CHAPTER 2.1.3.

INFECTION WITH RANAVIRUS

1. Scope

For the purpose of this chapter, ranavirus disease is considered to be systemic clinical or subclinical infection, in the major families of Anura and Caudata, with a member of the genus *Ranavirus.* It does not include epizootic haematopoietic necrosis virus, which is the aetiological agent for epizootic haematopoietic necrosis (EHN).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Ranaviruses belong to the genus *Ranavirus* of the Family *Iridoviridae.* The type species is frog virus 3 (FV3) (Chinchar *et al.,* 2005). Other species include Bohle virus (BIV), epizootic haematopoietic necrosis virus (EHNV), European catfish virus (ECV), European sheatfish virus (ESV) and Santee-Cooper ranavirus. There are many other tentative species in this genus. Since the recognition of disease caused by EHNV in finfish in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in amphibians. Ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe, Asia and Australia (Chinchar, 2002; Drury *et al.,* 1995; Fijan *et al.,* 1991; Hyatt *et al.,* 2002; Speare & Smith, 1992; Wolf *et al.,* 1968; Xia *et al*., 2009; Zupanovic *et al.,* 1998b). Ranaviruses have large (150–170 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). They possess common antigens that can be detected by several techniques.

Species	No. isolates	Examples	Geographic source
Ambystoma tigrinum virus	2	*Ambystoma tigrinum* virus, Regina ranavirus	North America
Bohle iridovirus	1	Bohle iridovirus	Australia
Frog virus 3	12	Frog virus 3	Europe, North & South America
		Box turtle virus 3	Europe, North & South America
		Bufo bufo United Kingdom virus	Europe, North & South America
		Bufo marinus Venezuelan iridovirus 1	Europe, North & South America
		Lucké triturus virus 1	Europe, North & South America
		Rana temporaria United Kingdom virus	Europe, North & South America
		Redwood Park virus	Europe, North & South America
		Stickleback virus	Europe, North & South America
		Tadpole oedema virus	Europe, North & South America
		Tadpole virus 2	Europe, North & South America
		Tiger frog virus	Europe, North & South America
		Tortoise virus 5	Europe, North & South America
Tentative species	3	*Rana esculenta* iridovirus	Europe, North & South America
		Testudo iridovirus	Europe, North & South America

2.1.2. Survival outside the host

All ranaviruses are probably extremely resistant to drying; EHNV can survive for months in water, in frozen fish tissues for more than 2 years (Langdon, 1989), and in frozen fish carcases for at least a year (Whittington *et al.,* 1996). Santee-Cooper ranavirus remains viable in frozen fish tissues for at least 155 days (Plumb & Zilbert, 1999). Less is known about other ranaviruses, but given their similarity to

EHNV they are presumed to have comparable stability. ATV was infectious for salamanders if present in moist but not dry pond sediment, but the duration of infectivity is unknown.

2.1.3. Stability of the agent (effective inactivation methods)

Ranaviruses (as exemplified via EHNV) are susceptible to 70% ethanol, 200 mg litre^{-1} sodium hypochlorite or heating to 60°C for 15 minutes (Fijan *et al.,* 1991). If desiccated first, EHNV may survive heating to 60°C for 15 minutes (unpublished observations). 10^7 plaque-forming units per ml of a ranavirus of amphibian origin was inactivated within 1 minute in a solution of 150 mg litre^{-1} chlorhexidine (0.75% Nolvasan®[1]), 180 mg litre^{-1} sodium hypochlorite (3% bleach) or 200 mg litre^{-1} potassium peroxymonosulfate (1% Virkon®) (Bryan *et al.,* 2009).

2.1.4. Life cycle

The route of infection is unknown but amphibians are susceptible experimentally following bath exposure injection and or exposure following laboratory induced abrasions. (Cunningham *et al.,* 2007; 2008).

