DEVELOPMENT OF CELL CULTURE SYSTEMS FROM SELECTED SPECIES OF FISH AND PRAWNS

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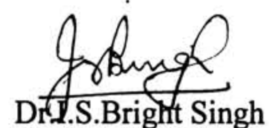
April 2000

Certificate

This is to certify that the research work presented in this thesis entitled **'Development of Cell Culture Systems from Selected Species of Fish and Prawns'** is based on the original work done by Mr.G. Sunil Kumar under my guidance, in the School of Environmental Studies, Cochin University of Science and Technology, Cochin 682 016, in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.



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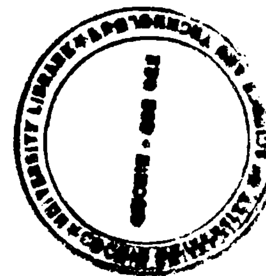

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1.1 Animal cell culture

Development of animal tissue culture was a natural outcome of some of the techniques of embryology, which were in use during 19th century. Wilhelm Roux performed an experiment to maintain the medullary plate of a chick embryo in warm saline for a few days during the year 1885 and this is the first recorded instance of a successful explantation. In 1898, Ljunggren demonstrated that by reimplantation the human skin could survive *in vitro* if stored in ascitic fluid. In 1903, Jolly performed experiments, which marked the first detailed observations on cell survival and cell division *in vitro*. He could maintain leucocytes from the salamander in hanging drops for upto a month. In 1906, Beebe and Ewing recorded a genuine attempt at tissue culture; they described the cultivation of an infectious canine lymphosarcoma in blood from resistant and susceptible animals. It was extremely difficult to repeat the experiments during those periods as the media available were generally unsatisfactory. It was Loss Harrison's experiment in 1907, which offered a reproducible technique that made the true beginning of tissue culture. Harrison explanted small pieces of tissue from the medullary tube region of frog embryos in to clots of frog lymph. When kept in aseptic conditions the fragments survived for some weeks and axons (nerve fibers) grew out from the cells.

Thereafter, the traditional techniques of tissue culture were rapidly established. Burrows, studying the process along with Harrison, introduced the use of a plasma clot in place of a lymph clot. Burrows and Carrel shortly afterwards undertook investigation in to the effects of tissue extracts on growth and Carrel made the discovery that the embryo extracts had a strong growth

promoting effect on certain cells. The techniques of growing tissues in plasma clots supplemented with embryo extracts then became a standard practice. Interestingly, the culture used to be prepared on a cover slip inverted over the cavity of a depression slide.

The greatest difficulty experienced by everyone in performing tissue culture at that time was the avoidance of bacterial contamination. Abxis Carrel, a Nobel Price Winner in experimental surgery was largely responsible for bringing the aseptic techniques in tissue culture, which he was using, for surgical operations. One of the main achievements of the Carrel School was the continuous cultivation of rapidly growing and dividing cells over long periods of time.

The perfection of our present methods of cell culture owes a great deal to the group at the National Cancer Institute in the United States, headed by Dr. Wilton Earle. This group was the first to grow cells directly on glass in large numbers.

Dr. David Thomson in 1914 and later by Dr. T.S.P. Strangeways and Honor Fell was successful in maintaining small fragments of tissues in a state as close as possible to their state *in vivo*, the technique known as 'organ culture'. At an early stage in the development of animal tissue culture Warren and Margeret Lewis started to investigate the factors in the medium necessary for growth and survival (1911-1912). This type of work was undertaken later by Carrel, Baker, Fischer, Parker, Healy, Morgan, White, Waymouth and Eagle and all these activities resulted in the development of our present day media.

Steinhardt, Israeli and Lambert showed as early as 1913 that vaccinia virus could survive for several weeks in explanted cornea. In 1925, Parker and Nye demonstrated multiplication of vaccinia virus in tissue culture of rabbit and also

with the Rous sarcoma virus were reported by Carrel and Rivers and Carrel in the next two years.

Maitland and Maitland in 1928 developed a very simple tissue culture method for virus multiplication. This consisted of suspended fragments of tissues in fluid medium and it led to many interesting studies in the ensuing years. However, during 1949 Enders and his colleague showed conclusively that the poliomyelitis virus could be cultivated *in vitro* in Gey's 'Hela' cell line. This observation was made at a time when cell culture techniques had undergone some remarkable developments. With the added practical interest the number of people in the field increased rapidly and the whole subject enveloped with extraordinary speed in the next years. Three types of cell cultures are commonly developed.

1.1.1 Primary cell cultures

These are the cell cultures obtained from the animal tissue that have been cultivated *in vitro* for the first time. They are characterized by the same chromosome number as parent tissue, cultivated *in vitro* for the first time, have wide range of virus susceptibility, usually not malignant, six chromatin retarded and do not grow as suspension cultures. The primary cell cultures commonly used in virology are PMK (Primary Monkey Kidney - Rhesus monkey kidney), PAGMK (Primary African Green Monkey Kidney), PHAM (Primary Human Amnion), PRK (Primary Rabbit Kidney), PHK (Primary Hamster Kidney) and PHEK (Primary Human Embryonic Kidney) (Anon, 1975).

1.1.2 Diploid cell lines

A diploid cell line is the one, which arises from a primary cell culture at the time of subculturing. A diploid cell line denotes a line having atleast 75% of the cells in the population with the same karyotype as the normal cells of the species from which the cells were originally obtained.

Diploid cell lines are characteristic in having in atleast 75% of the cells with cells with diploid set of chromosomes, growth in suspension culture are unsuccessful, cells usually are normal and limited to several subculturing, spectrum of virus susceptibility same as that of primary cell cultures, produce more acid in medium than a heteroploid cell lines (Anon, 1975). Diploid cell lines commercially used in virology are WI-38 (Human embryonic lung), WI-26 (Human embryonic lung) and HEX (Human embryonic kidney).

1.1.3 Heteroploid cell lines

These are cells that have been subcultivated with less than 75% of the cells in the population having a diploid chromosome constitution. An established cell line is a heteroploid cell line which demonstrate the ability to indefinite serial subcultivation. Characteristically heteroploid cell lines have heteroploid set of chromosomes (25% or more of cells), with the sex chromosomes not usually retarded, with the successful development of suspended cultures. Many of the cells in a heteroploid cell lines are malignant with unlimited cell multiplication. Spectrum of virus infectivity is different compared to the corresponding primary cell cultures. Acid production in the tissue culture medium is less than that of the diploid cell lines and are with unlimited serial subcultivation. Established cell lines commonly used in virology are Hela (carcinoma of human cervix), HEP-2 (carcinoma of human larynx), FL (Normal human amnion), KB carcinoma of human nasopharynx, Vero (African Green Monkey Kidney) (Anon, 1975).

1.2 Importance of cell lines in general

Tissue cultures have been extensively used in biomedical research. The main applications are in three areas. 1. Karyological studies, 2. Identification and study of hereditary metabolic disorders and 3. Somatic cell genetics. Other applications are in virology and host-parasite relationships. The ability of tissue

culture to support the growth of viruses and to reveal their presence by lesions which are in some cases specific has been applied in virology for three main purposes: 1. The study of host-parasite relationship, 2. The detection and identification of viruses and 3. The production of viruses for vaccine manufacture. Eventhough the greatest volume of work in the study of host-parasite relationship with tissue culture has been done with viruses, a number of other intracellular organisms have also been investigated. Rikketsia in particular have been the subject of intensive study. A considerable amount of work has also been done with mycobacteria especially the tubercle and leprosy bacilli in tissue culture. Many parasitic protozoans have been successfully cultured in tissue culture cells, including the parasites of several tropical diseases. Undoubtedly a great deal remains to be done in this field especially with regard to facultatively intracellular pathogenic organisms (Paul, 1975).

1.3 Fish cell culture

The literature on fish cell and tissue culture is extensive and overwhelming. Understandably it was the need of virology, which stimulated the development of fish cell cultures to the present level. Wolf and Quimby (1969) published the first comprehensive review of fish cell and tissue cultures. That work was followed by Clark's (1972) comparative presentation, which included information on reptilian, amphibian and teleostean cell and tissue culture. The most comprehensive reference on all tissue culture entitled 'Tissue Culture Methods and Applications' was published by Kruse and Patterson, (1973). The book includes brief description of trypsinization of marine fish tissue (Sigel and Beasley, 1973); preparation of marine fish leucocyte culture (Sigel *et al.*, 1973), from fresh water fishes (Mc Kenzie and Stephenson, 1973). Ahne and Bachmann (1974) published details of their standardized procedures for preparation of primary cell cultures of two fresh water teleosts such as carp and trout. Still another prime reference is the Journal of Tissue Culture Methods formerly the TCA manual, a publication of Tissue Culture Association. The serial publication

was begun in 1975 and now contains hundreds of specific methods, techniques and procedures by recognized authorities on various aspects of tissue culture. Wolf and Quimby (1976a,b,c and 1978) described five specific methods currently available for fish: Primary culture of fish cells initiated from trypsinized tissues, culture of fish leucocytes, subculture of fish cell lines and systematic management of animal cell lines. In 1980, Wolf and Mann made a current listing of cell lines of fishes available world over and found that some 61 cell lines representing 17 families and 36 species of fish are available. Nicholson (1982), attempted for an update in fish cell culture. As of the end of 1993, 159 fish cell lines have been established for isolating fish viruses (Fryer and Lannan, 1994). Most of these cell lines are derived from the tissues of fresh water fish and only 34 cell lines originated from marine fish. However, only the following cell lines are with American Type Culture Collection. They are CAR (Goldfish, fin), CHH1 (*Onchorhynchus keta,* Chum, heart), CHSE-214 (Fish-Salmon, Embryo), FHM (Fish-Minnow-Skin), GF-Grunt Fin (Fish-Blue stripped Grunt, Fin), RTG-2 (Fish-Trout, Rainbow gonad), BB- *Ictalunus nebulosus* (Bull head brown catfish, trunk), BF-2 (Fish-Blue gill fry , Caudal trunk). In India a cell line from the gill of Mrigal, *Cirrhinus mrigala* (Sathae *et al.*, 1995), the primary cell culture from kidney of *H. fossilis* (Singh *et al.*, 1995), larvae *P. reticulata* (Kumar *et al.*, 1998), caudal fin of Rohu, *L. rohita* (Lakra and Bhondae, 1996) and heart tissue of Major carp(Rao *et al.*, 1997) are the only reports available. National Centre for Cell Science, Pune, maintains established cell lines such as BB (trunk), FHM (skin), GF (Grunt Fin), RTG-2 (Gonads) and RTH-149 (Hepatoma), which would be available on demand.

1.3.1 Media

In general the growth media routinely employed for fish cell cultures are usually the same as those used for animal cell culture. The two most widely used growth media are Eagle's Minimum Essential Medium (MEM) and Leibovitz Medium (L-15) supplemented with 5-10 % Fetal Bovine Serum (FBS). In some

cases with certain marine fish cell lines such as Grunt Fin Line, GF (Clem *et al.*, 1961), it may be necessary to increase the NaCl concentration of standard media. However, this is not true for most marine fish cell lines. Nevertheless in preparing primary cell cultures from marine species it is perhaps wise to prepare two sets of cultures, one with standard growth medium and another with increased salt concentration.

FBS is usually used as a supplement to the basal medium, calf serum (Wilcox 1982) can also be used. Shea and Berry (1983) have reported the use of an undefined serum-free medium for the growth of five fish cell lines.

The optimum pH for growth is between 7.2 and 7.4. Fish cells require CO_2 either from bicarbonate in sealed vessels or from a CO_2 incubator. Organic buffers such as HEPES can also be used in the medium. One of the specialities of fish cell lines is that they can be maintained for prolonged period without fluid change.

1.3.2 Growth Temperature

According to Nicholson (1985) fish cell cultures grow over a wide range of incubation temperatures. For cells from cold water species, temperatures of 15 to 20°C are usually optimum. At the same time they can be maintained at temperatures ranging from 2 to 27 °C. Most warm water fish cell cultures do not tolerate relatively low incubation temperatures. But even they may grow at 37°C, the optimum temperature generally lies between 25 and 35°C.

1.3.3 Culture Vessels

All fish cell lines so far developed are anchorage dependent and must be maintained as monolayer cultures on some solid substratum. Besides standard culture vessels microcarrier beads which yield two to three times greater number of cells can also be used (Nicholson, 1980). In every instance fish cell lines have

been adapted to grow in suspensions with some alterations in properties (Lidgerding, 1981).

1.3.4 Long Term Storage

Most fish cell lines can be kept for extended periods under frozen conditions in liquid nitrogen or in ultra-cold freezers using standard methodologies. When such equipments are not available they can be maintained for two to six months by storing at temperatures well below optimum without any fluid change. In this way Salmonid cell lines have been maintained at 4 to 6°C for six months. But this is not true with regard to cell lines from warm water species (Nicholson,1985).

