CHAPTER III

MATERIALS AND METHODS

Materials :

1. Donor fish.

Anabas testudineus (Climbing perch).

Obtained from natural swamps.

Sizes 2 to 3 cm (10 fishes) and 10 to 11 cm (6 fishes).

Cyprinus carpio (Common carp).

Obtained from commercial ponds.

Sizes 5 to 6 cm (8 fishes) and 18 to 20 cm (1 fish).

Lebeo rohita (Rohu).

Obtained from State fish hatcheries.

Sizes 2 to 3 cm (50 fish).

Ophicephalus lucius (Snakehead fish).

Obtained from natural swamps.

Sizes 6 to 9 cm (6 fishse).

2. Stock of viruses.

Infectious pancreatic necrosis virus (IPNV).

Obtained from Stirling University, Scotland, UK.

Viruses from Snakehead fish (O. striatus) (SHV).

Figure 1. Anabas testudineus (Climbing perch).

Figure 2. Cyprinus carpio (Common carp).

Figure 3. Lebeo rohita (Rohu).

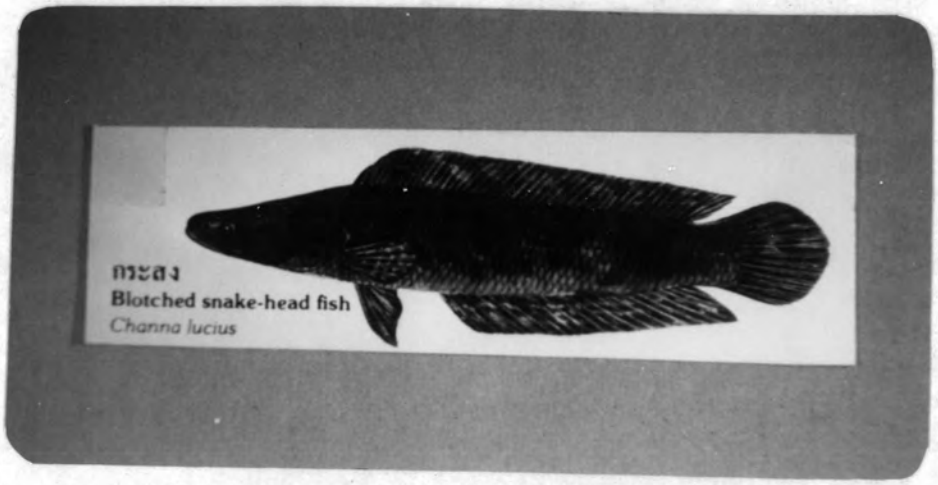


Figure 4. Ophicephalus lucius (Snakehead fish).

Viruses from Carp (Hampala sp.) (CV).

3. Cell line.

BF-2.

Obtained from Stirling University, Scotland, UK.

Methods :

A. Freshwater Fish Cell Culture-Preparation.

1. Collection of tissue.

a) Rinsed the fishes with tap water several times.

b) Killed donor fishes by cutting off the back of the heads with a sharp blade.

c) Removed scales from the caudal trunks.

d) Rinsed the caudal trunks with tap water several times.

e) Steriled the surface of the caudal trunks by immersing them in freshly prepared 50 % clorox solution for 3 minutes for large fish and 30 seconds for small fish.

f) Then removed the solution from (e) and rinsed the caudal trunks several times (5-6 times) with sterile distilled water.

g) Immersed them in PBS pH 7.4 which contained kanamycin 500 ug/ml for 2 hours at room temperature.

2. Isolation of cells from tissues.

a) Transfered the caudal trunks to a petridish or 50 ml sterile beaker and added about 20 % (v/v) PBS pH 7.4 to facilitate mincing.

b) Minced for 3 to 5 minutes or until fragments were about 1-2 mm in size.

c) Washed in PBS pH 7.4, allowing fragments to settle for one minute or more, then decanted or aspirated the supernatant fluid. Repeated the washing 2 or 3 times or until the PBS pH 7.4 remained clear. Aspirated all supernatant fluid.

d) Transferred minced tissue to trypsinized flask, added stirring bar and for each piece of minced tissues added five volumes of 0.25 % trypsin solution in PBS pH 7.4.

e) Placed a flask of digestive tissues on magnetic stirrer and stir at a low speed to avoid foaming.

f) After 30 minutes, removed the flask from the stirrer and set at an angle for several minutes to settle, then decanted as much supernatant trypsin digestive solution as possible.

g) Added five volumes of fresh trypsin solution and resumed stirring the same as (e). After 30 minutes repeated (f), and collected the supernate.

h) Repeated (g).

i) Harvested the dispersed cells by pouring the supernatant solution digestion through sterile gauze into a wide-mouthed erlenmeyer flask and added 2 % fetal bovine serum.

