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 *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 species, 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*).

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 fish species.

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.

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

None known.

2.2.6. Vectors

None demonstrated.

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. 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).

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 $TCID_{50}$ 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). European perch from distinct geographical areas with and without a history of EHNV have been tested under experimental conditions and have demonstrated susceptibility to EHN (Becker *et al.,* 2016). 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.

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 (Becker *et al.,* 2019; 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 and sporadic outbreaks involving small numbers of European perch (Becker *et al,* 2019; Whittington *et al*., 2010).

See WAHIS (https://wahis.woah.org/#/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 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 to 60°C for 15 minutes (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 that 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 should be sampled preferentially i.e. European perch where these are available, otherwise rainbow trout or the other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample epidemiological units 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 apparently healthy fish collected in such a way that all parts of the farm or affected waterbody 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, because there is no evidence of reproductive tract infection.

3.4. Non-lethal sampling

Not applicable.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0 General information (diseases of fish).

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

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.2.5 of Chapter 2.3.0. *General information* (diseases of fish).

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.3.0 *General information* (diseases of fish).

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.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for 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, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ = Methods are most suitable with desirable performance and operational characteristics.

++ = Methods are suitable with acceptable performance and operational characteristics under most circumstances.

+ = Methods are suitable, but performance or operational characteristics may limit application under some circumstances.

Shaded boxes = Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

WOAH Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology					++	++	++	1				
Cytopathology												
Cell culture	+	+	++	1	++	++	+++	1	+	+	++	1
Immunohistochemistry					+	+	+	1				
Real-time PCR	+++	+++	+++	1	+++	+++	+++	2	++	++	++	1
Conventional PCR	+	+	+	1	++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation												
Bioassay												
LAMP												
Ab-ELISA			+	1								
Ag-ELISA	+	+	+	1	+	+	+	1				
Other antigen detection methods												
Other methods												

LV = level of validation, refers to the stage of validation in the WOAH 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; IFAT = indirect fluorescent antibody test.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
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. The nuclei of infected cells are frequently located peripherally and are distorted in shape.

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 lines for virus isolation

EHNV replicates 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; and BF-2, EPC, or CHSE 214 incubated at 22°C 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 WOAH 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).

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 bovine serum [FBS] 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% FBS 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 days 3 and 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.

The identity of viruses in cell culture is determined by PCR and amplicon sequencing.

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 for the detection of ranaviruses (Jaramillo *et al.,* 2012; Pallister *et al,* 2007; Stilwell *et al.,* 2018), EHNV can only be detected when these methods are combined with methods that specifically detect EHNV.

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).

PCR assays should always be run with the controls specified in Section 2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each diagnostic sample should be tested in duplicate, i.e. by testing two aliquots.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

The ranavirus real-time screening protocol in use at the WOAH Reference Laboratory is based on Pallister *et al.,* 2007. 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) detects multiple ranavirus species using hydrolysis probe detection chemistry.

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Pallister *et al*., 2007); GenBank Accession No.: DQ457105			
Ranavirus/MCP	Fwd: RANA CON: CTC-ATC-GTT-CTG-GCC-ATC-A Rev: RANA CON: TCC-CAT-CGA-GCC-GTT-CA Probe: RANA CON Pr FAM-CAC-AAC-ATT-ATC-CGC-ATC-MGB	900 nM for each primer, 250 nM for probe	45 cycles of 95°C/15 sec; 60°C/60 sec
Method 2 (Jaramillo *et al.,* 2012); GenBank Accession No.:			
Ranavirus/MCP	C1096 GAC-TGA-CCA-ACG-CCA-GCC-TTA-ACG C1097 GCG-GTG-GTG-TAC-CCA-GAG-TTG-TCG	12.5 pM for each primer	40 cycles of 95°C/30 sec; 58°C/30 sec
Method 3 (Stilwell *et al*., 2018); GenBank Accession No.:			
Ranavirus/MCP	Fwd: RanaF1: CCA-GCC-TGG-TGT-ACG-AAA-ACA Rev: RanaR1 ACT-GGG-ATG-GAG-GTG-GCA-TA Probe: RanaP1 FAM-TGG-GAG-TCG-AGT-ACT-AC-MGB	900 nM for each primer, 250 nM for probe	40 cycles of 95°C/30 sec; 60°C/45 sec

4.4.2. Conventional PCR

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. Both MCP1 and MCP2 target a region within the capsid protein gene (Marsh *et al.,* 2002).