2.2. Host factors

2.2.1. Susceptible host species

Natural ranavirus infections are known from most of the major families of Anura and Caudata (Carey *et al.* 2003a; 2003b; Cullen & Owens, 2002; Daszak *et al.,* 2003).

2.2.2. Susceptible stages of the host

Susceptible stages of the host are all age classes, larvae, metamorphs and adults.

2.2.3. Species or subpopulation predilection (probability of detection)

Not known.

2.2.4. Target organs and infected tissue

Amphibian target organs and tissues infected with ranaviruses may vary. Three examples are given: i) BIV: liver, kidney, spleen, lung and other parenchymal tissues (Cullen & Owens, 2002). ii) FV3 infects proximal tubular epithelial cells in the kidney, the liver, and the gastrointestinal tract (Robert *et al.,* 2005). iii) United Kingdom ranavirus (RUK) infects epithelial cells, fibroblasts, lymphocytes, melanomacrophages and a small proportion of endothelial cells in many tissues, as well as hepatocytes and Kuppfer cells in the liver, the epidermis and dermis (Cunningham *et al.,* 2008). *Ambystoma tigrinum virus* is found in skin, spleen, liver, renal tubular epithelial cells, and lymphoid and haematopoietic tissues of salmanders.

2.2.5. Persistent infection with lifelong carriers

Not known.

2.2.6. Vectors

Amphibians can become infected in the same way as fish and, as such, details associated with EHNV are included here. Possible vectors include nets, boats and other equipment, or in amphibians used for bait by recreational fishers. Birds are potential mechanical vectors, as ranaviruses can be carried in the gut, on feathers, feet and the bill. It should be noted that ranaviruses are likely to be inactivated at typical avian body temperatures (40–44°C). Nevertheless, it is possible that ranaviruses (as evidenced by EHNV) can be spread by regurgitation of ingested material within a few hours of feeding is possible (Whittington *et al.,* 1996). In addition amphibians have been shown to be infected by exposure to sediment from sites where ranavirus die-offs have occurred.

2.2.7. Known or suspected wild aquatic animal carriers

Not known.

1 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*.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Ranavirus infections can occur from animal-to-animal contact, ingestion of infected, dying and dead individuals (e.g. Cullen & Owens, 2002; Picco & Collins, 2008). Viruses can also be spread between widely separated river systems and impoundments. Transmission is understood to occur by means other than water (refer above); mechanisms include translocation of live fish or bait by recreational fishers (e.g. Pico *et al*., 2007).

2.3.2. Prevalence

Ranavirus infections have been reported on five continents including Asia (Gray *et al.,* 2009); its prevalence, based on intensive widespread serosurveillance, antigen detection, is not known.

2.3.3. Geographical distribution

Ranaviruses have been recovered from free-living or farmed, healthy or diseased frogs in America, continental Europe, the United Kingdom and Asia (Ariel *et. al*., 2009;Chinchar, 2002; Cunningham *et al.*, 1996; Drury *et al.*, 1995; Fijan *et al.*, 1991; Fox *et al.*, 2006; Green *et al.,* 2002; He *et al.*, 2002; Wolf *et al.*, 1968; Zhan *et al.*, 2001; Zupanovic *et al.*, 1998b) as well as diseased free-living spotted salamanders *Ambystoma maculatum* in North America (Docherty *et al.*, 2003; Jancovich *et al.*, 2003). Bohle iridovirus (BIV), which is distinct from FV3, was isolated originally from diseased ornate burrowing frog *Limnodynastes ornatus* tadpoles in far north Queensland, Australia (Speare & Smith, 1992). It has not been isolated since, although there is serological evidence of ranavirus infection in cane toads *Bufo bufo* in that region (Whittington *et al.*, 1996). Another distinct species of ranavirus, *Ambystoma tigrinum* virus (ATV), is responsible for die-offs in the tiger salamander *A. tigrinum* (Jancovich *et al.*, 2005). Viruses closely related to FV3 have also been recovered from reptiles. Wamena iridovirus (WIV) was isolated in Australia from diseased green pythons *Chondropython viridis* smuggled from West Papua (Irian Jaya) while THIV (TV-CH8) was recovered from diseased Hermann's tortoises *Testudo hermanni* in Europe. Both WIV and THIV had >97% nucleotide sequence homology with FV3 in the regions of MCP that were examined (Hyatt *et al.*, 2002; Marshang *et al.*, 1999).