1.4 Importance of Fish Cell Cultures

1.4.1 Isolation and identification of fish viruses

Until recently the most widespread use of fish cell cultures have been for the isolation and characterization of fish viruses. The first fish cell line (RTG-2, Wolf and Quimbay, 1962) was developed from trout and used to facilitate the isolation of infectious pancreatic necrosis virus (IPNV). Over the years, fish health management with an emphasize on disease diagnosis became a high priority world over and that was especially true in North America and Europe. Consequently most early cell lines were derived from cold water species (Wolf and Mann, 1980). Initially, from warm water species relatively few such cell lines were developed with the exception of catfish related species, which are farmed in the southern parts of the United States (Wolf and Quimby, 1969).

Over the past three decades along with advancement of aquaculture of warm water species, disease problem has also cropped up extensively. That triggered so much enthusiasm among workers to try for developing new cell lines

from the warm water species. Consequently in recent years there has been a rapid increase in the number of continuous cell cultures derived from such species. Specific examples include carp, loach, tilapia, perch, milkfish, grouper, snakehead fish, sea bream and eels (Nicholson, *et al.*, 1987 and Chi *et al.*, 1999). These new cell lines are being used to isolate previously undetected and unknown viruses and for comparative studies of these viruses.

1.4.2 *In vitro* models for studying virus replication

Cell cultures are useful models for studying the replication and genetics of these viruses, the establishment and maintenance of persistent infection and virus carrier states, effects of antiviral drugs and the production of experimental vaccines. Fish cell culture have been used to study the relationships of virus infection and alteration in host cell macromolecule synthesis, Lothrop and Nicholson, (1974) demonstrated that IPNV infection specially inhibits cellular DNA synthesis early in the replication process by a mechanism resulting in a reduction of number of chromosomal sites active in DNA synthesis but not affecting the rate of polymerization at active sites.

Fish cell cultures have been the principal systems for elucidating genome expression and replication and virion morphogenesis of variety of fish viruses (Moss and Gravell, 1969; Kelly and Loh, 1973, Piper *et al.*, 1973, Tu *et al.*, 1974; Scherrer and Cohen, 1975; Dobos, 1977; Dobos *et al.*, 1977; Mac Donald and Dobos, 1981; Dobos and Roberts, 1982; Mertens and Dobos, 1982; Berry *et al.*, 1983; Kelly *et al.*, 1983; Kimura *et al.*, 1983; Hsu *et al.*, 1985, Kurath and Leong, 1985; Winton *et al.*, 1985)

In vitro systems also provide both fundamental and practical information on the stability of fish viruses under various environmental conditions and the effects of various inhibitors on the replication of these viruses (Midgus & Dobos, 1980; Kimura *et al.*, 1983 and Buck and Loh, 1985).

In vitro cell culture techniques have been used to investigate unique viruses that do not replicate in standard fish cell lines, but require highly differentiated cells. One examples is viral erythrocytic necrosis (VEN), an iridovirus infection of red blood cells of several species of marine and anadromous fishes (Johnston and Davies, 1973; Walker and Shirburne, 1977; Reno *et al.*, 1978).

1.4.3 Cytogenetics

In vitro cultures of fish cells have been utilized for determining karyotypes (Chen and Ebeling, 1975; Legundne, 1975; Yamamoto and Ojima, 1973) and other aspects of cytogenetics such as chromosomal polymorphism and speciation (Roberts, 1968, 1970; Hartley and Herne, 1982; Park and Kang, 1979; Wiley and Meisner, 1984) chromosomal abnormalities and evolution (Thorgaard, 1976; Etlinger, 1976, 1977 and 1978).

1.4.4 Cellular physiology and differentiation

Various cellular and physiological processes and differentiation can be studied by using primary and continuous fish cell cultures. Organ culture of pituitary glands derived from *Tilapia* and Rainbow trout, monolayer pituitary cell cultures from *Tilapia*, rainbow trout and dwarf bream have been used to study the production of the growth hormone prolactin. Also pituitary organ cultures from rainbow trout and cell cultures from trout, carp and gold fish have been employed for *in vitro* systems for studying the mechanisms of production and regulation of gonadotropin (Nicholson, 1982). Similarly various types of liver tissue cultures have proved to be productive as *in vitro* systems for studying basic physiological processes of that organ. Also catfish hepatocytes cultures have been used to investigate the ability of individual cells to exhibit temperature acclimation. Cultured kidney tissue has been useful in comparing testosterone depended

change *in vivo* and *in vitro* in the structure of the renal glomoruli of teleost fishes (de Ruiter, 1981). *In vitro* cultures of retinal cells of fish have been particularly fruitful in facilitating studies of the functions of the cells. Similarly *in vitro* propagation of brain and spinal cord tissue and cell cultures has permitted certain type of studies on neurogenesis and differentiation (Anderson *et al.*, 1987a). Gonadal cell and organ culture has contributed to studies on the effects of testosterone on spermatogenesis (De Clercq *et al.*, 1977). RTG-2 has been used to study the synthesis of heat shock proteins (Morsen *et al.*, 1986) and a number of studies have been reported using fish cell cultures to study the growth and differentiation of fish chromatophores (Akiyama *et al.*, 1987).

1.4.5 Toxicology

Fish cell cultures are of high value in the field of toxicology, both as *in vitro* systems for studying the metabolism of various toxicants and as indicator models for testing the cytotoxicity of aquatic pollutants. Generally, the genotoxicity of environmental contaminants to aquatic and marine species has been tested using *in vivo* assays that require facilities for large numbers of fish and result in the eventual sacrifice of the test animals. Increasing evidence suggests that both primary cultures and more importantly established cell lines may also be sensitive and more feasible assay systems for screening aquatic pollutants for cytotoxicity (Zakour et al., 1984).

1.4.6 Carcinogenesis

Fish cell cultures have been utilized for more detailed investigations of the processes leading to the proliferation and differentiation of tumor and tumor cells (Kuhn *et al.*, 1974 and Matsumoto *et al.*, 1980). An increasing number of reports are appearing in the literatures describing the value of fish cell cultures for testing and evaluating the effects of carcinogens (Klaunig, 1984).

1.4.7 Immunology

There is currently considerable interest in fish immunology both from the practical viewpoint of developing vaccines for important fish pathogens and in comparative immunology and the evolutionary aspects of the immune system. Again cell and organ cultures have facilitated studies of the immune response in fish (Anderson *et al.*, 1986). *In vitro* systems have been used to study the effects of various substances such as antibiotics (Groundel *et al.*, 1985) on the modulation of the cells of the immune system.

1.4.8 Education

The potential usefulness of fish cell and tissue cultures as teaching tools should not be overlooked. Most fish cell cultures are relatively easy to initiate and or maintain, and grow over a wide temperature range. They can be propagated at ambient room temperature and most continuous lines can be stored for long periods at temperatures of 4^0C to 15^0C. All of these characteristics provide advantages for fish cells in comparison to mammalian cells for use in high school and college classrooms.

1.5 Invertebrate Cell Culture

Inspite of the rapid progress achieved in the *in vitro* cell culture development from finfishes very little could be achieved in the area of shellfish cell culture. However, the recent episodes of viral diseases in commercial cultures of shellfishes triggered considerable interest in developing tissue culture systems from the susceptible species as diagnostic tools (Leudeman and Lightner, 1992). However, the development of *in vitro* cell cultures utilizing tissues from Crustacea is in the early experimental stage. Meanwhile, some encouraging results have recently been achieved with shrimps (Chen *et al.*, 1986; Ellender *et al.*, 1979; Chen and Kou 1989). Recently an in vitro culture of embryonic cells

from the freshwater prawn *Macrobrachium rosenbergii* has been developed by Frerichs (1996). Kasornchandra *et al.*, (1998) developed a primary shrimp cell culture from the hematopoeitic tissue of *Peneaus monodon* and could isolate and titrate the White Spot Virus. The media generally employed are L-15, M199 and MEM supplemented with NaCl to get a higher osmolarity. Requirement of higher osmolarity in the medium can be cited as an important deviation from that of fish tissue culture media. Hsu *et al.*, (1995) developed an *in vitro* subculture system from the lymphoid tissue of *P. monodon* using L-15 medium supplemented with 10 % FBS, $5 gL^{-1}$ NaCl, pH 7.6 to 8.1 with final osmolarity at 472 mM kg^{-1}. According to them several growth factors such as epidermal growth factors, transforming growth factor β, insulin-like growth factor, fibroblastic growth factor may be required for an effective development of a cell culture system from prawns.

1.6 Future prospects in developing new cell culture for finfish and shell fishes

Application of cell cultures, whether it is primary or established are manifold. Besides they are widely been used in virology, they are employed in cytogenetics, cellular biology and differentiation, toxicology, carcinogenesis, immunology and also in education.

Eventhough, 157 cell lines have been reported so far, India could not contribute even a single cell line so far and it still remain a highly neglected field. Several of the viral etiologies in fish prawn culture systems in Indian waters could not be elucidated, primarily because an appropriate genuine cell line could not be made available. Indian waters with very high diversity of fish and prawn populations, 2200 species of fishes, 160 species of prawns and 80 species of crabs and about 60 species of molluscs the scope of developing new cell lines is also very high. But it has to be remembered that for every fish and prawn species, which is attempted, the media, methods, growth factors, incubation conditions and

all have to be standardized. Eventhough the work is stupendous, undoubtedly it is a highly rewarding venture.

1.7 Summary

Animal cell culture in general and fish cell culture in particular has been reviewed. There are three kinds of animal cell culture such as primary cell culture, diploid cell lines and heteroploid cell lines. Cell lines find application in various field of biomedical research. As on today there are reports of 159 fish cell lines derived mostly from fresh water species of fish and only 34 from marine species. However there are only seven certified cell lines available with ATCC. National Centre for Cell Sciences, Pune, maintains four such cell lines to make available for researchers. Eventhough several reports are available on the development of prawn cell cultures no established cell line is available world over. Application of fish/prawn cell lines are 1. Isolation and identification of fish viruses, 2. As *in vitro* models for studying virus replication, 3. Cytogenetics, 4. Cellular physiology and differentiation, 5. Toxicology, 5. Carcinogenesis, 6. Immunology, and 7. In education. With the existing diversity of fresh and prawn population of Indian waters there is very high scope for developing new cell lines, which would satisfy the requirement of various biomedical researches.

CHAPTER 2

CELL CULTURE SYSTEM FROM THE EMBRYONIC TISSUE OF POECILIA RETICULATA

CHAPTER 2

CELL CULTURE SYSTEM FROM THE EMBRYONIC TISSUE OF *POECILIA RETICULATA*

2.1 Introduction

a) Biology, Reproduction, Ecology and Distribution of *Poecilia reticulata*

Guppy (*Poecilia reticulata*) is one of the most popular aquarium fishes next to gold fish and is native to the northern part of South America and the nearby islands of the West Indies (Edward *et al.*, 1977). The fish has been distributed far wide throughout the tropical and warm water temperate zones of the world as controller of the larvae of malaria carrying mosquito just like the mosquito fish. Females are about 2.25 inches long, the males shorter and are extremely variable in colour, particularly on their short dorsal and annual fins. The prime focus of the selective breeding has always been the colorful fins. The fish is extremely voracious and it is said that it can eat its own weight daily. The diet includes worms, crustaceans, insects, plant matter etc. As an aquarium fish the guppy is hardy and present few problems with regard to feeding and breeding, producing young ones so easily and rapidly. Males are always attentive to the females and the females can retain the sperm after getting fertilized for longer periods so that a fertile female may have as many as half a dozen broods even if no male is present. Gestation period ranges from 4-6 weeks and the females produce 20-50 (generally only 20) young ones and repeat the process thereby four times a year. The newly born fries are about an eight of an inch long (Edward *et al.*, 1977). They prefer a water temperature ranging from 22 to 28^{0}C. It is very

adaptable being found in clear ponds, brooks, marshes and streams. It can tolerate brackish water and a temperature as high as 30^0C (Wheeler, 1985).

b) Significance of using *P. reticulata* as donor fish

Several specialties make guppies an excellent donor fish for cell culture development. Primarily, it is not difficult to be maintained in aquaria and in captive conditions also it breeds prolifically. The most outstanding feature of the fish is the ovoviviparous nature, because of which it becomes rather easy and comfortable to dissect out aseptically the young ones from body cavity after disinfection of body surface. It is easy to locate pregnant females, which have a dark area just behind anal fins, and their sides are noticeably swollen.

c) Cell cultures developed from embryonic/larval tissue of *Poecilia reticulata*

Wolf and Mann (1980) made an effective listing of poikilothermic vertebrates cell lines and viruses in which there is the documentation of 61 cell lines reported from 17 families and 36 species of fish. They have listed a cell line GE-4 developed from the normal embryo of *Poecilia reticulata*, which was composed of fibroblastic cells and 22^0C as the optimum temperature. It is also reported that the cell line has been used for the isolation of IHNV and IPNV. The cell line was developed by Dr. Calnek B.W., Department of Avian and Aquatic Animal Medicine, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York, and had been reported that the originator was willing to supply the starting culture. Li et al., (1985) developed another cell line from the larvae/ embryo of *P. reticulata* named as GFT consisting of epithelial-like cells having an optimum temperature of 18^0C and susceptibility to IPNV. However, in the ATCC listing of the established cell lines these cell lines do not figure out. Now, after 15 to 20 years, it is doubtful whether these cell lines do exists. No further information was available regarding the GE-4 cell lines, as the work was not published.

