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ISOLATION OF FISH VIRUSES AND ITS CONFIRMATION IN CELL CULTURE

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Viruses are obligate intracellular parasites that require living cells in order to replicate. Cultured cells, eggs and laboratory animals may be used for virus isolation. Although embroyonated eggs and laboratory animals are very useful for the isolation of certain viruses, cell cultures are the sole system for virus isolation in most laboratories. The development of methods for cultivating animal cells has been essential to the progress of animal virology. Cell lines vary greatly in their susceptibility to different viruses. It is of utmost importance that the most sensitive cell lines are used for a particular suspected virus.

Specimens for isolation on cell culture should be transported to the laboratory as soon as possible upon being taken. Swabs should be put in a vial containing virus transport medium. Bodily fluids and tissues should be placed in a sterile container. Upon receipt, the specimen is inoculated into several different types of cell lines depending on the nature of the specimen and the clinical presentation. The maintenance media should be changed after 1 hour or if that is not practicable, the next morning. The inoculated tubes should be read at least every other day for the presence of cytopathic effect.

Certain specimens, such as urine and faeces, may be toxic to cell lines that may produce a CPE-like effect. If toxic effects are extensive, it may be necessary to passage the inoculated cells. Cell lines that are contaminated by bacteria should either be put up again or passed through a bacterial filter. Cell lines should be kept for at least one to two weeks (longer in the case of CMV). Cell lines should be added with fresh maintenance medium at regular intervals or if required should the culture medium become too acidic or alkaline. When CPE is observed, it may be advisable to passage infected culture fluid into a fresh culture of the same cell type.

Transportation and collection of samples

Pools of organs or of ovarian fluids are placed in sterile vials and stored at 4°C or on ice until virus extraction is performed in the laboratory. However, freezing of samples for testing for subclinical carriers should be avoided. Organ samples may also be transported to the laboratory by placing them in vials containing cell culture medium or Hanks' balanced salt

solution (HBSS) with added antibiotics to suppress the growth of bacterial contaminants (one volume of organ in at least five volumes of transportation fluid). Suitable antibiotic concentrations are: gentamicin (1000 μg ml^{-1}) or penicillin (800 IU ml^{-1}) and streptomycin (800 μg ml^{-1}). Antifungal compounds, such as Mycostatin® or Fungizone®, may also be incorporated into the transport medium at a final concentration of 400 IU ml^{-1}. Serum or albumin (5–10%) may be added to stabilise the virus if the transport time will exceed 12 hours.

Selection of tissue samples

The selection of tissues for virus assays varies according to the size and life stage of fish. The following tissues are the minimum that should be taken for virus assays.

Size/maturity of fish tissue assayed

- Under 4 cm- Entire fish (remove yolk sac if present)
- 4-6 cm- Entire viscera (includes kidney)
- Over 6 cm- Kidney and spleen
- Sexually mature- Ovarian fluid, kidney, and spleen

For fish 4 to 6 cm or over 6 cm, it is recommended (OIE 2013) that brain tissue also be included. The addition of gill filaments to the sample pool may also increase the sensitivity of detection for some viruses.

After tissues and fluid are removed from the fish, they can be pooled; however, no more than five fish should be in one pooled sample of tissue or fluid. Approximately equal volume or weight proportions should be maintained for each specimen in a pool.

Storage of samples

- The samples should be maintained between 4 and 10°C according to the virus (es) suspected. Samples should not be frozen.
- The samples should not be stored longer than 48 hours.
- Tissues may be stored in a buffered solution that contains antibiotics, antifungal, or both. The pH should be maintained within 7.4 to 7.8 or within the range that the suspected viruses (es) are stable.

Preparing samples for virus assays

- The preparation of samples involves the homogenization of tissues and bacterial and fungal decontamination of tissues and fluids.
- Homogenization can be accomplished in several ways; however, sonication is not acceptable for tissues. After homogenization, cellular material should be removed by centrifugation.
- Decontamination can be accomplished either with the use of antibiotics and antifungal or by filtration of the supernatant of centrifuged tissues samples.

- The antibiotics and antifungal that are used should be wide spectrum in their activity, and their concentrations should be effective in decontamination but not adversely affect cell cultures.