j) Centrifuged the dispersed cells at room temperature 200 g for 10 min.

k) Removed the supernatant and added PBS pH 7.4, shook gently, pipetting.

l) Repeated (j, k), 2-3 times.

m) Resuspended the pellet by gently pipetting in several ml of growth medium (see appendix I) depending on volume of the pellet, diluted into 1:200 to 1:400 volume.

n) Dispensed cells into culture vessel, at the volume of 5 to 7 ml per 25 cm^2 flask or 75 cm^2 flask and incubated at 28 °C.

o) Observed the cells every day, 3 to 7 days or more, until a monolayer was formed (see diagram in figure 5).

p) A subculture was done after confluent cell sheet was formed.

B. Subculturing and Propagation of Fish Cell Cultures (Wolf and Quimby 1976a).

1. Observed the culture medium cells for clearance of microbial contamination after cell culture

Caudal trunks

Treated with 50 % clorox, 1-3 min.

Rinsed several times with sterile water

Immersed in PBS pH 7.4 containing kanamycin 500 ug/ml

↓ 2 hrs

Removed tissues and minced 3-5 min.

Washed in PBS pH 7.4 2-3 times

Trypsinized in 0.25 % trypsin in PBS pH 7.4

↓ 30 min.

1st supernatant digestive fluid decanted

2nd supernatant digestive fluid collected and pooled

until successful

↓ 2-3 hrs.

Added 2 % fetal bovine serum

Poured through sterile gauze

Spun 200 g., 10 min.

Resuspened in PBS pH 7.4 and spun again (2-3 times)

Resuspened cells by diluting with growth medium (200-400x)

Transferred to tissue culture flask

Incubated at 28 °C, 3-7 days

Figure 5. Diagram showing method for freshwater fish cell culture-preparation.

monolayers were formed.

2. Decanted old medium, washed monolayer cells with PBS pH 7.4

3. At a volume of about 1 ml per 75 cm^2 flask or about 0.5 ml/25 cm^2 flask, added trypsin-versene solution. Allowed solution to contact cell sheet properly for 15-30 seconds and decanted. The cell sheets were separated.

4. Added 5 ml of growth medium to 25 cm^2 flask and pipetted cells 2 to 3 times.

5. Added enough additional growth medium to make at least two daughter cultures of a size similar to that of the original (5 ml) or it could be splitted into 1:3.

6. Incubated at 28 °C, 3 to 5 days.

7. After that, prepared the cells using the same method (1-6).

C. Growth Curve and Growth Optimal Temperature of the Freshwater Fish Cell Culture.

1. Did in the same manner as B1 to B4 for preparation of the cell cultures from A. testudineus, C. carpio, and O. lucius uesd for studying growth of the cells.

2. Adjusted the viable cells with growth medium by staining with 0.01 % neutral red.

3. Seeded the culture flasks (25 cm^2) with $1x10^6$ cell/ml in growth medium and divided into 5 groups. Each incubated at one of the following temperature : 37°, 28°, 22°, 16°, 4° C.

D. Studying Viral Multiplication in the Freshwater Fish Cell Cultures.

1. Preparation of stock virus, IPNV, CV, SHV.

a) Did in the same manner as B1 to B6 for preparation of BF-2 cell line used for stock virus.

b) Incubated cells at 28°C, 1 to 2 days.

c) Checked under an inverted light microscopy that the BF-2 cells had formed 80-90 % confluent cell monolayer and were not contaminated.

d) Removed old culture medium, inoculated with 1 ml of virus and absorbed at 22°C, 1 hr. Then added 5 ml of maintenance medium.

e) Incubated at 22°C, observed every day. When it produced an 80-90 % cytopathic effect (CPE) centrifuged the culture fluid at low speed, collected supernatant and dispensed in small volumes and stored at -80°C.

2. Titration of stock virus.

a) Preparation of ten-fold dilution of virus.

1) Set up a row of 10 small

sterile tube in a rack. Added 0.9 ml maintenance medium to each tube with sterile techniques.

2) Added 0.1 ml of virus suspension (C1) to tube #1.

3) With a new 1.0 ml pipette, mixed the contents of this first tube.

4) Removed 0.1 ml of 10^{-1} dilution from tube #1 to tube #2.

5) With a new pipette, mixed the contents of tube #2 as in step (3). Continued in this manner until 0.1 ml of solution had been removed and discarded from tube #10 (Figure 6).

b) Titration of virus in microplate.

1) Prepared BF-2 cells used for titration of virus in the same manner as B1 to B6.

2) Adjusted cells to a concentration of approximately $2x10^6$ cells/ml, added them into microplate 0.1 ml/well.

3) Incubated at 28 °C for 1–2 days.