This situation strongly justifies the efforts, which have been made here to develop a cell line from the embryo of *Poecilia reticulata*.

2.2 Materials and Methods

2.2.1 Development of an appropriate protocol for disinfecting the surface of *Poecilia reticulata* to remove the embryonic tissue aseptically

Among all groups of animals it is extremely difficult to obtain a particular tissue from aquatic animals aseptically without being contaminated by the native flora for cell culture development. Therefore, the first attempt made was to develop a viable protocol for disinfecting the animal surface, so that the internal tissues could be removed aseptically. Accordingly a protocol was developed for disinfecting *P. reticulata* to obtain the embryonic tissue for cell culture.

The embryonic fishes were collected from nearby canal and were maintained in a tank in laboratory and fed with a specially prepared diet. The animals with bulged abdomen were transferred to a beaker containing autoclaved, aerated tap water and were starved for 3 days with frequent changes of water in order to facilitate emptying the intestine. Sodium hypochlorite was the disinfectant of choice and a series of dilutions, ranging from 100 to 1000ppm available chlorine were prepared and the animals were sacrificed and disinfected by immersing in dilutions of sodium hypochlorite (BDH, Bombay) for 15 minutes in closed glass beakers. Subsequently they were washed repeatedly with sterile tap water and a swab was taken from fishes exposed to all dilutions of sodium hypochlorite and inoculated onto nutrient media plates and thioglycolate broth as listed below. Subsequently they were immersed in 70% ethanol for 5 minutes and were washed repeatedly with sterile tap water. Swabs were again taken from the animal surface and inoculated the above nutrient.
The media and their composition used were as follows:

Sabouraud Dextrose Agar:

Peptone	1 g
Dextrose	2 g
Agar	2 g
Final pH	7 ± 0.2
Distilled water	100 ml

The above medium was autoclaved at 15 lbs. for 15 minutes and poured in to sterile plates.

Nutrient Agar:

Peptone	0.5 g
Beef extract	0.5 g
Sodium Chloride	0.5 g
Yeast extract	0.1 g
pH	7.5 ± 0.2
Agar	2.0 g
Distilled water	100 ml

The above medium was autoclaved at 15 lbs. for 15 minutes and poured in to sterile plates.

Blood Agar:

Peptone	0.5 g
Beef Extract	0.5 g
Yeast Extract	0.1 g
Sodium chloride	0.5 g

Final pH	7.5 ± 0.2
Blood (Human)	10.0 ml
Agar	2 g
Distilled water	90 ml

The above medium was autoclaved at 15 lbs for 15 minutes and at around 50^0C, defibrinated blood (10% v/v) (Expired human blood from Blood Bank, Medical Trust Hospital, Cochin) was added and poured in to plates.

Thioglycollate Broth:

Casein enzymatic hydrolysate	1.5 g
Yeast Extract	0.5 g
Dextrose	0.55 g
Sodium Chloride	0.25 g
L-cystine	0.05 g
Sodium thioglycollate	0.05 g
Resazurin Sodium	0.0001 g
Agar	0.075 g
Final pH (at 25^0C)	7.1 ± 0.2

The above medium was boiled and dispensed in to culture tubes and was autoclaved at 10 lbs for 10 minutes.

2.2.2 Screening of commercially available media to select the most suitable one for further use

2.2.2.1 Preparation of media

So far there is no specially designed medium for developing fish tissue cultures and for that matter for any group of aquatic animals. Earlier workers have

been utilizing some of the media meant for mammalian and avian tissue culture for the development and maintenance of cell lines from fishes with slight modifications. But the criteria of selecting any such medium is not spelt out anywhere. In this context, it was decided to screen 21 commercial media available in India to select the most appropriate ones for further amendments and application. Details of composition of medium, mode of preparation and application are described below.

The common ingredients applicable to all media are sodium bicarbonate, L-glutamine, antibiotic mixture and phenol red.

Sodium bicarbonate (HiMedia laboratories, Bombay)

3.5g sodium bicarbonate was dissolved in 100 mL autoclaved double distilled water and added a few drops of 1% (w/v) phenol red solution which yielded a purple colouration. Carbon dioxide was passed through it for about 3 minutes till the colour of the solution changed to orange red indicating pH of about 7.2. It was then transferred into screw capped tubes having very little space at the top and was autoclaved at 10 lbs for 10 minutes. Tubes with light pink colouration alone were used.

L-Glutamine (HiMedia Laboratories, Bombay)

3.0g glutamine was dissolved in 50ml double distilled water and after complete dissolution it was filtered through a cellulose - acetate membrane of 0.22μ pore size (Sartorius India Pvt. Ltd.) in a laminar bio hood. The contents were transferred in small aliquots of 1-2ml in screw capped tubes and were maintained in a deep freezer for further use.

Antibiotic Mixture

1. Bezylpenicillin injection I.P (Alembic Chemicals Works, Vadodara)
   Benzyl penicillin injection I.P 1000000 units (600mg) buffered with sodium citrate I.P.

2. Streptomycin injection (Sarabhai Chemicals, Vadodara)
   Ambistryn –S (1g or 750mg)

Dissolved in 5 ml each in sterile distilled water and mixed together and filtered through membrane filter (0.22μ porosity, Sartorius India Pvt. Ltd.) and dispensed in small aliquots and maintained in a freezer).

Media composition and Preparation

1. Nutrient Mixture F-10 (HAM) AT 084 (HiMedia) Modified w/o glutamine and sodium bicarbonate (9.65gL^{-1})

Dehydrated medium	0.965g
Double distilled water	95.4ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	3.4 ml
Glutamine	1.0ml
Antibiotic mixture	0.2 ml

2.Hank's Balanced Salt solution TS 1003 (HiMedia) (HBSS) without sodium bicarbonate (9.76gL^{-1})

Dehydrated medium	0.976g
Double distilled water	98.8ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with

Sodium bicarbonate	1ml
Antibiotic mixture	0.2 ml

3. Dulbecco's Modified Eagle Medium, AT 006 (HiMedia) with L-glutamine, 1 g glucose per L, without sodium bicarbonate and antibiotics (9.98 gL^{-1})

Dehydrated medium	0.998g
Double distilled water	89.3ml
Filter sterilized	0.22µ membrane filter, subsequently supplemented with
Sodium bicarbonate	10.57 ml
Antibiotic mixture	0.2 ml

4. Earle's Balanced Salt solution (EBSS) TS 1002 (HiMedia) without sodium bicarbonate (8.72 gL^{-1})

Dehydrated medium	0.872g
Double distilled water	98.8ml
Filter sterilized	0.22µ membrane filter, subsequently supplemented with
Sodium bicarbonate	1 ml
Antibiotic mixture	0.2 ml

5. BME-Basal Medium (Eagle) AT 040 (HiMedia) with Hank's salts, L-glutamine and NEAA, without $NaHCO_3$ (10.39 gL^{-1})

Dehydrated medium	1.039g
Double distilled water	98.8ml
Filter sterilized	0.22µ membrane filter, subsequently supplemented with
Sodium bicarbonate	1 ml
Antibiotic mixture	0.2 ml

6. Medium 199 dried AT 014 (HiMedia) with Earle's salts, L-glutamine without $NaHCO_3$ (9.6 gL^{-1})

Dehydrated medium	0.90g
Double distilled water	93.5ml
Filter sterilized	0.22µ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.29ml
Antibiotic mixture	0.2 ml

7. RPMI-1640 AT 028 (HiMedia) with L-glutamine without $NaHCO_3$ and antibiotics (10.3 gL^{-1})

Dehydrated medium	1.03g
Double distilled water	93.5ml
Filter sterilized	0.22µ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.29 ml
Antibiotic mixture	0.2 ml

8. Minimum Essential Medium (MEM) AT 018 (HiMedia) (Modified) (Autoclavable) for suspension culture with spinner salts (11.5 gL^{-1})

Dehydrated medium	1.15g
Double distilled water	86.4ml
Filter sterilized	0.22µ membrane filter, subsequently supplemented with
Sodium bicarbonate	12.4ml
L-Glutamine	1.0ml
Antibiotic mixture	0.2 ml

9. BME- Basal Medium (Eagle) AT 010 (HiMedia) Modified autoclavable with Earle's salts, NEAA without L-glutamine, antibiotics and $NaHCO_3$ (10.1 gL^{-1})

Dehydrated medium	1.01g
Double distilled water	95.87ml
Autoclaved at	10 lbs 10min, subsequently supplemented with
Sodium bicarbonate	2.93 ml (7.5% solution)
L-Glutamine	1.0ml
Antibiotic mixture	0.2 ml

10. Nutrient Mixture F-12 HAM Modified AT 086 (HiMedia) without L-Glutamine and $NaHCO_3$ (10.48 gL^{-1})

Dehydrated medium	1.048g
Double distilled water	95.4ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	3.36 ml
L-Glutamine	1.0ml
Antibiotic mixture	0.2 ml

11. Lactalbumin hydrolysate Medium AT 052 (HiMedia) (ELH) Dried with Earle's Balanced Salt solution without $NaHCO_3$ (13.64 gL^{-1})

Dehydrated medium	1.364g
Double distilled water	98.8ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	1ml
Antibiotic mixture	0.2 ml

12. Minimum Essential Medium (MEM) AT 045 (HiMedia) (Eagle) modified (autoclavable) with Hank's salts, NEAA phenol red, without L-glutamine, $NaHCO_3$ and antibiotics (11.7 gL^{-1})

Dehydrated medium	1.17g
Double distilled water	94.1ml
Autoclaved at	10 lbs 10 min, subsequently supplemented with
Sodium bicarbonate	4.7 ml (7.5%)
Antibiotic mixture	0.2 ml

13. Lactalbumin Hydrolysate Medium AT 053 (HLH) (HiMedia) with Hank's Balanced Salt solution without NaHC03 (14.8 gL^{-1})

Dehydrated medium	1.48g
Double distilled water	98.8ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	1ml
Antibiotic mixture	0.2 ml

14. MaCoy's 5a Medium AT 057 (HiMedia) without $NaHC0_3$ (12 gL^{-1})

Dehydrated medium	1.2g
Double distilled water	93.52ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.29 ml
Antibiotic mixture	0.2 ml

15. MaCoy's 5a Medium AT 071 (HiMedia) Modified for suspension culture with L-glutamine, without calcium chloride and $NaHCO_3$ (11.5 gL^{-1})

Dehydrated medium	1.15g
Double distilled water	93.55ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.25 ml
Antibiotic mixture	0.2 ml

16. LY Medium AT 012 (HiMedia) without $NaHCO_3$ (18.3 gL^{-1})

Dehydrated medium	1.83g
Double distilled water	93.45ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.35ml
Antibiotic mixture	0.2 ml

17. Waymouth Medium MB 752/1 AT 091 (HiMedia) with L-Glutamine, without $NaHCO_3$ (13.84 gL^{-1})

Dehydrated medium	1.384g
Double distilled water	93.8ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.0ml
Antibiotic mixture	0.2 ml

18. Glassgow's Modified Eagle Medium AT 058 (HiMedia) Minimum Essential Medium (MEM) AT 018 (HiMedia) (Modified) (Autoclavable) for suspension culture with spinner salts, with L-glutamine and NEAA without $NaHCO_3$ and NaH_2PO_4 (12.5 gL^{-1})

Dehydrated medium	1.255g
Double distilled water	91.93ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	7.87ml
L-Glutamine	1.0ml
Antibiotic mixture	0.2 ml

19. L-15 (Leibovitz) Medium AT 011(HiMedia) with L-glutamine, without antibiotics (14.1 gL^{-1})

Dehydrated medium	1.09g
Double distilled water	99.8ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Antibiotic mixture	0.2 ml

20. Medium 199 Dried AT 015 (HiMedia) With Hank's salts, L-Glutamine without $NaHC0_3$

Dehydrated medium	0.96g
Double distilled water	93.5ml
Filter sterilized	0.22μ membrane filter, subsequently supplemented with
Sodium bicarbonate	6.3 ml
Antibiotic mixture	0.2 ml

21. Mimimum Essential Medium (MEM) AT 017 (Eagle's) (Modified), without L-Glutamine, $NaHCO_3$ and antibiotics

Dehydrated medium	1.03g
Double distilled water	93ml

Autoclaved	10 lbs 10 min, subsequently supplemented with
Sodium bicarbonate	5.5 ml
L-Glutamine	1.0ml
Antibiotic mixture	0.2 ml

2.2.2.2. Preparation of tissue and mode of culture to screen out the most appropriate media

The developing embryo which were found twitching in PBS 1X (NaCl 8g; KCl 0.2g; Na_2HPO_4 1.15g; KH_2PO_4 0.2g; Double Distilled Water 1000mL) on removing from the abdomen were used for the experiment oriented towards the screening of an appropriate media. For each medium, minimum of 10 numbers of embryos were selected and they were minced into small pieces of less than $1mm^3$ using surgical scalpel blade by keeping on a sterilized rubber cork to avoid crushing of tissues, in a laminar biohood. Then they were transferred to a tissue culture bottle provided with 0.5ml fetal bovine serum (FBS). Twenty two such bottles were prepared and were supplemented with 3.5 ml each of the growth media to examine how the explants responded to the different media contributed. The bottles were stoppered with rubber corks and were incubated at room temperature 28 ± 0.5^0C for 48 hours without disturbance before making the first observation for the attachment of explants and proliferation of cells. The bottles were observed under inverted microscope (Carl Ziess, Germany) and the performance were assessed qualitatively.