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 1 (Marsh *et al.,* 2002): amplicon size MCP-1 is 321 bp and amplicon size MCP-2 is 625 bp			
MCP-1 Gene location: 266-586 MCP-2 Gene location: 842-1466	M151: AAC-CCG-GCT-TTC-GGG-CAG-CA M152: CGG-GGC-GGG-GTT-GAT-GAG-AT M153: ATG-ACC-GTC-GCC-CTC-ATC-AC MI54: CCA-TCG-AGC-CGT-TCA-TGA-TG	250 ng of each primer	35 cycles of 94°C/30 sec, 50°C/30 sec, 72°C/1 min NOTE: the annealing temperature may be increased to 60 or 62°C to reduce non-specific amplification when the assay is used to test fish tissues

4.4.3. Other nucleic acid amplification methods

Not applicable.

4.5. Amplicon sequencing

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. *In-situ* hybridisation

Not applicable.

4.7. Immunohistochemistry

4.7.1. 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]. The primary affinity purified anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the WOAH 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.

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 anti-EHNV antibody 1:/1500 Lot No. M708) and negative control reagent (non-immune serum at a dilution of 1/1500) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides.

vii) Biotin-labelled secondary link antibody: 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).

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

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 serum on the same section.

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

4.8. Bioassay

Not applicable.

4.9. Antibody- or antigen-based detection methods

An antigen ELISA for detection of EHNV and an EHNV antibody detection ELISA have been reported (Whittington & Steiner, 1993). Indirect ELISA for detection of antibodies induced following exposure to EHNV has been described for rainbow trout and European perch (Whittington *et al.*, 1994; 1999; Whittington & Reddacliff, 1995). The same antibodies are suitable for immunohistochemistry on fixed tissues and for detection of ranavirus antigen in cell culture. Reagents and protocols are available from the reference laboratory. It should be noted that polyclonal antibodies used in all related methods (immunoperoxidase, antigen-capture ELISA and immunoelectron microscopy) cross-react with all known ranaviruses except Santee Cooper ranaviruses (Ahne *et al.,* 1998; Cinkova *et al.,* 2010; Hedrick *et al*., 1992; Hyatt *et al.*, 2000).

4.10. Other methods

The sensitivity and specificity of these assays in relation to a standard test are not known and interpretation of results is difficult. Protocols and specific anti-immunoglobulin reagents required to conduct these tests are available from the reference laboratory.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the most appropriate method of screening healthy fish populations for EHNV; however, the available methods are not specific for EHNV. Any real-time PCR positive samples should be tested by conventional PCR and sequence analysis to distinguish EHNV from other ranaviruses.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status[2]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological links to an infected population. Hydrographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equates to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

2 For example transboundary commodities.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with EHNV shall be suspected if at least one of the following criteria is met:

i) EHNV-typical CPE in cell culture

ii) Positive real-time or conventional PCR result

iii) Positive EHNV antigen ELISA

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with EHNV is considered to be confirmed if at least one of the following criteria is met:

i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR followed by sequence analysis of the amplicon

6.2 Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with EHNV shall be suspected if at least one of the following criteria is met:

i) Histopathology consistent with EHNV

ii) EHNV-typical CPE in cell cultures

iii) Positive real-time or conventional PCR result

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with EHNV is considered to be confirmed if, in addition to the criteria in Section 6.2.1, at least one of the following criteria is met:

i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR followed by sequence analysis of the amplicon

