopoietic 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).