2.3.4. Mortality and morbidity

Mortality and morbidity vary from species to species. Laboratory infections and field data show that mortality can range from low (e.g. 0%) to 100% of infected animals of an experimental group depending on species, virus and age and health status of the host. following short infection times (Harp & Petranka, 2006; Hyatt *et al.* 1998; Pearman *et al.*, 2004) However other experiments involving different host species and ranaviruses gave variable results (Brunner *et al.*, 2004; 2007; Cunningham *et al.*, 2007).

2.3.5. Environmental factors

Natural epizootics of amphibian ranaviruses appear to be similar for piscine ranaviruses (e.g. EHNV). Epizootics appear to be seasonal and can be related to poor husbandry (captive populations) and overcrowding (wild and captive). It has been assumed that for some amphibians such as salamanders (references) disease is related to the annual appearance of large numbers of non-immune young animals and their subsequent exposure to the virus in shallow waters (Brunner *et al.*, 2004; 2007; Green *et al.,* 2002; Greer & Collins, 2008; Greer *et al.*, 2008; Jancovich *et al.*, 1997; 2001; Rojas *et al.*, 2005).

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

Not tested.

3. Sampling

3.1. Selection of individual specimens

A simple method for preparation of tissues for cell culture and enzyme-linked immunosorbent assay (ELISA) has been validated in fish (Whittington & Steiner, 1993; Whittington *et al.*, 1999).

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

Large amphibians: (*>60 mm length*) remove 0.1 g liver, kidney, spleen (± 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 amphibian (*30–60 mm length*): scrape all viscera into the tube.

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

3.2. Preservation of samples for submission

For cell culture and ELISA, freeze tubes containing tissues at temperatures from –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 animals on the sensitivity of diagnostic tests has not been evaluated. However, tissues for virus isolation are commonly pooled in lots of 5 or 10 individuals per test.

3.4. Best organs or tissues

Liver, kidney, spleen, lung, skin.

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 two syndromes in frogs associated with ranavirus infection: a chronic ulcerative syndrome and an acute haemorrhagic syndrome (Cunningham *et al.*, 1996). Salmanders infected with *Ambystoma tigrinum* virus develop ulcerative dermatitis and enteritis. Affected larvae have small multifocal

haemorrhages affecting subcutaneous tissue on the plantar surface of feet, the inguinal area, and the vent area, with ventral oedema and the skin may contain pale foci (Bollinger *et al.*, 1999; Docherty *et al.*, 2003).

4.1.2. Behavioural changes

Field and behaviour changes differ between species, life stage and severity of disease. Changes include lordosis, erratic swimming, lethargy and loss of equilibrium (Gray *et al.*, 2009).

4.2. Clinical methods

4.2.1. Gross pathology

There may be no gross lesions or nonspecific lesions. There are two syndromes in frogs associated with ranavirus infection: ulcerative syndrome and haemorrhagic syndrome (Cunningham *et al.*, 1996). In salmanders infected with *Ambystoma tigrinum* virus there may be ulcerative dermatitis, pale foci in the skin, small multifocal haemorrhages affecting subcutaneous tissue on the plantar surface of feet, the inguinal area, the vent, the subserosal surface of the intestine, and the liver may be pale and swollen; there may be ventral oedema (Bollinger *et al.*, 1999; Docherty *et al.*, 2003).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

BIV and FV3 cause multifocal multi-organ haemorrhage and necrosis (Cullen & Owens, 2002; Robert *et al.*, 2005). Salmanders infected with *A.tigrinum* virus develop necrosis in many tissues including spleen, liver, renal tubular epithelial cells, and lymphoid and haematopoietic tissues (Bollinger *et al.*, 1999). Amphophilic intracytoplasmic inclusion bodies may be present in cells in many organs together with single cell or variable sized areas of focal necrosis (Bollinger *et al.*, 1999; Docherty *et al.*, 2003). In skin there may be foci of spongiosis and ballooning degeneration, erosion and ulceration and hyperplasia of epidermal epithelial cells which may have intracytoplasmic inclusion bodies (Bollinger *et al.*, 1999).