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with EHNV are provided in Tables 6.3.1. and 6.3.2 (no data are currently available). This information can be used for the design of surveys for infection with EHNV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR	Diagnosis	Clinically diseased fish (multiple species) from disease outbreaks and experimental infections	Pool of kidney, liver and spleen from individual fish	European perch (*Perca fluviatilis*), river blackfish (*Gadopsis marmoratus*), golden perch (*Macquaria ambigua*), trout cod (*Maccullochella macquariensis*), freshwater catfish (*Tandanus tandanus*), Macquarie perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*)	94.3%* (*n* = 105)	100% (*n* = 441)	Virus isolation in BF-2 cell culture	Jaramillo *et al.,* (2012)
Real-time PCR	Diagnosis	Clinically diseased fish (multiple species) from disease outbreaks and experimental infections	Pool of kidney, liver and spleen from individual fish	European perch (*Perca fluviatilis*), river blackfish (*Gadopsis marmoratus*), golden perch (*Macquaria ambigua*), trout cod (*Maccullochella macquariensis*), freshwater catfish (*Tandanus tandanus*), Macquarie perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*)	95%* (*n* = 106)	100% (*n* = 80)	Virus isolation in BF-2 cell culture	Stilwell *et al.,* 2018

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study;
PCR: = polymerase chain reaction. Note: these assays detect multiple ranaviruses in addition to EHNV that infect amphibian hosts. *A positive result requires characterisation using sequencing to confirm that the result indicates the presence of EHNV.

6.3.2. For surveillance of apparently healthy animals: not available

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study.

7. References

AHNE W., BEARZOTTI M., BREMONT M. & ESSBAUER S. (1998). Comparison of European systemic piscine and amphibian iridoviruses with epizootic haematopoietic necrosis virus and frog virus 3. *J. Vet. Med.* [*B*], **45**, 373–383.

AHNE W., OGAWA M. & SCHLOTFELDT H.J. (1990). Fish viruses: transmission and pathogenicity of an icosahedral cytoplasmic deoxyribovirus isolated from sheatfish *Silurus glanis*. *J. Vet. Med.* [*B*], **37**, 187–190.

AHNE W., SCHLOTFELDT H.J. & THOMSEN I. (1989). Fish viruses: isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). *J. Vet. Med.* [*B*], **36**, 333–336.

ARIEL E. & BANG JENSEN B. (2009). Challenge studies of European stocks of redfin perch, *Perca fluviatilis* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), with epizootic haematopoietic necrosis virus. *J. Fish Dis.,* **32**, 1017–1025.

Ariel E, Holopainen R, Olenen NJ & Tapiovaara H (2010). Comparative study of ranavirus isolates from cod (*Gadua morhua)* and turbot (*Psetta maxima*) wuth reference to other ranaviruses. Archives of Virology **155**, 1261-1271

ARIEL E., NICOLAJSEN N., CHRISTOPHERSEN M.-B., HOLOPAINEN R., TAPIOVAARA H. & BANG JENSEN B. (2009). Propagation and isolation of ranaviruses in cell culture. *Aquaculture,* **294**, 159–164.

BECKER J.A., GILLIGAN D., ASMUS M., TWEEDIE A. & WHITTINGTON R.J. (2019). Geographic distribution of Epizootic haematopoietic necrosis virus (EHNV) in freshwater fish in south eastern Australia: lost opportunity for a notifiable pathogen to expand its geographic range. *Viruses*, **11**, 315 doi:10.3390/v11040315

BECKER J.A., TWEEDIE A., GILLIGAN D., ASMUS M. & WHITTINGTON R. J. (2016). Susceptibility of Australian Redfin Perch *Perca fluviatilis* Experimentally Challenged with Epizootic Hematopoietic Necrosis Virus (EHNV). *J. Aquat. Anim. Health*, **28**, 122–130.

BLOCH B. & LARSEN J.L. (1993). An iridovirus-like agent associated with systemic infection in cultured turbot *Scophthalmus maximus* fry in Denmark. *Dis. Aquat. Org*., **15**, 235–240.

BRYAN L.K., BALDWIN C.A., GRAY M.J. & MILLER D.L. (2009). Efficacy of select disinfectants at inactivating Ranavirus. *Dis. Aquat. Org.,* **84**, 89–94.

CHINCHAR V.G. (2002). Ranaviruses (family Iridoviridae): emerging cold-blooded killers – brief review. *Arch. Virol.*, **147**, 447–470.

CHINCHAR G., ESSBAUER S., HE J.G., HYATT A., MIYAZAKI T., SELIGY V. & WILLIAMS T. (2005). Family Iridoviridae. *In:* Virus Taxonomy. Classification and Nomeclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses, Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. & Ball L.A., eds. Academic Press, San Diego, California, USA, 145–161.

