CHAPTER 7

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The beginning of animal cell culture techniques dates back to 1885 when Wilhelm Roux performed an experiment to maintain the medullary plate of a chick embryo in warm saline for a few days. However, fish cell culture techniques started developing since 1950s only that too along with the advancements in aquaculture. As on today 159 fish cell lines have been reportedly established, out of which only seven are available commercially with ATCC and four are maintained at the National Centre for Cell Sciences, Pune. From Indian waters no substantial contribution could be made so far to this field inspite of having a very high biodiversity of fish and prawns. Strikingly, no permanent prawn cell line has been made available so far world over. The present work deals with the development of primary cell cultures and diploid cell lines from two fishes such as *Poecilia reticulata* and *Clarias gariepinus* and a primary cell culture from a species of prawn, *Penaeus indicus*. The overall achievements made are summarized as follows:

- An appropriate protocol for disinfecting the surface of *Poecilia reticulata* to remove the embryonic tissue aseptically was developed. This consisted of starving the animal in sterile tap water for three days with frequent change of water, exposure to Sodium hypochlorite containing 300ppm available chlorine for 15 minutes, washing repeatedly with sterile distilled water and dipping in 70% ethanol for 5 minutes and again washing repeatedly with sterile distilled water.

- On screening 21 commercially available media to select the most suitable one for the development of cell culture from embryo/ larvae of *P. reticulata*, three
media such as MEM (Eagle) modified, L-15 (Leibovitz) medium and the medium M199 were found to give promising results.

- On examining the efficacy of tissue derived growth factors in developing cell cultures from embryo and larvae of *P. reticulata*, the combination of medium M199 prepared in fish muscle extract (FME) supplemented with 10% (v/v) fetal bovine serum (FBS) and prawn muscle extract (PME) was found to give better attachment of explants, cell proliferation and monolayer formation. In this medium the cell line developed could be passaged eight times.

- Efficacy of mitogen and growth factors as additives in media for enhanced growth and monolayer formation of the cell culture developed from embryo/larvae of *P. reticulata* was assessed. Medium M199 prepared in FME supplemented with PME (10% v/v), FBS (10% v/v), 0.02 µg mL⁻¹ Lectin-1, 0.02 µg mL⁻¹ Lipopolysaccharide, 0.02 mg mL⁻¹ Glucose D, 0.5% (v/v) ovary extract (OE) 0.5% (v/v) was the most appropriate combination in which the cell culture could be passaged eleven times.

- An appropriate sub culturing techniques and solutions were standardized. The protocol developed consists of the following steps: The medium is decanted off, gently washed twice with PBS devoid of Calcium and Magnesium, rinsed twice with TPVG containing 0.05% Trypsin and allowed to act upon the cell sheath till the cells get rounded off and the cells are dislodged by gentle tapping of the bottle. Trypsin activity was stopped by adding 0.5mL FBS and 2mL growth medium and cells fully dislodged by agitating with Pasteur pipettes and the entire content is transferred to a new tissue culture bottle, and the process completed by adding rest of the medium and the growth factors.

- Besides adopting explant method for developing cell cultures from the embryonic tissue of *P. reticulata*, the method of cell dissociation also was employed to generate a diploid cell line. The cell culture developed by this
method consisted of both epitheloid and fibroblastic cells, which could be successfully subcultured.

- Carbazole, a well known carcinogen was tried on the cell cultures developed to cause *in vitro* transformation. But the compound was toxic to the cells even though an initial stimulation could be obtained.

- Duration required for effecting subculturing in this standardized media was 10 - 16 days and the cell line in this way could be passaged 13 times.

- Optimum temperature of growth of the cell culture developed (PRL-1) from embryonic/ larval tissue of *P. reticulata* is 25°C.

- In order to pave the way for developing cell cultures from liver, spleen, kidney, testis and ovary of *Clarias gariepinus* an appropriate protocol for disinfecting the animal surface was developed. According to this method the animals are starved for two to three days and sacrificed by giving a hard blow on the forehead. Surface disinfection was achieved by dipping in sodium hypochlorite solution to have 600 ppm chlorine for 15 minutes. The surface is rinsed with sterile tap water and exposed the surface to 70% ethanol. The animal surface is once again rinsed with sterile tap water.

- By screening 21 commercially available growth medium an appropriate medium for each tissue was segregated. Liver, spleen, kidney preferred MEM with Earle’s salt with sodium bicarbonate, L-glutamine and antibiotic mixture. Meanwhile, testis and ovary preferred L-15 (Leibovitz-15) with L-glutamine and without antibiotics.

- Since a primary cell culture system could be developed by employing media described above with respect to each tissue, the next attempt was to develop an effective protocol to passage the cell culture. This was necessitated by the
fact that cell dislodgment solutions containing 0.2% trypsin was toxic to fish cells. By a series of experiments the following cell dislodgment solutions could be segregated from each category of cell lines.

Liver : TPVG containing 0.05% trypsin.
Spleen: TPYG containing 0.10% trypsin.
Kidney: TPVG containing 0.05% trypsin.
Testis : Cocktail of cell dissociation solution containing TPVG (0.025% trypsin), non-enzymatic solution-1(50% V/V) and non-enzymatic solution-2 (50%V/V).
Ovary: Cocktail of cell dissociation solution containing TPVG(0.0125% ) non-enzymatic solution-1(25% V/V) and non-enzymatic solution-2 (25%V/V).

