AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

SEPTEMBER 2010 REPORT

USA Comments

CHAPTER 2.1.2.

INFECTION WITH RANAVIRUS

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2.3.4. Mortality and morbidity

Mortality and morbidity vary from species to species. Laboratory infections show that >75–100% of infected animals of an experimental group following short infection times (Harp & Petranka, 2006; 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).

General Comment to Article 2.3.4 : The second sentence of this paragraph is incomplete. We are not clear if the >75-100% of the experimentally infected animals become morbid or die.

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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 (e*pithelioma papulosum cyprini* [Fijan *et al.*, 1983]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 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 FV-3 (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. Raanvirus is detected by a biotinylated anti-species antibody and a streptavidin–peroxidase conjugate. The addition of a substrate results in ‘brick-red’ staining in areas labelled with antibodies.

Samples: tissue homogenates.

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

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

i) CHSE-214, 24-well plates are seeded the day before use with 250,000 cells/well (or 4 million cells in 40 ml of growth medium per plate) in 1.5 ml of growth medium (Earle's MEM with non-essential amino acids [EMEM], 10% FCS, 10 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid [HEPES], 2 mM glutamine, 100 IU penicillin and 100 µg streptomycin) and incubated in 5% 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.

General Comment about Articles 4.3.1.2.1 and 4.3.1.2.2:
Formatting of these sections is inconsistent. The first half of Article 4.3.1.2.1 and Article 4.3.1.2.2 use lower case Roman numerals, while the Cell culture/artificial media section of Article 4.3.1.2.1 describes the laboratory protocol in paragraph form.