CINKOVA K., RESCHOVA S., KULICH P. & VESELY T. (2010). Evaluation of a polyclonal antibody for the detection and identification of ranaviruses from freshwater fish and amphibians. *Dis. Aquat. Org.,* **89**, 191–198.

CRANE M.S.J., YOUNG J. & WILLIAMS L. (2005). Epizootic haematopoietic necrosis virus (EHNV): growth in fish cell lines at different temperatures. *Bull. Eur. Assoc. Fish Pathol*., **25**, 228–231.

DRURY S.E.N., GOUGH R.E. & CALVERT I. (2002). Detection and isolation of an iridovirus from chameleons (*Chamaeleo quadricornis* and *Chamaeleo hoehnelli*) in the United Kingdom. *Vet. Rec.,* **150**, 451–452.

FIJAN N., MATASIN Z., PETRINEC Z., VALPOTIC I. & ZWILLENBERG L.O. (1991). Isolation of an iridovirus-like agent from the green frog (*Rana esculenta* L.). *Veterinarski Arhiv,* **61**, 151–158.

HEDRICK R.P., MCDOWELL T.S., AHNE W., TORHY C. & DE KINKELIN P. (1992). Properties of three iridovirus-like agents associated with systemic infections of fish. *Dis. Aquat. Org.,* **13**, 203–209.

HOLOPAINEN R., OHLEMEYER S., SCHÜTZE H., BERGMANN S.M. & TAPIOVAARA H. (2009). Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Dis. Aquat. Org.,* **85**, 81–91.

HYATT A.D., GOULD A.R., ZUPANOVIC Z., CUNNINGHAM A.A., HENGSTBERGER S., WHITTINGTON R.J., KATTENBELT J. & COUPAR B.E.H. (2000). Comparative studies of piscine and amphibian iridoviruses. *Arch. Virol.,* **145**, 301–331.

HYATT A.D., WILLIAMSON M., COUPAR B.E.H., MIDDLETON D., HENGSTBERGER S.G., GOULD A.R., SELLECK P., WISE T.G., KATTENBELT J., CUNNINGHAM A.A.& LEE J. (2002). First identification of a ranavirus from green pythons (*Chondropython viridis*). *J. Wildl. Dis.,* **38**, 239–252.

JARAMILLO D., TWEEDIE A., BECKER J.A., HYATT A., CRAMERI S. & WHITTINGTON R.J. (2012). A validated quantitative polymerase chain reaction assay for the detection of ranaviruses (Family Iridoviridae) in fish tissue and cell cultures, using EHNV as a model. *Aquaculture*, **356–357**, 186–192.

LANGDON J.S. (1989). Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. *J. Fish Dis*., **12**, 295–310.

LANGDON J.S. & HUMPHREY J.D. (1987). Epizootic Hematopoietic Necrosis a New Viral Disease in Redfin Perch *Perca fluviatilis* L. in Australia. *J. Fish Dis.*, **10**, 289–298.

LANGDON J.S., HUMPHREY J.D. & WILLIAMS L.M. (1988). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *J. Fish Dis.*, **11**, 93–96.

LANGDON J.S., HUMPHREY J.D., WILLIAMS L.M., HYATT A.D. & WESTBURY H.A. (1986). First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. *J. Fish Dis.*, **9**, 263–268.

MAO J., THAM T.N., GENTRY G.A., AUBERTIN A. & CHINCHAR V.G. (1996). Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. *Virology*, **216**, 431–436.

MAO J.H., HEDRICK R.P. & CHINCHAR V.G. (1997). Molecular characterisation, sequence analysis and taxonomic position of newly isolated fish iridoviruses. *Virology*, **229**, 212–220.

MARSH I.B., WHITTINGTON R.J., O'ROURKE B., HYATT A.D. & CHISHOLM O. (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. *Molec. Cell. Probes*, **16**, 137–151.

PALLISTER J., GOULD A., HARRISON D., HYATT A., JANCOVICH J. & HEINE H. (2007). Development of real-time PCR assays for the detection and differentiation of Australian and European ranaviruses. *J. Fish Dis.,* **30**, 427–438.