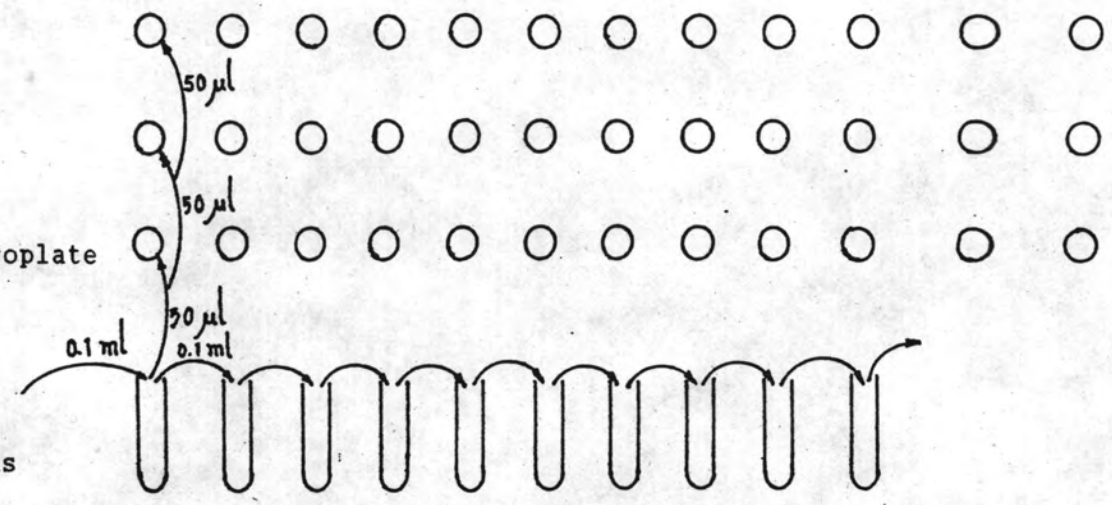
4) Checked under an inverted light microscope that BF-2 cells had formed a confluent cell monolayer and freed from microbial contamination.

5) Mixed the content of tube #1, remove 150 ul of this 10^{-1} dilution in each well in row 1.

6) Mixed the contents of tube #2, removed 150 ul, added 50 ul of this 10^{-2} dilution to

Well # 1 2 3 4 5 6 7 8 9 10 11 12

Dilution 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} cell control



Tube # 1 2 3 4 5 6 7 8 9 10

Maintenance media.....0.9 ml.....

Figure 6 Diagram of viral dilution and inoculation.

each well in row 2.

7) Repeated the procedure above for all the wells up to row 10. Rows 11 and 12 were used for cell control.

8) Absorbed at 22 °C for 60 minutes.

9) Added maintenance medium 50 ul to well and incubated at 22 °C.

10) Observed for changes (CPE) each day for 5 days.

11) Read a last well that gave 50 % cytopathic effect as the dilution end point.

12) Calculated for concentration of the viruses by Reed and Meunch method.

3. The viruses (IPNV,CV,SHV) were studied in experiments with cells which were prepared from O. lucius, C. carpio and A. testudineus.

a) Conducted the preparation of the freshwater fish cell cultures in the same manner as B1 to B5.

b) Adjusted cell concentration to approximately $2x10^6$ cell/ml. Added 1 ml to leighton tube, incubated at 28 °C, for 1-2 days. Then, checked the cell in the same manner as C, 1.c.

c) Then, inoculated with 0.1 ml of the virus (2) which had been stored at -80 °C. After thawing, it was diluted in a growth medium to a concentration of

appoximately $10^{2.5}$ $TCID_{50}$/50 ul.

d) Absorbed virus to the cells at 22°C for 1 hr and added 1 ml maintenance medium to the inoculum and incubated at 22°C.

e) Observed for changes (CPE) each day for 5 days.

f) The virus from (e) was then carried through five serial passages in each of the freshwater fish cell cultures. Steps (a) to (e) were repeated.

g) Titrated the virus in the same manner as (2) and used the same monolayer as in the virus culture.

E. Microbial Checking of the Cell.

a) Checked microbial contamination of bacterium and fungus.

b) Subcultured cells in the same manner as B1 to B6, using a growth medium without antibiotics.

c) After three passages, inoculated thioglycollate broth with these cells and streaked on Sabouraud's agar plate.

d) Incubated at 37°C for 3 to 7 days, and check for contamination.

F. Cell Culture Preservation.

a) Did in the same manner as B1 to B4 for preparation of the freshwater fish cell culture.

b) Adjusted the cells to a concentration of approximately $1x10^6$ cells/ml in growth medium which contained 20 % fetal bovine serum and 10 % DMSO.

c) Dispensed the cells in 1 ml amounts into sterile vial and stored at -80 °C.