2.2.3 Efficacy of tissue derived growth factors in developing cell cultures employing the segregated media from the embryonic tissue of P*oecilia reticulata*

Among the twenty one media screened, three media such as Minimum Essential Medium (MEM), Eagle's Modified without L-glutamine sodium bicarbonate and Leibovitz 15(L-15), and medium 199(M199) were found to be

comparatively better in supporting attachment of explants and growth of cells. Singh *et al.*, (1995) have demonstrated that the above media supplemented with 20% Foetal Bovine Serum (FBS) and 20% Fish Muscle Extract (FME) can be used for the development of a primary cell culture from the kidney of *Heteropneustus fossilis*. Based on this observation, experiments were carried out to evaluate the requirement of FME and Prawn Muscle Extract (PME) in addition to FBS as the tissue derived growth factors.

The overall attempt to assess the usefulness of FME and PME as supplements to media in the development of primary cell cultures and cell lines from *P. reticulata* were made in three phases. In phase I, Minimum Essential Medium (MEM) with Earle's salts and without L-glutamine, sodium bicarbonate and antibiotics; Leibovitz medium (L-15) with glutamine and without antibiotics, and Medium 199 with Hank's Balanced salts, L-glutamine and without sodium bicarbonate (Hi Media Laboratory, Bombay), were employed simultaneously. The above three media were prepared in all-glass double distilled water. The MEM was autoclaved at 10 lbs for 10 minutes, where as other media were filter sterilized using the membranes of 0.22µ porosity. The above three media were made appropriately completed by adding sodium bicarbonate, (not in L-15), L-glutamine and antibiotic mixture as per the description given under the section 2.2.2.1. At the time of application these media were amended with Foetal Bovine Serum (FBS) (Sigma Chemical Company, USA) to a final concentration of 10 per cent.

In phase II, the above media were supplemented with 20% (v/v) fish muscle extract (FME). To prepare FME, 10g fish *Arius maculatus* muscle was macerated in 200 ml PBS (1X) and centrifuged at 6000rpm to remove the debris. The supernatant was inactivated in a water bath at 56^0C for 30 min and centrifuged to remove the coagulated proteins and sterilized by passing through Seitz filter (0.45µ porosity) and was stored at 4^0C.

In phase III, contrary to the mode of preparation of medium described above, the dehydrated media were reconstituted in FME itself prepared in distilled water. The media was subsequently amended with FBS to a final concentration of 20% (v/v) and supplemented with prawn (*Penaeus indicus*) muscle extract (PME) to a final concentration of 20%. The PME was prepared in all-glass double distilled water following the same protocol employed for preparing FME and was stored at 4^0C.

Preparation of fish and tissue removal

Preparation (disinfection) of fish and removal of embryonic and larval tissues for carrying out the above three phases of experiments were performed following the procedure described under the section 2.2.1.

Preparation of tissue and mode of culture

The general procedure followed in the preparation of tissue and mode of culture were as given under section 2.2.2.2. The experiments were carried out in three successive stages. Whenever sufficient cells were found growing, attempts were made to subculture them.

2.2.4 Application of growth factors as additives in media for enhanced growth and monolayer formation

From the above sets of experiments, the medium M199 prepared with FME supplemented with 20% FBS and PME was found to be superior than any other media and combinations in supporting a primary cell culture from the embryonic/larval tissue of *Poecilia reticulata*. However, there was a difficulty experienced in subculturing and maintaining the cell culture developed for a prolonged period. Therefore, it was decided to try with various additives as supplements in the above medium, which would enhance growth, confluent

monolayer formation and subsequent establishment of cell lines. The growth factors tried were,

1. Lectin 1
2. Lectin 2
3. Lipopolysaccharide
4. Glucose
5. Sucrose
6. Trehalose
7. Ovary extract
8. Prawn Shell Extract
9. Chitin
10. Prawn haemolymph
11. Fish Skin Extract
12. Clam haemolymph

Preparation/Procurement of Growth factors

1. Lectin 1

5mg lectin 1 [From *Phaseolus vulgaris* (Red Kidney Bean) (Phytohaemagglutinin), Sigma Chemical Company, USA, Product no L9132] was dissolved in 1mL sterile PBS aseptically and 0.1mL from the above was made upto 10mL, filter sterilized and maintained at 4^0C till use. An appropriate quantity of the above solution was incorporated aseptically into the growth medium to obtain a final concentration of 0.02 μg lectin 1 mL^{-1}.

2. Lectin 2

Lectin 2 from *Canvalia ensiformis* (concanavallin A) (Sigma Chemical Co., USA, Product No. C 5275). The mode of preparation was the same as

described above. An appropriate quantity to obtain a final concentration of 0.02μg lectin 2 mL^{-1} was incorporated into the growth medium aseptically.

3. Lipopolysaccharides (LPS)

1mg Lipopolysacharides (LPS) (Sigma Chemical Co., USA, Product No. L 2654) was dissolved in 1mL sterile PBS aseptically. From the stock, 0.1ml was made up to 10mL sterile PBS and filter sterilized. An appropriate quantity of the solution was incorporated in to the growth medium aseptically so as to obtain a final concentration of 0.02 μg mL^{-1}.

4. Glucose D (Qualigens, Bombay)

200mg glucose D was dissolved in 10 mL PBS, autoclaved at 10 lbs pressure for 10 minutes and stored at 4^0C till use. From this an appropriate quantity to obtain the final concentration of 0.2mg mL^{-1} growth medium was added aseptically.

5. Sucrose (SRL, Bombay)

200 mg sucrose was dissolved in 10mL PBS, autoclaved at 10 lbs pressure for 10 minutes and stored at 4^0C till use. From this an appropriate quantity to obtain the final concentration of 0.2mg mL^{-1} growth medium was incorporated aseptically.

6. Trehalose dihydrate

200mg trehalose (Koch-Light Laboratories Ltd., Coinbrook Bucks, England) was dissolved in 10 mL PBS, autoclaved at 10 lbs pressure for 10 minutes and stored at 4^0C till use. From this an appropriate quantity was added to the growth medium to obtain the final concentration of 0.2mg mL^{-1} aseptically.

7. Ovary extract

Adult females of *Clarias gariepinus* weighing around 600g with bulged abdomen were used for harvesting the ovarian tissue. The animal was sacrificed by giving hard blow on its head and the surface was disinfected with 70% ethanol. The body cavity was cut open at the ventral side and the ovary was removed aseptically, weighed and stored at -35^0C. For the preparation of ovary extract 10g ovarian tissue was macerated in 100mL PBS aseptically, centrifuged thrice at 10,000 rpm and Seitz filtered and finally passed through a membrane (Sartorius India Pvt. Ltd.) of 0.22μ and stored at 4^0C. The growth media were supplemented with 0.5% ovary extract (OE).

8. Prawn Shell Extract

Shells removed from freshly caught prawns *Penaeus indicus* were washed thoroughly with sterile distilled water. 10g of the above was cut in to small pieces and macerated using autoclaved glass wool (Himedia) in 100mL PBS. The preparation was centrifuged at 1000 rpm thrice and the supernatant was first filtered through Seitz filter and subsequently through membrane filter of 0.22μ pore size and maintained at 4^0C. The growth media were supplemented with 10% prawn shell extract.

9. Chitin

Chitin flakes were prepared following Madhavan and Nair (1974). The flakes were grounded to fine powder and sterilized by autoclaving. The polymer was dried at 80^0C and added to tissue culture bottles 10mg each and added 5 ml growth medium.

10. Prawn haemolymph

Juvenile prawns collected from a prawn farm (Maradu, Cochin) were used for the collection of haemolymph. The area in between eyestalk and beneath the rostral spine was disinfected with 70% ethanol using cotton swab. To prevent blood clotting 0.015 (w/v) cysteine hydrochloride was used. Blood was collected using capillary tube specially designed for that purpose. (Anon, 1999). The tube was rinsed with the anticoagulant and inserted gently in to the rostral sinus located in the area described above. Blood, which rose to the tube by capillary action, was transferred in to Eppendorff tubes rinsed with the anticoagulant. An equal quantity of PBS was added to the haemolymph and centrifuged at 10000 rpm at 4^0C for 15 minutes which would sediment both bacteria, haemocytes and cell debris. The supernatant was passed through membrane of 0.22μ porosity and stored at 4^0C.

11. Fish Skin Extract

The fish *Arius maculatus* was sacrificed by plunging in ice cold water. The surface was disinfected with sodium hypochlorite diluted to contain 200ppm available chlorine for 10 minutes and subsequently disinfected with 70% ethanol, washed repeatedly with autoclaved distilled water. The skin was removed using surgical scalpel without having any muscles attached and was washed once more with sterile distilled water and was maintained at -35^0C. To prepare the extract, 10g skin tissue was macerated with sterile glass wool in PBS using mortar and pestle and centrifuged thrice repeatedly at 10,000 rpm at 4^0C. The supernatant was passed through Seitz filter first and then through the membrane of 0.22μ porosity and stored at 4^0C. The growth media were supplemented with the skin extract to the final volume of 10% (v/v).

12. Clam haemolymph

The black clam *Villorita villorita* were collected from Cochin backwater and maintained in a plastic trough with seawater having 15ppt. Prior to collection of blood, the shell surface was washed thoroughly with autoclaved seawater (15ppt) and the shells were opened by inserting a scalpel blade through the broad edge of the shell and by tilting it, haemolymph was collected from the adductor muscle using tuberculin syringe aseptically. An equal quantity of PBS was added to the haemolymph, centrifuged thrice at 10000 rpm for 10 minutes each and passed through membrane of 0.22µ porosity and stored at 4^0C.

Sterility test

Sterility of all the preparations made from animal sources was tested by inoculating 0.5 ml each suspension into nutrient and Saboraud dextrose agar slants and in to thioglycollate broth. The preparations were incubated for 7 days and checked for growth and turbidity. Sterility of the preparations containing lectins and lipopolysaccharides was required to be tested, as they were prepared aseptically.

2.2.5 Development of an appropriate subculturing technique

Experiments with the addition of different growth factors as described above resulted in the fixation of percentage of additives in the medium M199 prepared in Fish Muscle Extract. The additives were Fetal Bovine Serum, Prawn Muscle Extract, Ovary Extract, Lectin 1, Lipopolysaccharide and Glucose. By employing this medium ready attachment of explants and fast proliferation of cell and monolayer formation could be observed.

Attempts were made to subculture the primary cell culture formed in the above medium by using the enzymatic solution Trypsin Phosphate Versene

Glucose (TPVG), non enzymatic cell dissociation solution 1 (C-5789,Sigma Chemical Co, USA), non enzymatic cell dissociation solution 2 (Sigma Chemical Co, USA) and their various combinations, cell scrapers and by simple agitation using pasteur pipette.

2.2.5a Trypsin Phosphate Versene Glucose (TPVG)

Composition (gL^{-1})

NaCl	: 8
Na2HP04	: 1.15
K2H2P04	: 0.2
EDTA (Difco)	: 0.2
Glucose	: 0.5
Trypsin (1:250)(Sigma USA)	: 1.0
Phenol red	: 0.01
Double Distilled water	: 1000ml
pH	: 7.2

The above components were filter sterilized through cellulose-acetate membranes of 0.22μ porosity aseptically and were dispensed in to small vials and were maintained at 4^0C.

2.2.5b Non- enzymatic cell dissociation solution 1 (C.5789- Sigma Chemical Co., USA)

This non-enzymatic cell dissociation solution was prepared in Hank's Balanced Salt solution without calcium and magnesium. This is a specifically prepared solution for gentle dislodging of adherent cell types from plastic or glass surfaces.

2.2.5c Non-Enzymatic cell dissociation solution 2 (C-5914 Sigma Chemical Co., USA)

This was prepared in buffered saline and was specially prepared as the non-enzymatic formula for gentle dislodging of adherent cell types from plastic or glass surfaces.

