**USA COMMENTS in RED font**

CHAPTER 2.3.2.

**Infection with epizootic
haematopoietic necrosis virus**

**1. Scope**

Infection with epizootic haematopoietic necrosis virus means infection with the pathogenic agent *epizootic haematopoietic necrosis virus* (EHNV) of the Genus *Ranavirus* of the Family *Iridoviridae*.

**2. Disease information**

**2.1. Agent factors**

**2.1.1. Aetiological agent**

EHNV is a species of the genus *Ranavirus* in the Family *Iridoviridae* (Chinchar *et al.,* 2005). In addition to fish, ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (Chinchar*,* 2002; Drury *et al.,* 2002; Fijan *et al.,* 1991; Hyatt *et al.*, 2002; Speare & Smith, 1992; Whittington *et al.*, 2010; Wolf *et al*., 1968; Zupanovic *et al*., 1998). 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 (Chinchar *et al.,* 2005).

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 (European catfish virus, ECV) (Pozet *et al*., 1992), sheatfish (*Silurus glanis*) in Germany (European sheatfish virus, ESV) (Ahne *et al.,* 1989; 1990), turbot (*Scophthalmus maximus*) in Denmark (Bloch & Larsen, 1993), and cod (*Gadus morhua*) in Denmark (Cod iridovirus, CodV) (Ariel *et al.,* 2010). EHNV, ECV, ESV, and CodV share >98% nucleotide identity across concatenated sequences across the RNR-α, DNApol, RNR-β, RNAse II and MCP gene regions (Ariel *et al*., 2010).

EHNV and ECV can be differentiated using genomic analysis (Ahne *et al.,* 1998; Holopainen *et al*., 2009; Hyatt *et al.*, 2000; Mao *et al*., 1996; 1997; Marsh *et al*., 2002). This enables epidemiological separation of disease events in finfish in Australia (EHNV) and Europe (ECV), and differentiation of these from ranavirus occurrences in amphibians.

**2.1.2. Survival and stability in processed or stored samples**

EHNV can persist in frozen fish tissues for more than 2 years (Langdon, 1989) and frozen fish carcases for at least a year (Whittington *et al.*, 1996).

**2.1.3. Survival and stability outside the host**

EHNV is resistant to drying and remained infective for 97 days at 15°C and 300 days at 4°C in water (Langdon, 1989). 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.

For inactivation methods, see Section 2.4.5.

**2.2. Host factors**

**2.2.1. Susceptible host species**

Species that fulfil the criteria for listing as susceptible to infection with EHNV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

| **Family** | **Scientific name** | **Common name** |
| --- | --- | --- |
| Esocidae | *Esox lucius* | Northern pike |
| Galaxiidae | *Galaxias olidus* | Mountain galaxias |
| Ictaluridae | *Ameiurus melas* | Black bullhead |
| Melanotaeniidae | *Melanotaenia fluviatilis* | Crimson spotted rainbow fish |
| **‎**Percidae | *Perca fluviatilis* | European perch |
| *Sander lucioperca* | Pike-perch |
| Percichthyidae | *Macquaria australasica* | Macquarie perch |
| Poeciliidae | *Gambusia holbrooki* | Eastern mosquito fish |
| *Gambusia affinis* | Mosquito fish |
| Salmonidae | *Oncorhynchus mykiss* | Rainbow trout |
| Terapontidae | *Bidyanus bidyanus* | Silver perch |

**~~2.2.2.~~****~~Species with incomplete evidence for susceptibility~~**

~~Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with EHNV according to Chapter 1.5 of the~~ *~~Aquatic Code~~* ~~are: none known.~~

~~In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: Atlantic salmon (~~*~~Salmo salar~~*~~), freshwater catfish (~~*~~Tandanus tandanus~~*~~), golden perch (~~*~~Macquaria ambigua~~*~~), Murray cod (~~*~~Maccullochella peelii~~*~~) and purple spotted gudgeon (~~*~~Mogurnda adspersa~~*~~).~~

**RATIONALE:** Remove Section 2.2.2. because the listing of species that cannot demonstrate susceptibility to EHN should not be included in the OIE Code. Listing of “species with incomplete evidence for susceptibility” may cause confusion that such species should be regulated in the same manner as “susceptible species,” without having undergone the same level of scientific demonstration of susceptibility.

**2.2.3. Likelihood of infection by species, host life stage, population or sub-populations**

Natural infections and disease have been limited to European perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) in Australia. The disease is more severe in European perch and in juveniles compared with adult fish (Whittington *et al.,* 2010). There are no descriptions of infection of eggs or early life stages of any other fish species.