4.2.4. Wet mounts

Not applicable.

4.2.5. Smears

Not tested.

4.2.6. Fixed sections

Refer to Section 4.3.

4.2.7. 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 (ranaviruses can vary greatly in size ranging from approximately 150 nm to >170 nm) non-enveloped 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 ranavirus. Formalin-fixed paraffin-embedded sections can also be stained using

an immunoperoxidase method (see below) to identify ranavirus antigen associated with necrotic lesions.

Electron microscopy: Ultrathin routine sectioning methods can be used for preparation of tissues and cell cultures (Eaton *et al.*, 1991) 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 (Hyatt, 1991).

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 (Eaton *et al.*, 1991).

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 amphibian tissues for virus isolation and ELISA

A simple method for preparation of tissues for cell culture and ELISA has been described (Whittington & Steiner, 1993; Whittington *et al.*, 1999) (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 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.

Cell culture/artificial media

Cell culture is the gold-standard test but is costly and time consuming. Ranaviruses grow well in many fish cell lines including BF-2 (bluegill fry ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (epithelioma papulosum cyprini [Fijan *et al.*, 1983]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15°C to 22°C (Crane *et al.*, 2005), 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 (see Section 4.3.1.2.2). *Ambystoma tigrinum* virus produces CPE like that of EHNV in FHM, RTG and bullfrog tongue cells at 25°C (Docherty *et al.*, 2003). Others have used frog embryo fibroblasts at 27°C or FHM cells to isolate or propagate the United Kingdom isolates of FV3 (Cunningham *et al.*, 1996, 2007).

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% 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 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 EHNV. 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 (Hyatt *et al.*, 2000).

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

Principle of the test: ranaviruses replicate within cultured cells. The addition of a mild detergent permeabilises the cells allowing an affinity purified rabbit antibody to bind to intracellular viral proteins. Ranavirus 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-ethane-sulfonic acid [HEPES], 2 mM glutamine, 100 IU penicillin and 100 µg streptomycin) and incubated in 5% CO_2 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-EHNV 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 de-ionised 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: non-granular, non-focal, more generalised, pale, pinkish staining may occur throughout the culture. This background staining could be caused by any number of reasons, e.g. non-specific antibody reaction with non-viral 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_2HPO_4	11.5 g
KCl	2.0 g
KH_2PO_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 $Na_2HPO_4.2H_2O$ 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 ranavirus using antigen-capture ELISA

Antigen-capture ELISA has been validated to detect EHNV in cell cultures and directly in fish tissue homogenates. The same assay can be applied to amphibian tissues. The analytical sensitivity is 10^3 to 10^4 $TCID_{50}$ 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 (Drury *et al.*, 1995; Marsh *et al.*, 2002; 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 (Hengstberger *et al.*, 1993; Hyatt *et al.*, 1991; Reddacliff & Whittington, 1996). 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.

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 $well^{-1}$) 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}) (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^{-1}). 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^{-1}). 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 H_2O_2) (100 µl well^{-1}) 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^{-1}), 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 ($Na_2B_4O_7.10H_2O$)	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_2HPO_4	11.50 g
KH_2PO_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_2HPO_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_2O_2 per 22-ml aliquot.	

ABTS stop solution (0.01% NaN_3 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 #14-23-06[2]

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.	

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

2 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

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 (Hyatt, 1991). These collective data enable classification to the genus Ranavirus.