Previous results suggested that for enhanced growth and monolayer formation the growth medium has to be amended drastically incorporating several growth factors and mitogens. By way of comparative study for the development of cell cultures from liver, MEM (Eagle’s Modified) amended with 10% FBS, FME, and PME and 0.5% OE, 0.02μg mL-1 lectin 2, the same quantity of LPS and 0.2 mg mL-1 glucose; for the cell culture from spleen MEM amended with 10% FBS, FME, PME and 0.5% OE, 0.02μg mL-1 lectin 1, the same quantity of LPS, 0.2mg mL-1 glucose and 2% prawn haemolymph; for the cell culture from kidney MEM amended with 10% FBS, FME, PME, 0.05% OE, Lectin 0.02μg mL-1 and the same quantity of LPS; for the cell culture from testis L-15 was amended with 10% FBS, FME, PME and 0.05% OE, 0.02μg mL-1 LPS and 2% PHL; for the cell culture from ovary L-15 amended with 10% FBS, FME, PME and 0.5% OE, 0.02μg mL-1 lectin-2 and the same quantity LPS, 0.2 mg mL-1 glucose and 2% PHL were found to be the most suitable combinations.
In the above media the cell lines derived from various tissues exhibited different range of duration for completing growth so as to enable for subculturing. The number of passages each cell line could be subjected also varied. CGL-1, cell line from liver, could be passaged 9 times, and the shortest duration for subculturing was 6 to 9 days. CGS-1, the cell line developed from spleen could be passage 8 days and the minimum time required for subculturing was 8 to 9 days. CGK-1, the cell line derived from kidney, could be subcultured for eight times and the shortest duration required for subcultured was only 6 hours once in 5 to 9 days. CGO-1, the cell line developed from the ovarian tissue exhibited very rapid growth rate with a very short span of 2 to 3 days for completing growth. The cell line could be passaged for 16 times indicating that given adequate growth condition it can get transformed in to an established cell line by \textit{in vitro} transformation.

Optimum temperatures of growth of the cell cultures developed from all the five tissues were determined. Among them the cell cultures developed from liver, kidney and spleen grew well at 28°C and those from testis and ovary at 25°C. Generally the cell lines derived from homeothermic animals require 37°C and optimum range of 25 to 20°C is the property of all cell lines from poikilothermic animals.

An attempt was made to preserve the ovarian tissue at low temperatures in the presence of cryoprotectants so that the tissue can be retrieved at any time and a cell culture could be developed. Among various options, preservation of the tissue at $-35^\circ$C in the presence of 7.5% DMSO gave the best results as the tissue pieces stored could give rise to a primary culture.

An aqueous extract of the ovarian tissue of \textit{C. gariepinus} was found to have attachment and growth factors. In this context an attempt was made to substitute FBS with the ovary extract in the subculturing growth and monolayer formation of RTG-2 cell line. In the presence of ovary extract
alone the growth rate got relieved; however, the cells attached to new bottle and formed monolayer. It appears that the OE can be used as a partial substitute of FBS in the maintenance of cell lines.

➢ To pave the way for developing a cell culture system from prawn and to have reproducibility, a protocol for disinfecting the surface of *Penaeus indicus* was developed. According to this the animals caught from wild are starved for a day in filtered sea water (20 ppt) and sacrificed by plunging in ice cubes and disinfected in sodium hypochlorite prepared in cold (4°C) sea water (20 ppt) having 400 ppm available chlorine for 10 minutes. The animals after thoroughly washing in autoclaved seawater are immersed in cold 70% ethanol for 3-4 minutes and washed subsequently with sterile seawater. All operations are carried out at 4°C.

➢ Three Commercially available growth media such as L-15 (Leibovitz-15) MEM (Eagle’s) and medium M199 were screened against hepatopancreas which was removed from the animal at 4°C. The best results in terms of explant attachment and proliferation of cells was obtained with MEM prepared in seawater (30 ppt) and filter sterilized.

➢ When the above medium was amended with 10%(v/v) FBS, FME, PHL and 0.02µg mL⁻¹ lectin a primary cell culture could be developed. This cell culture was mostly composed of fibroblastic cells.

➢ Thanks to the above mentioned efforts, six diploid cell lines such as PRL-1, CGL-1, CGS-1, CGK-1, CGT-1 and CGO-1 and one primary cell culture PIH-1 were developed. Among them the most promising ones were PRL-1 and CGO-1.
These two cell lines were used for isolating a virus from Blue gourami (Trichogaster trichopterus) (named as gourami virus, GV) and the white spot virus (WSV) from prawns.

These two viruses were initially isolated in RTG-2 and were under repeated passage. The diploid cell line PRL-1 was used for inoculating the GV and CGO-1 for inoculating WSV and in both cases characteristic CPE could be observed.

One of the biggest problems in animal tissue culture is contamination due to fungi. To mitigate this issue, six commercially available antifungal compounds were screened against an Aspergillus species and among them clotrimazole was found to be most active as it inhibited the fungus at a concentration of 10 μg mL\(^{-1}\). In tissue culture this can be added up to a level of 200 μg mL\(^{-1}\) without any outward expression of toxicity.

To sum up, through this piece of work viable techniques to develop six diploid cell lines from two species of fishes such as P. reticulata and C. gariepinus have been developed and standardized. Among them the cell line PRL-1 and CGO-1 were found to be promising, as they are likely to get established by \textit{in vitro} transformation in due course. These cell lines can be applied for virus isolation and can be used for various biomedical applications. Besides, a primary cell line from the hepatopancreas of P. indicus also could be developed which however, could not be subcultured. Clotrimazole has been found as an appropriate antifungal compound to be added in tissue cultures.