POZET F., MORAND M., MOUSSA A., TORHY C. & DE KINKELIN P. (1992). Isolation and preliminary characterization of a pathogenic icosahedral deoxyribovirus from the catfish (*Ictalurus melas*). *Dis. Aquat. Org.,* **14**, 35–42.

REDDACLIFF L.A. & WHITTINGTON R.J. (1996). Pathology of epizootic haematopoeitic necrosis virus (EHNV) infection in rainbow trout (*Oncorhynchus mykiss* Walbaum) and redfin perch (*Perca fluviatilis* L.). *J. Comp. Pathol.,* **115**, 103–115.

RIMMER A.E., BECKER J.A., TWEEDIE A. & WHITTINGTON R.J. (2012). Validation of high throughput methods for tissue disruption and nucleic acid extraction for ranaviruses (family Iridoviridae). *Aquaculture*, **338–341**, 23–28.

SPEARE R. & SMITH J.R. (1992). An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. *Dis. Aquat. Org.*, **14**, 51–57.

STILWELL N.K., WHITTINGTON R.J., HICK P.M., BECKER J.A., ARIEL E., VAN BEURDEN S., VENDRAMIN N., OLESEN N.J. & WALTZEKT.B. (2018). Partial validation of a TaqMan real-time quantitative PCR for the detection of ranaviruses. *Dis. Aquat. Org*., **128**, 105–116.

WHITTINGTON R.J., BECKER J.A. & DENNIS M.M. (2010). Iridovirus infections in finfish – critical review with emphasis on ranaviruses. *J. Fish Dis.,* **33**, 95–122.

WHITTINGTON R.J., KEARNS C., HYATT A.D., HENGSTBERGER S. & RUTZOU T. (1996). Spread of epizootic haematopoietic necrosis virus (EHNV) in redfin perch (*Perca fluviatilis*) in southern Australia. *Aust. Vet. J.*, **73**, 112–114.

WHITTINGTON R.J., PHILBEY A., REDDACLIFF G.L. & MACGOWN A.R. (1994). Epidemiology of epizootic haematopoietic necrosis virus (EHNV) infection in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum): findings based on virus isolation, antigen capture ELISA and serology. *J. Fish Dis.*, **17**, 205–218.

WHITTINGTON R.J. & REDDACLIFF G.L. (1995). Influence of environmental temperature on experimental infection of redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) with epizootic haematopoietic necrosis virus, an Australian iridovirus. *Aust. Vet. J.,* **72**, 421–424.

WHITTINGTON R.J., REDDACLIFF L.A., MARSH I., KEARNS C., ZUPANOVIC Z. & CALLINAN R.B. (1999). Further observations on the epidemiology and spread of epizootic haematopoietic necrosis virus (EHNV) in farmed rainbow trout *Oncorhynchus mykiss* in southeastern Australia and a recommended sampling strategy for surveillance. *Dis. Aquat. Org.,* **35**, 125–130.

WHITTINGTON R.J. & STEINER K.A. (1993). Epizootic haematopoietic necrosis virus (EHNV): improved ELISA for detection in fish tissues and cell cultures and an efficient method for release of antigen from tissues. *J. Virol. Methods,* **43**, 205–220.

WOLF K., BULLOCK G.L., DUNBAR C.E. & QUIMBY M.C. (1968). Tadpole edema virus: a viscerotrophic pathogen for anuran amphibians. *J. Infect. Dis.,* **118**, 253–262.

ZUPANOVIC Z., MUSSO C., LOPEZ G., LOURIERO C.L., HYATT A.D., HENGSTBERGER S. & ROBINSON A.J. (1998). Isolation and characterisation of iridoviruses from the giant toad *Bufo marinus* in Venezuela. *Dis. Aquat. Org.,* **33**, 1–9.

*
* *

NB: There is a WOAH Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV) (please consult the WOAH web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on infection with EHNV. The WOAH Reference Laboratory can supply purified EHNV DNA, heat-killed EHNV antigen and polyclonal antibodies against EHNV together with technical methods. A fee is charged for the reagents to cover the costs of operating the laboratory.

NB: FIRST ADOPTED IN 1995 AS EPIZOOTIC HAEMATOPOIETIC NECROSIS. MOST RECENT UPDATES ADOPTED IN 2023.