2.2.5d Application of enzymatic and non-enzymatic cell dislodgment solutions

The TPVG solution having four different concentrations of trypsin such as 0.2%, 0.1%, 0.05%, and 0.025% were used individually. A non-enzymatic solution 1 and 2 were used individually and besides it was applied after diluting by 50%. In addition to this, two combinations of TPVG and non-enzymatic solutions (NES) such as 1) TPVG (0.025% Trypsin) plus non-enzymatic solution 1, 50% and plus non-enzymatic solution 2, 50%; 2) TPVG (0.0125% trypsin) and non-enzymatic solutions 1 and 2, 25% each were also used.

2.2.5e General protocol adopted for subculturing

The medium was drained off and the cell sheath was washed twice with PBS, rinsed with dissociation solution twice. The efficacy of the preparation was observed and recorded on the basis of rounding of the cells. The bottles were tapped to assist the dislodgment of cells. Sufficient growth medium was added and gently agitated with pasteur pipette and transferred to a fresh tissue culture bottle and supplemented with rest of the quantity of medium and growth factors.

2.2.5f Physical methods of cell dislodgment

Two physical processes were also tried for the dislodgment of cells from tissue culture bottles.

1. **Simple agitation**

The spent medium was drained off from the bottle and added fresh medium. Agitated gently with Pasteur pipette without causing any damage to the cells. The dislodged cells were transferred to new bottle.

2. **Application of cell scraper/policeman**

This is a simple device for the dislodgment of cells from tissue culture bottles by gently scraping on the growth surface. The dislodged cells were transferred to the new tissue culture bottle.

2.2.6 Application of carbazole as mutagen in the cell culture system developed

Carbazole is a heterocyclic aromatic compound containing a dibenzopyrnole system produced during coal gasification. Coal tar produced at high temperature contains an average of 1.5% carbazole. This is a mutagen and a tumour accelerator which is available commercially. Since this is not water-soluble, filter sterilization is not possible. Therefore, the compound was chemically sterilized using solvent ether and suspended in sterile distilled water and diluted and added aseptically to the medium to get a final concentration of 20μg mL^{-1}. In the experimental design three sets of bottles were maintained, all with the modified M199 with the addition of cocktail of growth factors. One of the bottles was without carbazole, second one with carbazole at the time of seeding and the third one was with carbazole at every passage. Incubation and observation of cells were done as described elsewhere.

2.2.7 Duration required for effecting subculturing the monolayer formed in M199 at different levels of amendments

Generally, subculturing is done when the growth gets completed by way of contact inhibition. Normally if the growth conditions are optimum for the cell culture concerned, as the number of passages progressed the duration required for effecting subculturing gets shortened. This shall be considered as the indication that the diploid cell culture might get established. In this experiment four growth conditions were provided 1. Medium M199 prepared in FME and supplemented with 10% Prawn Muscle Extract 2. Medium M199 prepared in FME and supplemented with growth factors, in combination, found to be most effective as described earlier and 3. Medium 199 prepared in FME supplemented with all the above growth factors and carbazole at the time of seeding and 4. Medium M199 prepared in FME along with growth factors and carbazole at the time of every passage. Duration in days required for effecting subculture was determined and recorded. Besides, the total number of passages that each culture could be submitted also was recorded.

2.2.8 Optimum temperature of growth of cell culture system derived from the embryonic tissue of *Poecilia reticulata*

The temperatures selected were 5, 10, 15, 20, 25, 30 and 35^{0}C. A diploid cell culture at the 3rd passage was dislodged by employing TPVG containing 0.05% trypsin and cells were counted. An aliquots of 1mL cell suspension containing 10^4 cells mL^{-1} were dispersed into Leighton tubes, plugged with rubber cork and incubated in the above mentioned temperatures. The medium used was the complete one (M199 prepared in FME supplemented with 10% FBS, 10% PME, 0.5% OE, 0.02 µg mL^{-1} Lectin 1, 0.02 µg mL^{-1} LPS and 0.2 mg mL^{-1} Glucose. After 4 days of incubation in the above temperatures the cells were harvested and counted following the trypan blue exclusion method. For this, cells removed from the culture bottle were mixed with equal quantity of 0.1% trypan

blue (HiMedia) prepared in PBS (1X) and the cells were counted using haemocytometer. The healthy viable cells used to be round, refractile and relatively small in comparison with dark blue dead cells.

2.2.9 Development of diploid cell line by the method of cell dissociation

Preparations of fish and tissue removal were done as described under section 2.2.1. The embryonic tissue/larvae were washed repeatedly with PBS and cut in to small pieces of $1mm^3$ and washed with PBS devoid of calcium and magnesium (NaCl 8g; KCl 0.2g; Na_2HPO_4 1.15 g, KH_2PO_4 0.2g and double distilled water 1000 mL) in a screw capped tube. The fluid was decanted off and replaced with TPVG containing 0.20% trypsin and the enzymatic preparation to penetrate the tissues for 10 minutes. The fluid was decanted off, replaced with the growth medium M199 and agitated gently with Pasteur pipette and the turbid supernatant was transferred to a stoppered centrifuge tube and centrifuged at 4000rpm. The pellet was resuspended in growth medium and transferred to tissue culture bottle and completed by adding 10% FBS, 10% PME, 0.5% OE, $0.02\mu gmL^{-1}$ Lectin 1, $0.02\mu g$ LPS, $0.2mg\ mL^{-1}$ glucose D. The bottles were stoppered and incubated 26 ± 1^0C. Observations were made after 48 hrs and cell attachment, proliferation and monolayer formation were observed. On attaining sufficient growth, the primary cell culture was subcultured following the protocol described under the section 2.2.2.6e using TPVG containing 0.05% trypsin. Subsequent to this the subculturing was continued in the same way for 4 more times before they perished.

2.3 Results and Discussion

2.3.1 An appropriate protocol for disinfecting the surface of *Poecilia reticulata* to remove the embryonic tissue aseptically

Results of the experiments conducted to develop an appropriate protocol for disinfecting the surface of *P. reticulata* to remove the embryonic tissue aseptically as well as without loosing viability are presented in Table 2.1. As very well demonstrated 300ppm chlorine for an exposure period of 15 minutes and subsequent disinfection with 70% ethyl alcohol for 5 minutes was found to be effective as no bacterial or fungal growth could be obtained by swabbing from such surfaces on appropriate media plates and in thioglycollate broth. When chlorine was used its content had to be raised to 600ppm for effective surface disinfection. When concentration above 600ppm were used the explants generated from such tissues were found to be without the attachment property. When tissues of internal organs is to be used, external decontamination becomes an absolute necessity. According to Wolf and Ahne (1982) the donor fish can be soaked for several minutes in 1:10 solution of household bleach or any other hypochlorite solution that provides a final concentration of 500ppm available chlorine. Alternatively, according to them a 1:1000 aqueous solution of benzalkonium chloride (such as 10% Roccal or Zephiran) can be used. Chen *et al.*, (1983) used freshly prepared calcium hypochlorite solution for one minute and wiped with 70% ethyl alcohol at an interval of 30 minutes for four times for aseptically removing ovarian tissue from an hybrid of *Tilapia nilotica*. Meanwhile Singh *et al.*, (1995) used a protocol for disinfecting fish surface by dipping in cetrimide (1% v/v) for 5 minutes followed by repeated washing with sterile distilled water. The general procedure followed in the present study matches with the earlier ones and further the procedure adopted here is very efficient in providing healthy, contamination-free embryo and larvae from the body cavity. The protocol thus finalized is as follows: Starve the animal in autoclaved (aerated subsequently) tap water for three days with frequent changes of water, expose to sodium

hypochlorite containing 300ppm available chlorine for 15 minutes, wash repeatedly with sterile distilled water, dip in 70% ethanol for 5 minutes and wash repeatedly with sterile distilled water.

2.3.2 Screening of commercially available media to select the most suitable one for further use

Results of screening 21 commercially available media to select the most appropriate one for the embryonic/larval stages are summarized in Table 2.2. Among the 21 growth media used, only three media such as MEM (Eagle) modified without L-glutamine, $NaHCO_3$ and antibiotics; L-15 (Leibovitz) medium with L-glutamine and without antibiotics and the medium M199 with Hanks salts, L-glutamine and without $NaHCO_3$ were alone found to give promising results. For some time, most fish cell and tissue cultures have been carried out with Eagle's minimum essential medium (MEM), Eagle's basal medium (BME), Medium 199, or Leibovitz L-15. By far, the single most frequently used medium as per the literature has been Eagle's MEM (Wolf and Ahne, 1982). Leibovitz (L-15) medium merits particular attention for it was designed to maintain pH in the physiological range under normal atmosphere without added CO_2. In the Leibovitz medium, galactose is used as the energy source but in most other media glucose is used. However, in the present study Leibovitz was used in closed system. Interestingly in the present experiment also, the above two media stood out first along M199 which was not generally seen to be used elsewhere for fish cell line and can be considered as a new observation. Nicholson (1988) on writing an overview of fish cell cultures observed that the growth media routinely employed in the culture of fish cells are generally the same used for homeotherm cell lines and the most widely used media for fish cells are Eagle's Minimum Essential Medium (MEM) and Leibovitz Medium L-15 supplemented with 5-10% foetal bovine serum. But for the development and maintenance of a primary cell culture the plain media routinely used for homeotherms might not be sufficient, as the cell cultures developed here could

not be maintained for long by subculturing. Reasonably some more additives in the form of growth factors were required.

2.3.3 Efficacy of tissue derived growth factors in developing cell cultures

Eventhough the three media mentioned above were better compared with the other media tested based on the proliferation of cells and formation of confluent monolayer from the explants, they were not supporting enhanced growth after subculturing. Since the overall objectives of work was to develop a cell culture system and to maintain them indefinitely it was modified and upgraded by supplementing with tissue derived growth factors. Results of the series of experiments conducted in this direction are presented in Table 2.3. In the experiment which was conducted in three stages, the first stage was with growth media alone supplemented with FBS in 4:1 ratio, in the second stage in addition to FBS, FME was added at 3:1:1 ratio and in the third stage the media were prepared in FME and supplemented with FBS and PME at 3:1:1 ratio. Out of the three stages with the three combinations, the best combination was M199 prepared in FME supplemented with FBS and PME where more than 50% confluence (Fig 2.1) was obtained. Therefore for all subsequent works this combination was used.

The major change, which has been brought about in the preparation of this medium, is its preparation in FME and incorporation of PME. In the history of fish cell line development, application of tissue derived growth factors are not recommended. This was first introduced by Singh *et al.*, (1995) while developing a primary cell culture from the developing kidney of *Heteropneustus fossilis*. Latter this procedure was repeated by Lakra and Bhondae (1996) while developing a primary cell culture from the caudal fin of an Indian carp *Lobeo rohita*. In the present study this was found to be very much essential in the faster development of primary cell cultures suggesting that the original medium as such was incomplete.

Using this modified medium M199 prepared in Fish Muscle Extract supplemented with Prawn Muscle Extract and Fetal Bovine Serum, the cells could be passaged 8 times (Fig 2.2 and 2.3). After this the cells lost viability, understandably for prolonged maintenance and subculturing the medium has to be modified further.

2.3.4 Application of mitogens and growth factors as additives in media for enhanced growth and monolayer formation

Results of the experiment conducted to assess the requirement of mitogens and growth factors as additives in media are presented in Table 2.4. To assess the influence of growth factors, the criteria of merit considered were attachment of explants, time taken for attachment and beginning of cell proliferation in days and growth and monolayer formation. Based on this assessment incorporation of Lectin 1, Lipopolysacharides, glucose D and Ovary Extract in M199 which was prepared in fish muscle extract and supplemented with 10% PME and FBS were selected as the combination of media and growth factors to be applied as a cocktail of additives (Table 2.4). The cocktail preparation when provided, 90% of explants could be attached to the tissue culture bottles with in 24 hours and within three days cell proliferation could commence. More than 60% confluency could be obtained in this combination. Based on repeated attempts with this mode of culture, the number of passages could be increased to eleven (Fig. 2.4). The results were reproducible and employing the above medium and the cocktail of growth factors, the cell cultures could be developed more than 60 times and could be maintained for more than one year. However, inspite of the above modifications in media the diploid cell lines developed could not be established as *in vitro* transformation failed to take place.

In the cocktail of growth factors employed, lectins (concanavalin A) and lipopolysaccharides served as mitogens and enhanced the growth rate and multiplication of cells. Lectins are highly specific polyvalent carbohydrate-

binding proteins. In tissue culture certain lectins are used to induce the mitogenic activity (Goldstein and Hayeo, 1978). Lectins from various sources such as *Canavalia ensiformis, Phaseolus vulgaris* (leucoagglutinin PHA-L and phytohaemagglutinin-PHA-P) and *Triticum vulgaris* (wheat gram agglutinin) have been tested in tissue culture systems using ^{3}H - thymidine incorporation as a measure of mitogenic activity (Rosenberg and Lipsky, 1979).