The following compounds and concentrations should not be exceeded:

- Gentamicin 1000 µg/mL
- Penicillin 800 IU/mL
- Streptomycin 800 µg/mL
- Fungizone® 40 IU/mL
- Mycostatin® 400 IU/mL

The supernatant from centrifuged tissue samples can also be decontaminated by filtration through a 0.45 µm filter. Passing tissue culture medium supplemented with serum through the filter before filtration of the sample is passed is recommended to minimize virus adherence to the filter.

Inoculating the Samples

Selection of Cell Cultures

Each virus section should be consulted to determine the most sensitive cell line(s) for a given virus. The cells should be normal appearing, rapidly dividing, and mycoplasma-free. Stock cell cultures should be routinely tested for susceptibility to specific viruses and for the presence of *Mycoplasma* Penicillin (100IU/mL) streptomycin (100µg/mL) and antifungal agents such as Mycostatin$^{(R)}$ (50 IU/mL) can be used in media for cell culture and virus assay work.

Inoculation

Direct inoculation; transfer an appropriate volume of the antibiotic-treated or filtered homogenate on to 24 to 48 hour old cell monolayer in tissue culture flasks or multi-well plates. Inoculate at least 5 cm^2 of cell monolayer with 100 µL of the filtered supernatant. Alternatively, make a further tenfold dilution of the filtered supernatant in cell culture medium, buffered at pH 7.6 and supplemented with 2% foetal calf serum (FCS), and allow adsorption for 0.5–1 hour at 18–22°C. Then, without withdrawing the inoculate, add the appropriate volume of cell culture medium (1–1.5ml/5 cm^2 for cell culture flasks), and incubate at 20-25°C. The cell cultures used for sample inoculation should be 80-90% confluent and not older than 48 hours. A minimum of 50 µL of sample should be inoculated per l.0 $cm^2$of cell sheet. Un-inoculated controls must be used. Dilution of original samples should not exceed 1:10 for fluids and 1:100 for tissue samples.

Duration of Assay

The cell cultures should be incubated at 28°C and observed for cytopathological changes for a minimum of 14 days but 21 days incubation is recommended. Cell culture medium should be buffered or cells incubated so that a pH between 7.4 and 7.8 is maintained. A blind pass of 14 days is also recommended. The duration of the assay may need to be longer depending on

which viruses are suspected. When cytopathological changes occur, the cultures should be sub cultured or analyzed by serum neutralization or other confirmatory tests.

If effects have been observed after inoculation of antibiotic-treated homogenate, filter at least 1 ml of the organ homogenate supernatant through a 0.45 µm disposable cellulose acetate filter unit (or unit fitted with a similar low protein binding filter membrane).

Monitoring incubation

Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at 40×–100× magnification for 14 days. The use of a phase-contrast microscope is recommended.

- Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition of sterile bicarbonate buffer or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) to the inoculated cell culture medium for tightly closed cell culture flasks.
- If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately.
- If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be sub-cultured for a further 14 days. If the virus control fails to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

Viral Plate Observation

Following inoculation of plates, all wells will be monitored on the following day and every other day for the next two weeks for signs of cytopathic effects (CPE), toxicity, and contamination. Plates will be monitored twice during the following week. Total observation period will be 21 d. If no CPE, toxicity or abnormalities are observed within 21 d, the samples are discarded and recorded as negative.

Re-Inoculation

If toxicity, abnormal pH or CPE is observed, one of the replicate wells of that sample will be aseptically aspirated, diluted 1: 10 with medium, filtered through a *0.45µm* filter and re-inoculated onto another 24-well test plate in duplicate and monitored for CPE an additional 14 d. All observations will be documented and recorded by the observer and kept on file with the laboratory records. If no CPE is observed in 14 days after re- inoculation, the sample is discarded and recorded as negative. If CPE is observed, proceed to corroborative methods to confirm identity of suspect virus.

Cytopathic Effects (CPE) of Virus Infection in Tissue Culture Cells

IHNV-induced CPE

- Rounded and granular cells in grape-like clusters.

- Rounded, infected cells also accumulate at plaque margins and can be present within the plaque.

IPNV -induced CPE

- Spindle-shaped or "balloon-on-a-stick"-shaped cells.
- Pyknosis of nuclei (nuclei shrink in size and chromatin condenses).
- Plaques are stellate in a confluent cell monolayer and contain not only live cells but also normal looking cells.