Cell cultures and tissues

i) Fix tissues or cell cultures as described in Hyatt (1991). 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. Spurrs 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 × 3minutes, 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 ranavirus antigens in formalin-fixed paraffin-embedded tissue sections (He *et al.*, 2002). 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[3]. 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) De-paraffinise the section:

Pre-heat 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.

3 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.

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

Ranavirus 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 fish ranaviruses (EHNV, ECV) from amphibian ranaviruses (FV3, BIV) (Harp & Petranka, 2006). 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 (refer to Chapter 2.3.2 *Infection with epizootic haematopoietic necrosis virus*).

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 4.1.

Table 4.1. *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 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 $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 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 4.2.

Table 4.2. *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°C or 62°C to reduce non-specific 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.

Table 4.3. *Restriction endonuclease analysis of ranavirus MCP amplicons*

PCR Assay	Restriction enzyme	Predicted band sizes after restriction (bp)	Pattern applies to
MCP-1 (321bp)	*Pfl*M I	321	EHNV, BIV
		131, 190	FV3, WIV
MCP-2 (625bp)	*Hinc* II	100, 138, 387	EHNV
		100, 525	BIV, FV3
		100, 240, 285	WIV
	Acc I	238, 387	EHNV
		625	BIV, ESV, ECV, WIV
		164, 461	FV3, GV
	*Fnu*4H I	33, 38, 44, 239, 271	EHNV
		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	WIV

Restriction endonuclease analysis (REA)

PCR amplicons are subjected to REA with the enzymes described in Table 4.3. 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 PflM 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 4.3.

GV: Gutapo virus (Hyatt *et al*., 2000).

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 EHNV 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 EHNV DNA by PCR. This PCR procedure can be used for the specific detection of ranaviruses from redfin perch, rainbow trout, sheatfish, catfish, guppy fish (*Poecilia reticulata*), doctor fish (*Labroides dimidatus*) and a range of amphibian ranaviruses (Eaton *et al.*, 1991). 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 $MgCl_2$. 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 EHNV has been described (Drury *et al.*, 1995; Hyatt *et al.*, 2000) 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 ranaviruses Indirect ELISA for detection of antibodies induced following exposure to ranavirus has been described for *Bufo marinus* 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 ranavirus 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 *Principles and methods of validation of diagnostic assays for infectious diseases*), 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	Tadpoles	Metamorphs	Adults		
Gross signs	n/a	d	d	d	d	d
Histopathology	n/a	d	d	d	b	d

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Ova/milt	Tadpoles	Metamorphs	Adults		
Immunoperoxidase stain	n/a	c	c	c	b	b
Transmission EM	n/a	d	d	d	c	c
Immuno-EM	n/a	d	d	d	c	c
Cell culture	n/a	a	a	a	a	a
Antigen-capture ELISA	n/a	a	a	a	b	b
Antibody-capture ELISA	n/a	d	d	c	c	d
PCR-REA	n/a	d	a	d	c	a
PCR sequence analysis	n/a	d	d	d	c	a

EM = electron microscopy; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; REA: restriction endonuclease analysis; n/a: not applicable

6. Test(s) recommended for targeted surveillance to declare freedom from ranavirus

Statistically valid sampling practices need to be used but these cannot presently be defined for amphibians.

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.

Serology might also play a useful role in surveys to identify infected amphibian populations. Further research is required to confirm the validity of this approach.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Amphibian, apparently healthy, moribund or dead in which skin and or 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

Amphibian, apparently healthy, moribund or dead in which skin and or 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 ranavirus is demonstrated by the following means:

1. Characteristic CPE in cell culture and cell culture is positive for ranavirus 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, where PCR is used

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

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NB: There is an OIE Reference Laboratory for Infection with ranavirus
(see Table at the end of this *Aquatic Manual* or consult the OIE web site:
https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratory for any further information on
Infection with ranavirus

NB: FIRST ADOPTED IN 2011.