Meanwhile due to the lymphocytes-activating properties, lipopolysaccharides (LPS) isolated from the wall of Gram negative bacteria have been used extensively in experimental immunology (Anderson *et al.,* 1972) Lipopolysacharides of *E.coli* and *Salmonella* sp. purified, γ -irradiated have been tested in tissue culture using ^{3}H-thymidine incorporation as a measure of mitogenic activity (Rosenberg, 1979). As energy supplier, glucose has its own importance in the growth medium. According Alawa and Pascual (1987) glucose, sucrose and trehalose can serve as the energy sources in cell culture system. On studying with *in vitro* subculture system from lymphoid organ of *Penaeus monodon*, Hsu *et al*., (1995) observed that on supplementing growth medium with glucose, 80% of the cell got attached themselves and with trehalose and sucrose an attachment of 50% was seen. Ovary extract was another additive hitherto not found to have been used in tissue culture and specifically introduced in this study. The ovary extract is found to be sticky and is believed to contribute to the adherence of explants to the tissue culture bottle. Contrary to this, primary cultures of epithelial cells from rainbow trout gill were not found to respond to any of the growth factors employed (Part *et al*., 1993). This precisely indicate that there is no all or none rule as far as growth factors are concerned and this requirement has to be standardized for every fish species studied and every organ used for the cell culture development.

2.3.5 Development of an appropriate subculturing technique

For selecting an appropriate cell dislodgment solution, 12 combinations were tried such as various concentrations of TPVG, two types of non-enzymatic solution in four different concentration, simple agitation with Pasteur pipette, application of cell scraper and two cocktail solutions. Uniformly in all cases, percentage of dislodgment of cells, percentage of attachment in new bottles and types of cells formed were recorded. Results were varying from dislodgment solution to solution and on repeated application, TPVG containing 0.05% trypsin was found to be the most suitable preparation.

For successful development of a cell culture system, easy dislodgment of cells from tissue culture bottles and their passage to new bottles and subsequent development in to monolayers with least percentage of cell death are absolutely essential. In mammalian and avian cell culture systems 0.2% trypsin is generally used without any damage to the cells (Shipman Jr.1973). But the application of the same concentration trypsin, eventhough it is in the form of TPVG, lead to cell death invariably in all cases. That was the reason why different categories of cell dislodgment solutions were tried in the present study. As can be seen from the Table 2.5, 0.2% trypsin was toxic to cells that developed. Bols *et al.*, (1994) for developing a cell line from primary cell culture of rainbow trout gills, used trypsin and two commercially available non enzymatic all dissociation solutions (CDS) (C5789 or C 5915, Sigma) up to 60 seconds. In the present study also the above non enzymatic solutions were used but with little success. Driever and Rangini (1993) while making initial passages the cells were found to be very sensitive to EDTA. Hence cells were passaged by washing first with Hank's balanced salt solution (HBSS) devoid of calcium and magnesium, and for cell dissociation trypsin at 0.025% in HBSS of the above concentration was used. In the same way Tung (1991) used 0.1% Trypsin-EDTA in PBS for subculturing cell lines derived from spleen and kidney of Black porgy. Meanwhile Sathe *et al.*, (1995) exposed the cell cultures to TPVG for 30 seconds (But they have not mentioned the

content of trypsin in the preparation). Pant *et al.*, (1997) are of the opinion that just agitating with pipettes can dispense cells which are loosely attached to glass on plastic flask.

The foregoing discussion points out that trypsin is the enzyme of choice for sub culturing fish cell lines. But its concentration has to be regulated as done in the present case for every fish species.

To sum up, the final protocol of subculturing consist the following steps. Decant the medium, gently wash twice with PBS devoid of calcium and magnesium, rinse twice with TPVG containing 0.05% trypsin, allow to act upon the cell sheath for a couple of minutes judged by the rounding of the cells , tap the bottle to dislodge cells, trypsin activity is stopped by adding 0.5ml FBS and 2 ml growth medium, gently agitated using Pasteur pipette and transfer to a fresh bottle either plastic or glass and complete the process by adding rest of the medium and growth factors.

2.3.6 Development of diploid cell line by the method of cell dissociation.

A primary cell culture from the embryonic/larval tissue of *P. reticulata* could be developed by the cell dissociation method. The cell culture was constituted of both epitheliod and fibroblastic cells which could be successfully subcultured and passaged four times by the standardized protocol.

The most commonly used method, as per the existing literature, for initiating primary culture of fish cells is that of dispensing tissue by a stirred enzymatic digestion. For such digestion, the most widely employed enzyme is trypsin and usual final concentration is 0.25% using products with an activity of 1:250, (Wolf and Ahne, 1982). In the beginning of this work enzymatic disaggregation of the tissue using TPVG containing 0.20% trypsin was tried, but during none of such instances cells could attach to the culture bottle and

proliferate. It was not known at that time why did no cell culture could be obtained by this method. This failure lead to adopt the explant culture method for generating primary cell cultures. It was much later observations were made that trypsin with a concentration of 0.2% even though applied as TPVG was toxic to the cells as evidenced by the toxicity with the primary cell culture. On finding out that 0.05% is the safest concentration of trypsin, TPVG with the same concentration of trypsin was applied for the enzymatic disaggregation of the tissue.

As general principle tissues from most fish should not be digested at mammalian-avian temperatures (37^0C - 40^0C), instead lower temperature should be used (Wolf and Ahne, 1982). According to them a single overnight (12-16 hrs) processing at 4-5^0C has been effective for tissues of both warm water and cold water adult fish tissues. Embryonic tissues are easily digested, and overnight processing at 4^0C can be excessive. Therefore, in the present study, tissue pieces were exposed to TPVG containing 0.05% trypsin for just 10 min and often transferring the tissue to growth medium, the tissue disruption was achieved by pipetting gently. On developing cell cultures from embryos and melenoma of Poecilid fish, Kuhns *et al.*, (1979) made the observation that the cell yield by trypsinizing the tissue at 24^0C for four hours with a solution containing 0.25% trypsin was very high but the viability was very low. Whereas the less efficient conditions such a incubation in 0.01% trypsin plus 0.2M EDTA at 4^0C for 5 hrs yielded much higher proportion of viable cells (85% viable cells) even though the total cell output was low. Thus the mild protocol developed in this work is justified.

2.3.7 Application of carbazole in the cell culture developed

Effect of carbazole in the cell cultures developed is summarized in Table 2.6. Addition of carbazole at every passage was found to lead to death of cells at the 7th passage while with carbazole at the time of seeding and without carbazole

continued to 8th passage. Among the two the one without carbazole performed better indicating that carbazole was toxic to the cells and not wasteful as mutagen atleast for the present cell cultures. (Fig 2.7 and 2.8)

Generally after several subcultures a cell line either die out (finite cell line) or transform to become a continuous cell line. It is not clear in all cases whether the stem line of continuous culture pre-exists marked by the finite population or arises during serial propagation. Because of the time taken for such cell lines to appear (often several months) and the differences in their properties, it has been assumed that a mutational event (chromosomal rearrangement, translocation, partial or total non disjunction or point mutation) occurs, but the pre existence of immortalized cells, particularly in cultures of neoplasm cannot be excluded (Freshney, 1986). Viewing the present developments in the light of above statement it becomes clear that the embryonic and larval tissues do not consist of any pre existing immortalized cells which would later come out as an established cell line. On the contrary, the cells in general are all of a finite population. Therefore the only option left is to transform them by the application of mutagen or carcinogens to achieve neoplastic transformation. However, carbazole was found to be ineffective and for this, compounds such as polynuclear aromatic hydrocarbons, β-naphthyl amine, Benzo (a) pyrine and trace metals like nickel, arsenic and polonium can be thought of.

2.3.8 Duration required for effecting subculturing the monolayer formed in M199 at different levels of amendments.

Comparison of the duration required for effecting subculturing the monolayer formed in M199 at different levels of amendments is presented in Table 2.7. Among various combinations the one prepared in FME and supplemented with all selected growth factor mentioned earlier showed almost uniform duration for monolayer formation. As presented here 10-16 days were required to attain complete growth. The cell line could be passaged 13 times.

2.3.9 Optimum temperature of growth of cell culture system derived from *P. reticulata*

Response of the diploid cell culture at the 8th passage to varying temperature is presented in Fig 2.9. On experimenting from 5 to 35^{0}C for a period of 120 hours. Interestingly 25^{0}C was found to be the most appropriate for enhanced proliferation and out put of cells. According to Meguro *et al.*, (1991) the optimum temperature for growth and maintenance of the cell line derived from the fin of Japanese flounder ranges from 25 to 28^{0}C and 15^{0}C respectively. According to Nicholson (1985) the optimal growth temperature and the temperature range over which a particular culture will grow usually reflect the fish species and its natural environment. Temperature of 15 to 20^{0}C are usually optimal for cells from cold water species such as salmon and trout. According to him most warm water fish cell cultures do not tolerate relatively low incubation temperatures, but many grow even at 37^{0}C. Generally the optimum temperature for these cells lie between 25^{0}C and 35^{0}C.

2.4 Summary

A diploid cell line has been developed from the embryonic/ larval tissue of *Poecilia reticulata*. The cell line named PRL-1 could be subcultured 13 times in M199 prepared in FME supplemented with FBS and PME (10% each v/v), 0.02 μg mL^{-1} Lectin 1 and LPS, 0.2 mg mL^{-1} glucose and 0.5% (v/v) OE. For the development of PRL-1 as per the requirement a viable protocol for disinfecting the animal surface to remove embryonic/ larval tissue aseptically, a protocol for the development of primary cell culture by explant method, a formulation of cell dislodgment solution and a protocol for subculturing have been developed and standardized.

Table 2.1 Efficacy of the protocol developed for disinfecting the surface of *Poecilia reticulata* to remove the embryonic tissue aseptically

Growth on Nutrient Agar Plates				Growth on Sabouraud Dextrose Agar Plates				Blood Agar				Thioglycollate Broth			
A	**B**		**C**	**A**	**B**		**C**	**A**	**B**		**C**	**A**	**B**		**C**
++	100	++	+	+	100	++	+	++	100	++	+	++	100	++	+
++	200	++	+	+	200	+	+	++	200	++	+	++	200	++	+
+	300	++	-	+	300	+	+	+	300	++	-	+	300	++	-
+	400	++	-	+	400	+	-	+	400	++	-	+	400	++	-
+	500	+	-	+	500	+	-	+	500	+	-	+	500	+	-
+	600	-	-	+	600	-	-	+	600	-	-	+	600	-	-
+	700	-	-	+	700	-	-	+	700	-	-	+	700	-	-
-	800	-	-	+	800	-	-	-	800	-	-	-	800	-	-
-	900	-	-	-	900	-	-	-	900	-	-	-	900	-	-
-	1000	-	-	-	1000	-	-	-	1000	-	-	-	1000	-	-

\+ : Growth
++: Heavy growth
\- : No growth

100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 : Sodium hypochlorite concentrations in ppm.

A : Before disinfection
B : After treatment with sodium hypochlorite
C : After treatment with 70% ethanol

Table 2.2 Screening of commercially available media for developing primary cell culture system from the embryonic tissue of *Poecilia reticulata*

Sl.no	Name of Media	Response of tissue
1	Dulbecco's Modified Eagle Medium (AT006)	-
2	Nutrient Mixture F-10 (HAM) modified (AT084)	-
3	BME (AT040)	+
4	Medium 199 (AT 014)	++
5	Earle's Balanced Salt Solution (TS 1002)	-
6	RPMI-1640 (AT 028)	+
7	BME with NEAA (AT010)	-
8	MEM Eagle (Modified) (AT018)	-
9	Hank's Balanced Salt Solution (TS 1003)	+
10	Glassgow's Modified Eagle Medium (AT 058)	+
11	LY Medium (AT012)	-
12	Lactalbumin Hydrolysate Medium (ELH) (AT052)	-
13	MEM Eagle (With Phenol Red) (AT045)	+
14	Nutrient Mixture (F12 HAM) (AT086)	+
15	MaCoy's 5a Medium Modified (AT071)	+
16	Lactalbumin Hydrolysate Medium (ELH) (AT053)	-
17	MaCoy's 5a Medium (AT057)	-
18	Waymouth Medium MB (AT 091)	-
19	L-15 (Leibovitz) Medium (AT 011)	+++
20	MEM Eagle (AT017)	+++
21	Medium 199(AT015)	++++

+ : Attachment, growth and monolayer formation
- : No attachment and growth.