**RATIONALE:** Editorial clarification.

~~For the purposes of Table 4.1, larvae and fry up to approximately 5 g in weight may be considered to be early life stages, fingerlings and grower fish up to 500 g may be considered to be juveniles, and fish above 500 g may be considered to be adults.~~

**RATIONALE:** This information may be more appropriately placed in Diseases of Fish – General Chapter (2.3.0.)., or Table 4.1.

**2.2.4. Distribution of the pathogen in the host**

Target organs and tissues infected with the virus are kidney, spleen and liver. 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. Aquatic animal reservoirs of infection**

*Rainbow trout:* The high case fatality rate and low prevalence of infection with EHNV in natural infections in rainbow trout means that the recruitment rate of carriers is likely to be very low (<2%) (Whittington *et al.,* 1994). EHNV has been detected in growout fish but histopathological lesions consistent with infection with EHNV indicated an active infection rather than a carrier state (Whittington *et al.,* 1999). Anti-EHNV serum antibodies were not detected in fingerlings during or after an outbreak but were detected in a low proportion of growout fish, hence, it is uncertain whether these were survivors of the outbreak (Whittington *et al.,* 1994; 1999). There are data for European stocks of rainbow trout in experimental infections where potential carriers were identified (Ariel & Bang Jensen, 2009).

*European perch:* EHNV was isolated from 2 of 40 apparently healthy adult European perch during epizootics in juveniles in Victoria, Australia (Langdon & Humphrey, 1987), but as the incubation period extends for up to 28 days (Whittington & Reddacliff, 1995), these fish may have been in the preclinical phase.

**2.2.6. Vectors**

Birds are potential vectors for EHNV, it being carried in the gut, on feathers, feet and the bill (Whittington *et al.,* 1996).

**2.3. Disease pattern**

**2.3.1. Mortality, morbidity and prevalence**

*Rainbow trout:* It appears that under natural farm conditions EHNV is poorly infective, but once infected most fish succumb to the disease ~~but has a high case fatality rate~~. Infection with EHNV may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate. Infection with 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 (Whittington *et al.*, 1994; 1999). There is a low direct economic impact because of the low mortality rate. Differences in susceptibility between European and Australian stocks of rainbow trout may exist (Ariel & Bang Jensen, 2009).

**RATIONALE:** The statement that “ENHV is poorly infective but has a high case fatality rate” directly contradicts the subsequent sentences which refer to the low mortality rates. The proposed changes are to clarify that the pathogen is not that contagious, but once a fish is infected there is a high fatality rate.

*European perch:* There is a very high rate of infection and mortality in natural outbreaks that, over time, leads to loss in wild fish populations (Langdon & Humphrey, 1987; Langdon *et al.,* 1986; Whittington *et al.*, 1996). 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 (Whittington & Reddacliff, 1995). Differences in susceptibility between European and Australian stocks of European perch may exist (Ariel & Bang Jensen, 2009).

**2.3.2. Clinical signs, including behavioural changes**

Moribund fish may have loss of equilibrium, flared opercula and may be dark in colour (Reddacliff & Whittington, 1996). Clinical signs are usually more obvious in fingerlings and juvenile fish than adults of both rainbow trout and European perch. There may be clinical evidence of poor husbandry practices, such as overcrowding and suboptimal water quality manifesting as skin, fin and gill lesions (Reddacliff & Whittington, 1996).

**2.3.3 Gross pathology**

There may be no gross lesions in affected fish. 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 (Reddacliff & Whittington, 1996).

**2.3.4. Modes of transmission and life cycle**

*Rainbow trout:* EHNV has spread between rainbow trout farms by transfer of infected fingerlings and probably transport water (Langdon *et al.,* 1988; Whittington *et al.*, 1994; 1999). 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 or from wild European perch present in the same catchment.

**RATIONALE:** Insert “or” because reinfection from “successive batches” is not necessarily caused exclusively by “wild European perch present in the same catchment.”

*European perch:* The occurrence of infection with EHNV in European perch in widely separated river systems and impoundments suggested that EHNV was spread by translocation of live fish or bait by recreational fishers (Whittington *et al.,* 2010).

The route of infection is unknown. European perch and rainbow trout are susceptible to immersion exposure. The virus infects a range of cell types including hepatocytes, haematopoietic cells and endothelial cells in many organs (Reddacliff & Whittington, 1996). Virus is shed into water from infected tissues and carcasses as they disintegrate.

**2.3.5. Environmental factors**

*Rainbow trout:* Outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. 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 (Whittington *et al.*, 1994; 1999). The incubation period after intraperitoneal inoculation was 3–10 days at 19–21°C compared with 14–32 days at 8–10°C (Whittington & Reddacliff, 1995).