Herpesvirus-induced CPE

- Pyknosis of nuclei and cellular fusion (syncytia).
- Plaques tend to elongate and follow whorl lines of growth if on RTG-2 cells.
- They have relatively clear interiors, but living cells extend into the open area.

VHSV-induced CPE

- The VHSV isolates plaque very similarly to IHNV in EPC cells forming rounded and granular cells in grape-like clusters.
- Number of days following infection with virus that CPE is usually observed in freshly mono layered fish cell cultures.

LMBV-induced CPE

- CPE within 48 h after inoculation.
- Initial CPE - few pyknotic cells, which develop to form circular, cell free areas, with rounded cells at the margins.
- Advanced CPE - Pyknosis, rounding and detached cell sheet. Entire cell sheet affected.

Confirmatory identification

The confirmation of the etiological agent should be done using following methods,

- Confirmation of virus identity by neutralisation
- Confirmation of virus identity by the indirect fluorescent antibody test (IFAT)
- Confirmation of virus identity by enzyme-linked immunosorbent assay (ELISA)
- Confirmation of virus identity by polymerase chain reaction (PCR)

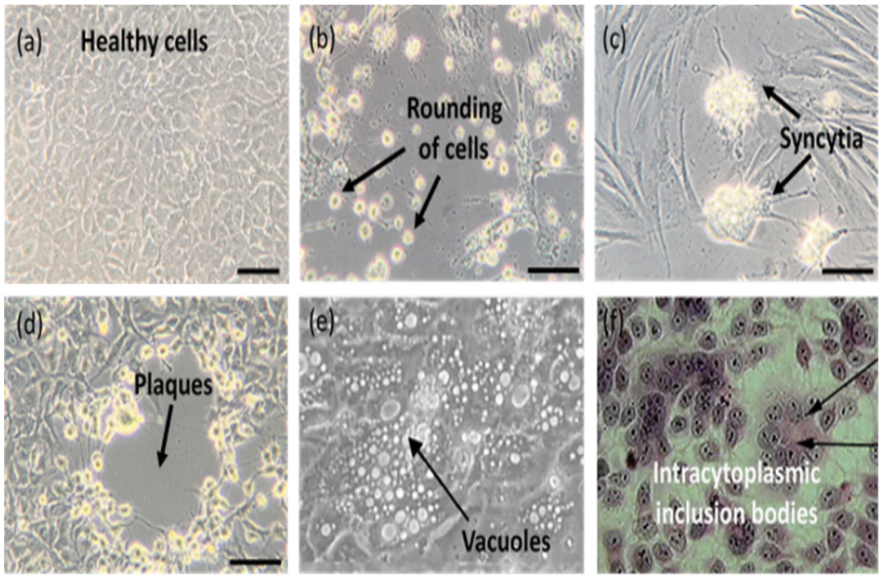
The most reliable method for confirmatory identification of a CPE is by PCR, followed by sequence analysis of the PCR product. The PCR methods recommended for identification of virus are the same methods recommended for direct detection in fish tissues. PCR products for final confirmation of the correct size should be identified as viral in origin by sequence analysis.

Virus preservation and storage

- Centrifuge infected cell cultures at 2–5°C and 2000–4000 *g* for 15 minutes, then dilute the virus containing supernatants in order to obtain virus titres averaging 1–2 × 10^6 PFU ml^{-1}.

- Dispense the resulting viral suspensions into sterile vials at volumes of 0.3–0.5 ml each.
- Freeze and store each series of standard virus stocks at –80°C or liquid nitrogen, and check the titre of each virus stock at regular intervals if it has not been used during that time period.
- *Lyophilisation:* long-term storage (decades) of the seeds of standard virus strains is achievable by lyophilisation. In this purpose, viral suspensions in cell culture medium supplemented with 10% foetal calf serum are mixed (v/v) with an equal volume of cryopreservative medium (20% lactalbumin hydrolysate in distilled water) before processing. Seal or plug under vacuum and store at 4°C, in the dark.

Cytopathic Effects (CPEs) displayed by viruses in susceptible host



Source: Aarattuthodi S, Dharan V, Koshy M (2021) Fish Cell Cultures -