Table 2.3 Efficacy of tissue derived growth factors in developing cell culture from the embryonic tissue of *Poecilia reticulata*

Phase	Sl.no	Media Composition	Performance of the explants in each medium and cell proliferation		
			Attachment (24-48hrs)	Attachment (72-120hrs)	Attachment (120-168hrs)
1[a]	1	MEM+FBS (4:1)	+	-	-
	2	L-15+FBS (4:1)	-	-	-
	3	M199+FBS (4:1)	+	-	-
2[a]	4	MEM+FME+FBS (3:1:1)	+	+	-
	5	L-15+FME+FBS (3:1:1)	-	-	-
	6	M199+FME+FBS (3:1:1)	++	++	-
3[a]	7	MEM in FME +FBS +PME (3:1:1)	+	+	+
	8	L-15 in FME +FBS +PME (3:1:1)	+	+	+
	9	M199 in FME +FBS +PME (3:1:1)	+++	+++	+++ >60% confluence

FBS : Fetal Bovine Serum
FME : Fish Muscle Extract
PME : Prawn Muscle Extract
[a] : Three phases of experiment

Table 2.4. **Importance of growth factors as additives in media for enhanced growth and monolayer formation of a cell culture system developed from the embryonic tissue of *Poecilia reticulata***

Phase No	Sl. No	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in days	Growth and monolayer formation @
1	1	M199	10%FBS+10%PME	60	6	+++
	2	M199	10%FBS+10%PME+Lec1*	63	4	+++
	3	M199	10%FBS+10%PME+Lec2*	55	7	++
2	4	M199	10%FBS+10%PME+LPS*	62	4	+++
	5	M199	10%FBS+10%PME+Glu•	65	4	+++
	6	M199	10%FBS+10%PME+Suc•	40	7	+
3	7	M199	10%FBS+10%PME+Tre•	55	8	+
	8	M199	10%FBS+10%PME+0.5% OE	60	4	+++
	9	M199	10%FBS+10%PME+0.5% PSE	35	12	-
4	10	M199	10%FBS+10%PME+0.5% Chit	35	15	-
	11	M199	10%FBS+10%PME+PHL	55	9	++
	12	M199	10%FBS+10%PME+Insulin	55	8	+
5	13	M199	10%FBS+10%PME+FSE	50	7	+
	14	M199	10%FBS+10%PME+CHL	45	8	+
	15	M199	10%FBS+10%PME+0.5% OE+ Lec1*+LPS*+Glu•	90	3	+++++

FBS - Fetal Bovine serum; PME -Prawn muscle extract; Lec1-Lectin1; Lec2-Lectin2; LPS - Lipopolysaccharides; Glu - Glucose; Suc - Sucrose; Tre – Trehalose; OE - Ovary Extract; PSE-Prawn Shell Extract; Chit.-Chitin; PHL - Prawn Haemolymph; FSE-Fish Skin Extract; CHL-Clam Haemolymph

* $0.02 \mu g\ mL^{-1}$

• $0.2 \mu g\ mL^{-1}$

+ : Growth and monolayer formed
- : No growth

Table 2.5 Development of an appropriate dissociation solution and sub culturing techniques for a cell culture system derived from the embryonic tissues of *Poecilia reticulata*

Sl.No.	Dissociation Solution	Dislodged cells in %	Attached cells in %	Types of cells
1	TPVG (0.2% trypsin)	100	0	EP/FB
2	TPVG (0.1% trypsin)	90	20	EP/FB
3	TPVG (0.05% trypsin)	90**	85**	EP/FB
4	TPVG (0.025% trypsin)	50	30	EP/FB
5	NES1	50	50	EP/FB
6	NES 2	60	45	EP/FB
7	NES 1 (50%)	45	40	EP/FB
8	NES 2 (50%)	60	65	EP/FB
9	Simple Agitation	30	25	EP/FB
10	With Cell Scraper	70	35	EP/FB
11	TPVG(0.025% trypsin) +NES1 50%+ NES2 50%	80	70	EP/FB
12	TPVG(0.0125%trypsin) +NES1 25%+ NES2 25%	80	75	EP/FB

NES 1 - Non Enzymatic Solution 1, NES 2 -Non Enzymatic Solution 2
TPVG- Trypsin Phosphate Versene Glucose
EP: Epithelioid cells, FB : Fibroblastic cells

* Most suitable preparation

Table 2.6 **Efficacy of carbazole as mutagen for *in vitro* transformation of the cell culture system developed from embryonic tissue of *Poecilia reticulata***

Nature	Without carbazole	With carbozole at the time of seeding	With carbazole at every passage
Type of cell	EP/FB	EP/FB	EP/FB
Time requirement for monolayer formation	4 days	2 days	2 days
Number of cells found at the time of subculturing:			
1st passage	4.3×10^{4}/mL	4.7×10^{4}/mL	4.7×10^{4}/mL
2nd passage	4.9×10^{4}/mL	4.8×10^{4}/mL	3.8×10^{4}/mL
3rd passage	4.7×10^{4}/mL	4.2×10^{4}/mL	3.0×10^{4}/mL
4th Passage	4.9×10^{4}/mL	4.3×10^{4}/mL	2.8×10^{4}/mL
5th passage	4.6×10^{4}/mL	3.9×10^{4}/mL	2.5×10^{4}/mL
6th passage	4.9×10^{4}/mL	4.0×10^{4}/mL	1.0×10^{4}/mL
7th passage	4.7×10^{4}/mL	2.4×10^{4}/mL	(Dead)
8th passage	4.9×10^{4}/mL	1.0×10^{4}/mL	
9th passage	Continuing……	(Dead)	

EP : epithelioid cells
FB : fibroblasitc cells

Table 2.7 **Duration required for effecting subculturing the monolayer formed in M199 at different levels of amendments**

Passage No.	Days required for monolayer formation			
	M199 in FME and PME	M199 in FME along with the growth factors	M199 in FME along with the growth factors and carbazole at the time of seeding	M199 in FME along with the growth factors and carbazole at the time of every passage
1	28	8	4	4
2	30	20	6	5
3	16	13	7	17
4	13	9	6	17
5	46	10	10	20
6	60	13	19	(dead)
7	40	10	17	
8	50	12	(dead)	
9	(dead)	13		
10		15		
11		16		
12		14		
13		15		

1. Fig. 2.1 Attachment of explants from the embryonic tissue of *P. reticulta*) and cell proliferation. Majority of cells was fibroblastic. (5X)

2. Fig. 2.2 Fibroblastic cells- first passage (10X)

3. Fig. 2.3 Formation of monolayer composed mostly of fibroblastic cells derived from Embryonic tissue of *P. reticulata*

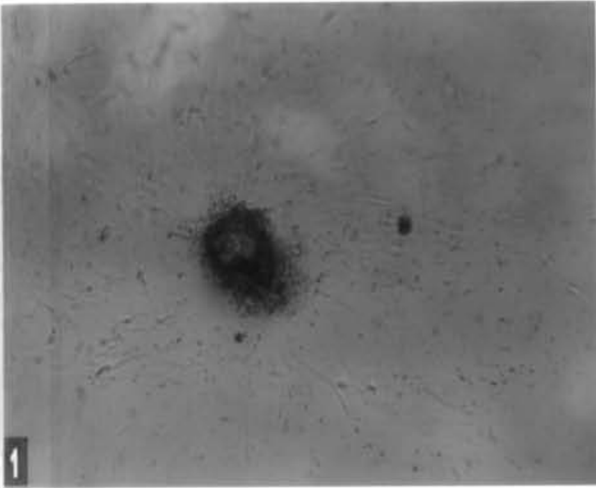
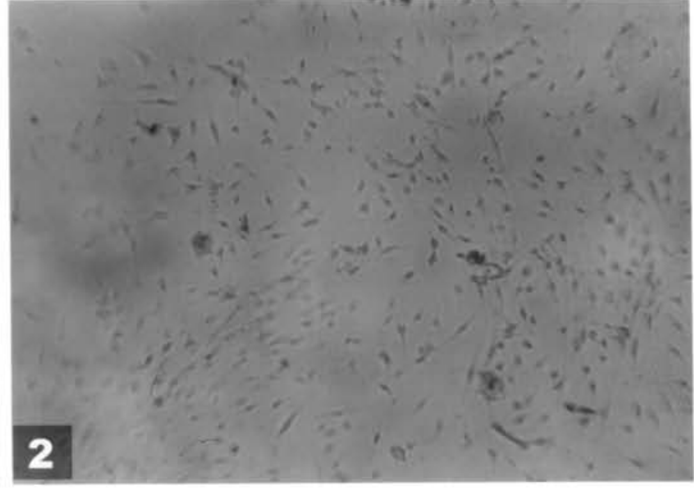
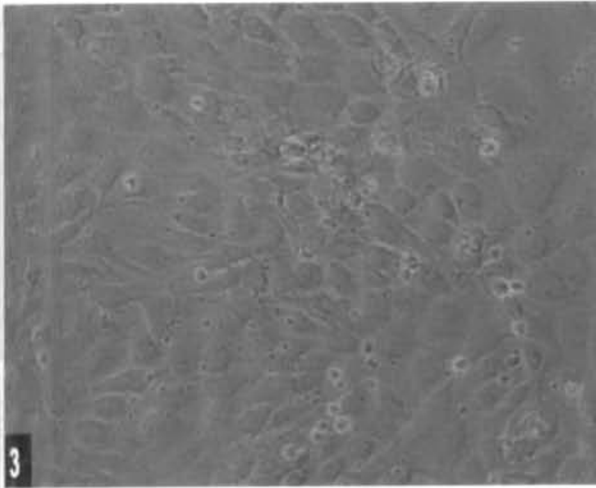
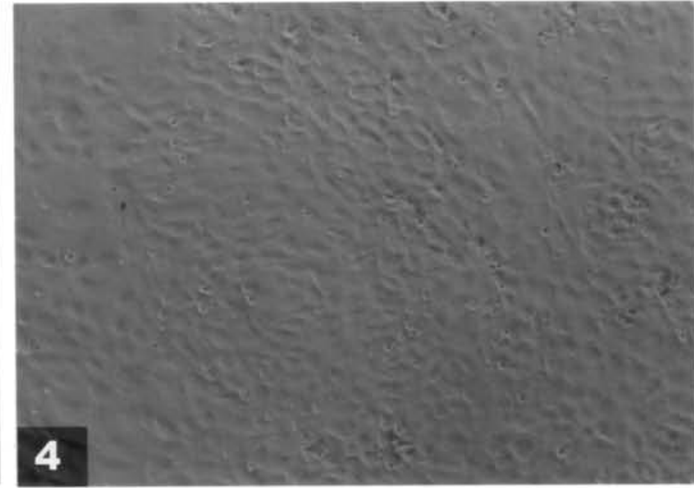
4. Fig. 2.4 Formation of sheet of cells (PRL-1) composed mostly of Fibroblastic cells

5. Fig. 2.5 A primary cell culture composed of fibroblastic and epitheloid cells from embryonic tissue of (*P .reticulata*). Derived by cell dissociation method (20X)

6. Fig. 2.6 Diploid cell line on subculturing the above primary cell culture (20X) both epitheloid and fibroblastic cells are seen