*European perch:* Natural epizootics of infection with EHNV affecting juvenile and adult European perch occur mostly in summer (Langdon & Humphrey, 1987; Langdon *et al.,* 1986; Whittington *et al.*, 1994). 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 (Whittington & Reddacliff, 1995). 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 (Whittington & Reddacliff, 1995). European stocks of European perch also displayed temperature-dependent susceptibility (Ariel & Bang Jensen, 2009).

**2.3.6. Geographical distribution**

Infection with EHNV has been reported from rainbow trout farms within two river catchments in New South Wales, Australia (Whittington *et al*., 2010). Infection with EHNV is endemic in south-eastern Australia, with a discontinuous distribution (Whittington *et al*., 2010).

See WAHIS (<https://wahis.oie.int/#/home>) for recent information on distribution at the country level.

**2.4. Biosecurity and disease control strategies**

Not available.

**2.4.1. Vaccination**

None available.

**2.4.2. Chemotherapy including blocking agents**

None available.

**2.4.3. Immunostimulation**

None available.

**2.4.4. Breeding resistant strains**

There has been no formal breeding programme for resistant strains of susceptible species. However, experimental trials using a bath exposure have shown that European perch from water bodies in New South Wales, Australia with previous EHNV infections showed lower mortality compared with European perch from neighbouring and distant water bodies in Australia that have no previous history of EHNV (Becker *et al.,* 2016).

**2.4.5. Inactivation methods**

EHNV is susceptible to 70% ethanol, 200 mg litre–1 sodium hypochlorite or heating to 60°C for 15 minutes (Langdon, 1989). Data for the inactivation of amphibian ranavirus may also be relevant: 150 mg/litre chlorhexidine and 200 mg/litre potassium peroxymonosulphate were effective after 1 minute contact time (Bryan *et al*., 2009). If it is first dried, EHNV in cell culture supernatant is resistant to heating (Whittington *et al.*, 2010).

**2.4.6. Disinfection of eggs and larvae**

Not tested.

**2.4.7. General husbandry**

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. Investigations on one rainbow trout farm indicated that ponds with high stocking rates and low water flow, and thus poorer water quality, may result in higher levels of clinical disease compared with ponds on the same farm with lower stocking rates and higher water flow (Whittington *et al.,* 1994). The mechanism of protection may be through maintenance of healthy integument (Whittington *et al.,* 1994).

**3. Specimen selection, sample collection, transportation and handling**

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

**3.1. Selection of populations and individual specimens**

Clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) The most susceptible species (e.g. rainbow trout and European perch) should be sampled preferentially. Other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present, the fish selected should 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.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with EHNV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

**3.2. Selection of organs or tissues**

Liver, anterior kidney and spleen from individual fish are pooled (Jaramillo *et al.,* 2012).

**3.3. Samples or tissues not suitable for pathogen detection**

Inappropriate tissues include gonads, gonadal fluids, milt and ova, ~~since~~ because there is no evidence of reproductive tract infection.

RATIONALE: Editorial for clarification.

**3.4. Non-lethal sampling**

No non-lethal samples (blood, fin, gill, integument or mucous) are suitable for ~~testing~~ EHNV testing.

**RATIONALE:** Editorial

**3.5. Preservation of samples for submission**

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

**3.5.1. Samples for pathogen isolation**

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 General information (diseases of fish).

**3.5.2. Preservation of samples for molecular detection**

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. Alternatively, samples can be preserved in a DNA storage medium compatible with most DNA purification methods (e.g.DNA/RNA Shield or RNA later). If material cannot be ~~fixed~~ preserved it may be frozen.

**RATIONALE:** There is more than one option for preservatives that may be used. Consider adding commonly used nucleic acid preservatives.

**3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation**

Tissue samples for histopathology should be fixed immediately after collection in 10% neutral buffered formalin. The recommended ratio of fixative to tissue is 10:1.

**3.5.4. Samples for other tests**

Not recommended for routine diagnostic testing.

**3.6. Pooling of samples**

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry or specimens can be pooled to provide the minimum amount of material needed for testing. If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.

**4. Diagnostic methods**

~~The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:~~

~~Key:~~

~~+++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;~~

~~++ = Suitable method(s) but may need further validation;~~

~~+ = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;~~

~~Shaded boxes = Not appropriate for this purpose.~~

Ratings against purposes of use. For each recommended assay a qualitative rating against the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, successful application by diagnostic laboratories, cost, timeliness, and sample throughput. For a specific purpose of use, assays are rated as:

Key:

+++ = Method(s) have desirable performance and operational characteristics.

++ = Method(s) have acceptable performance and operational characteristics under most

 circumstances.

+ = Method(s) – performance or operational characteristics may significantly limit application.

Shaded boxes = Not appropriate for this purpose.

**Rationale:** Proposed revisions reflect proposed changes in Annex 24, *Aquatic Manual* disease chapters Table 4.1., for all manual chapters.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

***Table 4.1.*** *OIE recommended**diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

| **Method** | 1. **Surveillance of apparently healthy animals**
 | 1. **Presumptive diagnosis of clinically affected animals**
 | 1. **Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis**
 |
| --- | --- | --- | --- |
| **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** |
| **Wet mounts** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Histopathology3** |  |  |  |  | ++ | ++ | ++ | 1 |  |  |  |  |
| **Cytopathology3** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Cell culture** | +++ | +++ | +++ | 2 | +++ | +++ | +++ | 2 |  |  |  |  |
| **Immunohistochemistry** |  |  |  |  | + | + | + | 1 |  |  |  |  |
| **Real-time PCR** | +++ | +++ | +++ | 2 | +++ | +++ | +++ | 2 |  |  |  |  |
| **Conventional PCR** | + | + | + | 1 | ++ | ++ | ++ | 1 |  |  |  |  |
| **Amplicon sequencing4** |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 3 |
| ***In-situ* hybridisation** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Bioassy** |  |  |  |  |  |  |  |  |  |  |  |  |
| **LAMP** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ab-ELISA** |  |  | + | 1 |  |  |  |  |  |  |  |  |
| **Ag-ELISA** | + | + | + | 1 | + | + | + | 1 |  |  |  |  |
| **Other antigen detection methods5** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other method5** |  |  |  |  |  |  |  |  |  |  |  |  |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;
Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
1For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). 2Susceptibility of early and juvenile life stages is described in Section 2.2.3.
3Histopathology and cytopathology can be validated if the results from different operators have been statistically compared. 4Sequencing of the PCR product.
5Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

**4.1. Wet mounts**

Not applicable.

**4.2. Histopathology and cytopathology**

*Light microscopy:* routine methods can be used for tissue fixation, such as in 10% buffered neutral formalin, paraffin embedding, preparation of 4–10 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 infection with EHNV. Formalin-fixed paraffin-embedded sections can also be stained using an immunoperoxidase method (see below) to identify EHNV antigen associated with necrotic lesions.

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 (Reddacliff & Whittington, 1996).

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.

**RATIONALE:** Editorial

**4.3. Cell culture for isolation**

**4.3.1. Preparation of fish tissues for virus isolation**

A simple method for preparation of fish tissues for cell culture and ELISA has been validated (Whittington & Steiner, 1993) (see sampling Section 3).

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 µg ml–1 streptomycin and 4 µg 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.

**4.3.2. Cell culture~~/artificial media~~**

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 [Cinkova *et al.,* 2010]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (Crane *et al.,* 2005). Incubation temperatures of 20°C or 24°C result in higher titres than 15°C; 22°C and BF-2 EPC or CHSE-214 cells are recommended to maximise titres, which might be important for the detection of low numbers of viruses in fish tissues (Ariel *et al.,* 2009). BF-2 cells are preferred by the OIE Reference Laboratory with an incubation temperature of 22°C. The procedure for BF-2 cells is provided below. A procedure for CHSE-214 cells is provided under immunoperoxidase staining below (Section 4.7). The identity of viruses in cell culture is determined by immunostaining, ELISA, immuno-electron microscopy, PCR or other methods.

**4.3.3. Cell culture technical procedure**

*Samples:* tissue homogenates.

Cells are cultured (in flasks, tubes or multi-well plates) with growth medium (MEM + 10% fetal calf serum [FCS] with 100 IU ml–1 penicillin, 100 µg ml–1 streptomycin and 2 µg 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 µg
ml–1 streptomycin and 2 µg 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 final 1/100 dilution of a 0.1 mg ml–1 tissue homogenate. A further 1/10 dilution is made representing a final 1/1000 dilution, and two cultures are inoculated. 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 EHNV. P2 is incubated as above, and a third pass is conducted if necessary.

**4.3.4. 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. Cell cultures can be tested for EHNV DNA using real-time PCR and conventional PCR with sequence analysis as described in Section 4.4. Antigen can be detected using immunocytochemistry in cell cultures with polyclonal antibodies and protocol available from the reference laboratory.

Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

**4.4. Nucleic acid amplification**

Although several conventional PCR or quantitative real-time PCR methods have been described (Jaramillo *et al.,* 2012; Pallister *et al,* 2007; Stilwell *et al.,* 2018) none has been validated according to OIE guidelines for primary detection of EHNV. However, identification of ranavirus at genus and species level is possible using several published PCR strategies. Samples can be screened by real-time PCR, but as the assays described are not specific for EHNV, identification of EHNV by conventional PCR and amplicon sequencing must be undertaken on any samples screening positive by real-time PCR. For testing by conventional PCR, two PCR assays using MCP primers are used with amplicon sequencing required to differentiate EHNV from ECV, FV3 and BIV (Marsh *et al*., 2002). Alternatively, PCR of the DNA polymerase gene and neurofilament triplet H1-like protein genes can be used (Holopainen *et al*., 2011) (this method is not described in this chapter).

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

**4.4.1. Real-time PCR**

Tissue samples can be homogenised by manual pestle grinding or by bead beating (Rimmer *et al.,* 2012). Commercially available nucleic acid extraction kits (e.g. spin columns, magnetic beads) may be used to extract DNA directly from tissues and from tissue homogenates and cell culture supernatants. Depending on the number of samples to be tested, in the OIE Reference Laboratory, nucleic acids are extracted with either the QIAamp Viral RNA Mini Kit (Qiagen) or MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer’s instructions. A negative extraction control, consisting of extraction reagents only, is included when test samples are extracted.

The ranavirus real-time screening protocol in use at the OIE Reference Laboratory, based on Pallister *et al.,* 2007 is as follows; Template (2 μl) is added to 23 μl reaction mixture containing 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM for each primer, 250 nM for probe, and molecular grade water. After 1 cycle of 50°C for 2 minutes and 95 °C for 10 minutes, PCR amplification consists of 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

Alternative real-time PCR assays can be used according to published protocols for detection of the major capsid protein gene sequence of EHNV and other ranaviruses. The assay described by Jaramillo *et al.* (2012) uses SYBR Green detection chemistry and the assay described by Stilwell *et al.* (2018) was designed to detect multiple ranavirus species using hydrolysis probe detection chemistry.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

***Table 4.4.1.1.*** *Ranavirus primer and probe sequences*

|  |  |  |
| --- | --- | --- |
| **Primer** | **Sequence** | **Reference** |
| RANA CON F | 5’-CTC-ATC-GTT-CTG-GCC-ATC-A-3’ | Pallister *et al.,* 2007 |
| RANA CON R | 5’-TCC-CAT-CGA-GCC-GTT-CA-3’ |
| **Probe** |  |
| RANA CON Pr | 5’-**6FAM**-CAC-AAC-ATT-ATC-CGC-ATC-**MGB**-3’ |
| **Primer** |  |  |
| C1096 | 5’-GAC-TGA-CCA-ACG-CCA-GCC-TTA-ACG-3’ | Jaramillo *et al.,* 2012 |
|  |  |
| C1097 | 5’-GCG-GTG-GTG-TAC-CCA-GAG-TTG-TCG-3’ |
| **Primer** |  |  |
| RanaF1 | 5’-CCA-GCC-TGG-TGT-ACG-AAA-ACA-3’ | Stilwell *et al.,* 2018 |
| RanaR1 | 5’-ACT-GGG-ATG-GAG-GTG-GCA-TA-3’ |
| **Probe** |  |
| RanaP1 | 5’-**6FAM**-TGG-GAG-TCG-AGT-ACT-AC-MGB-3’ |

**RATIONALE:** Revision suggested for consistency throughout the table.

**4.4.2. Conventional PCR**

*PCR and restriction endonuclease analysis* (*REA*): *technical procedure*

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

*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 (100 ng µl–1) and should be stored at –20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 4.4.2.1.

***Table 4.4.2.1.*** *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 (250 ng) of each working primer, 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 5 µl of 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 4.4.2.2.

***Table 4.4.2.2.*** *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.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

**4.4.3. Other nucleic acid amplification methods**

Not applicable.

**4.5. Amplicon sequencing**

Amplicons generated using the MCP-1 and/or MCP-2 primers sets can be sequenced. Amplicons should be gel-purified and sequenced using both the forward and reverse primer. Consensus sequence, generated after analysis of the quality of the sequence chromatograms, can then be compared to reference sequences, for example by BlastN search of the NCBI database.

**4.6. *In-situ* hybridisation**

Not applicable

**4.7. Immunohistochemistry**

*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 (Reddacliff & Whittington, 1996). 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 DAKO[[1]](#footnote-1). The

1. Dako Cytomation California Inc., 6392 via Real, Carpinteria, CA 93013, USA, Tel.: (+1-805) 566 6655, Fax: (+1-805) 566 6688; [↑](#footnote-ref-1)