7. Fig. 2.7 Cell proliferation seen from the margin of explants of embryonic tissue with out addition of carbazole (10X)

8. Fig. 2.8 Extensive cell proliferation seen from margin of explants in the presence of carbazole (10X)


1

2

3

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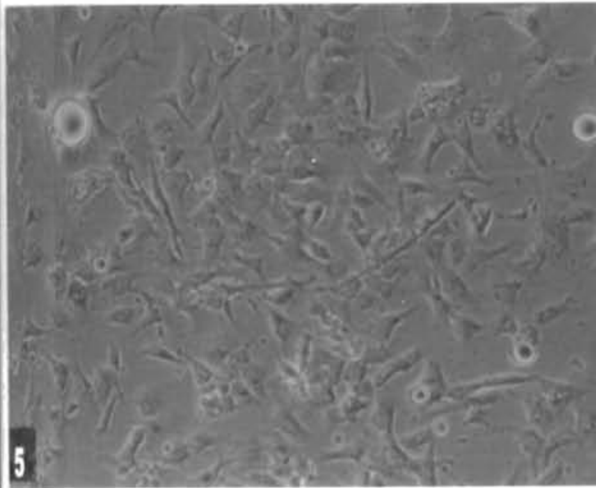
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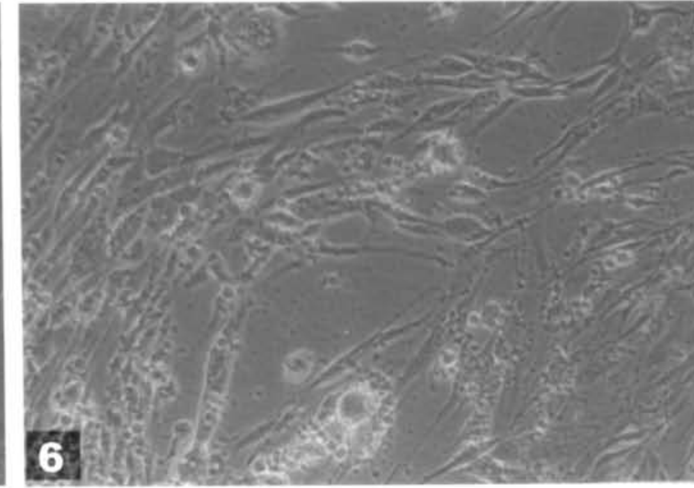
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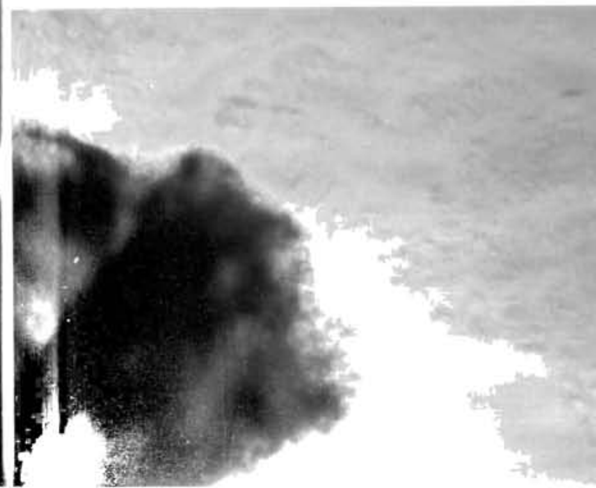
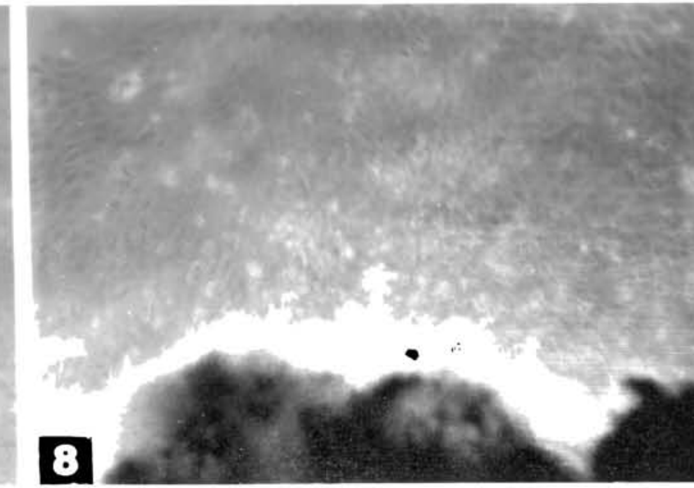


8

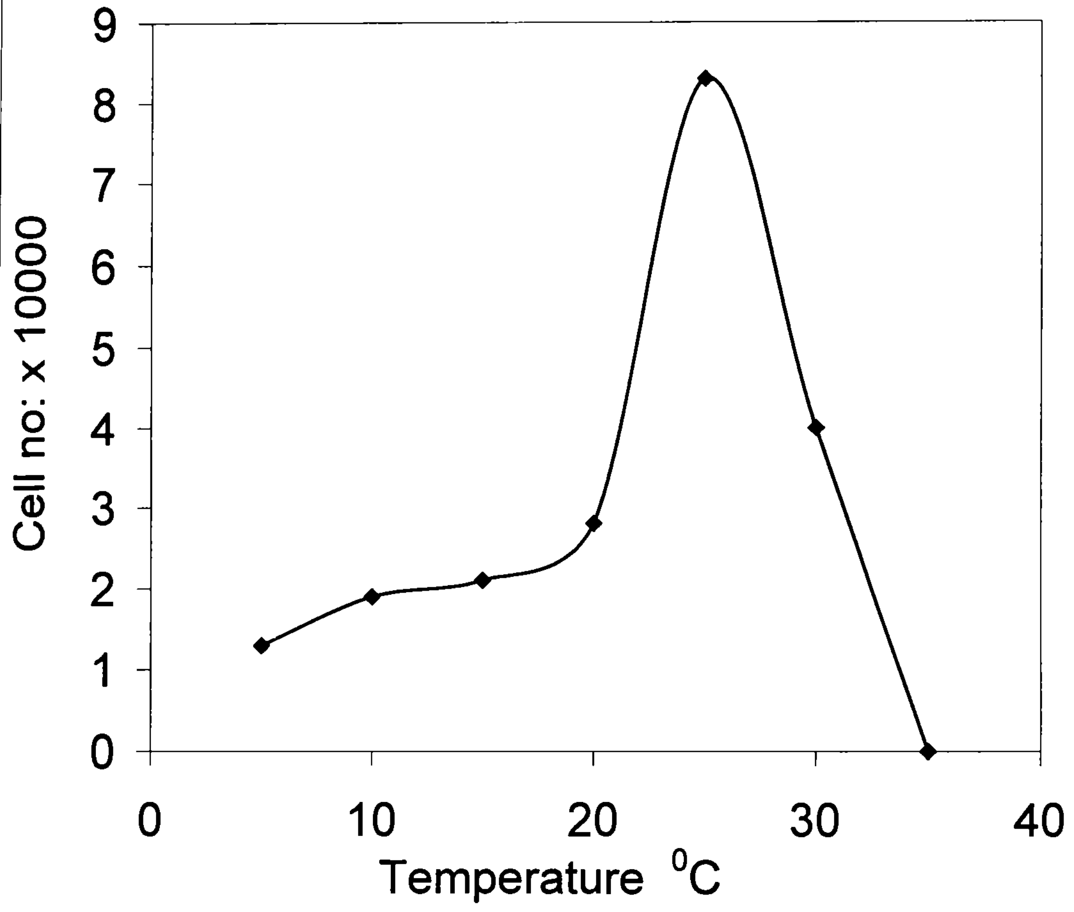


Figure 2. 9 Optimum temperature of growth of the cell culture developed from *Poecilia reticulata*

CHAPTER 3

CELL CULTURE SYSTEMS FROM LIVER, SPLEEN, KIDNEY, TESTIS AND OVARY OF CLARIAS GARIEPINUS

CHAPTER 3

CELL CULTURE SYSTEMS FROM LIVER, SPLEEN, KIDNEY, TESTIS AND OVARY OF *CLARIAS GARIEPINUS*

3.1 Introduction

a) Biology, reproduction, ecology and distribution of *Clarias gariepinus*

Clarias lazera (=*gariepinus*), known as the African catfish or sharp tooth catfish or the Nile catfish is a recent addition to aquaculture in Africa. *C. gariepinus* is omnivore often feeding on vegetable matter, aquatic invertebrates, small fishes, detritus etc. Though normally survives on dissolved oxygen, it comes to surface and breaths atmospheric air when the oxygen concentration of water becomes low. The fishes have been observed to reach over 130 cm in length and 12.8 kg in weight. A high degree of hardiness and the ability to feed on a variety of feed stuffs and attainment of good growth and survival in poorly oxygenated waters have made it an attractive fish for rural aquaculture. The species can grow even in brackish water in salinities of 10ppt and have been found to survive in salinities up to 29ppt (Pillay, 1990).

The animals mature under natural conditions at the size of about 32 cm (two or three years old) and spawn in flooded rivers. Under pond conditions they mature in about 7 months especially when they attain a weight of 400-600gm. They seem to spawn only once with the onset of the rainy season under the natural conditions. However, they can breed throughout the year under captivity. Observations confirm that spawning is stimulated by floods or increased levels of water in ponds due to rain or exchange with fresh water. Eggs are ejected out in several batches (15-50 in a batch) during the extended mating and spawning, at

about 17^{0}C and the eggs adhere to sedges and grass. Females can be identified by the rounded vent with a longitudinal clefts, and the males by elongated urogenital papilla. From the experimental studies it is concluded that the best means of stimulating ovulation in females is by injection of the hormone product desoxycorticosterone (DOCA). The suggested dose is a single intraperitonial injection of 5mg DOCA per 100g weight of fish. Injected females and matured males (which may not require any injection) are kept in separate tanks for about 10 hours after which they are placed together in a tank or cement cistern, usually in the evening hours. Spawning occurs during night, about 10-16 hours after injection. The eggs can easily be collected in the morning and hatchlings can be allowed in separate containers. The main problem with this method is that the couples often inflict fatal injuries on each other. To avoid this, the injected can be stripped and the eggs fertilized artificially. First the females are injected ones with the above dose of DOCA., usually in the morning, and the males in the evening. The female is stripped about 10 hours after the injection. Males cannot be hand-stripped because of the structural peculiarities of the seminal vesicle. If the pressure is excerted on the abdomen, the milt will pass to the dorsolateral lobes of the vesicle and not to the genital opening. Therefore the males have to be killed and the sperm collected directly from the vesicles. Injection helps to increase the yield of milt three to five times. Embryonic development is completed in about 24 hours after fertilization at the temperature around 26^{0}C. The yolksac is absorbed in 6 days and the larvae start feeding when about 3 days old.

Clarias lazera can be spawned by hypophysation as well, like many other species (Hogendoon, 1979). Acetone – dried carp pituitary at a dose of 4 mg per kg body weight is adequate to ripen females. As mentioned earlier, injection of males does not seem to help in stripping, and they have to be sacrificed to obtain milt. The females can be stripped 11-16 hours after injection. At 20^{0}C hatching of fertilized eggs occur in about 48 hours, where the hatchlings can be transferred from incubators to trough rearing.

The stocking rate of fry is generally 10000-20000 per.hectare. They are fed on natural food, and normally they reach a weight of around 10g in about three weeks. Thereafter, the fry start feeding on larvae of aquatic insects. Artificial feeds can be given to the fry at this stage. Though amphibians, aquatic insects and occasionally wild fish prey on the fry, the main reason for low survival in ponds appears to be the lack of appropriate feed. The fish have been reared in ponds heavily fertilized with pig manure and fed on different locally available feed stuffs. Brewery waste and peanut cake are used as the main supplementary fed in some centers. Richer (1976) pointed out the composition of a diet used to determine the growth rate of fingerlings.

Although a number of parasites have been identified from *C. lazera,* mortality due to major infections has not been reported. *Trichodiana* infection of fry has been observed in ponds and this can be controlled by treatment with 50 ppm formalin for about an hour.

b) Significance of using *C. gariepinus* as donor fish

By experience *C.gariepinus* has proved to be one of the best donor fresh water fishes of tissues and organs for cell culture development. The primary reason is the possibility for its captive breeding and the easiness with which young ones can be produced. In laboratory the fish can be easily maintained and by third month of age gonadal development starts. Seldom the animals are succumbed to diseases and that increases the success rate of aseptic collection of internal organs.

No cell line from *C. gariepinus* has been reported. However, from species of the genus *C. batrachus* three cell lines have been generated such as GIB(N-gill), GDII (N-gonad) and KIK (N-kidney) (Noga, 1977 and, Noga and Hartmann, 1977). They prefer a temperature ranging from 18-37^{0}C and 25^{0}C has been reported to be the optimum . All the cell lines were susceptible to channel

catfish virus (CCV) and GIB is composed of epithelioids, KIK, fibroblastic and GDII mixed (Wolf and Mann, 1980)

The present work deals with the development of cell cultures from liver, spleen, kidney, testis and ovary of *C. gariepinus*

c) Cell cultures developed from *C. gariepinus*

To date no report has been seen with regard to the development of cell cultures from *C. gariepinus*.

3.2 Materials and Methods

3.2.1 Development of an appropriate protocol for disinfecting the surface of *Clarias gariepinus* to remove the internal organs aseptically

As described in the earlier section an appropriate protocol for disinfecting the surface of donor fish has to be evolved for every fish species brought under study. This is because microbial flora on the surface varies with the habitat, food and feeding habits and several other physico-chemical factors of the environment. Since *Clarias gariepinus* can survive in muddy turbid water, there is every possibility for fish to carry a higher bacterial load on the surface. Therefore the fishes to be sacrificed were maintained and starved in clean tap water for 2 to 3 days and subjected for a disinfection protocol as described under section 2.2.1 The strength of the disaffection solution used and the percentage of alcohol employed and the type and composition of media used for testing the efficacy of the procedure were exactly the same.

3.2.2. Screening of commercially available media to select the most suitable ones for developing cell culture systems from different internal organs

3.2.2.1 Preparation of media

As described under section 2.2.2.1 twenty one commercially available media were used as prepared in the same composition with the same strength of sodium bicarbonate, L-glutamine and antibiotic mixture.

3.2.2.2 Preparation of fish and tissue removal

Females of *Clarias gariepinus* identified by the rounded vent with a longitudinal cleft were brought to the laboratory alive and maintained on a diet specially prepared. For removing internal organisms the animals were transferred to another container with running tap water and starved for three days. Small fishes were sacrificed by plunging in ice cold water and larger ones by giving a hard blow on the forehead. The animals were then dipped in sodium hypochlorite with 600ppm available chlorine for 15 minutes and washed thoroughly with sterile tap water. The surface was again washed with 70% alcohol for 5 minutes. The animal was pinned to the dissection board and the mucous from the body was removed by rubbing with sterile cotton pad. The body was cut open through the ventral side and the organs such as liver, spleen, ovary and kidney were removed to sterile PBS containing glucose and antibiotic mixture. For getting testis male fishes were selected, identified by the elongated urogenital papila. Generally for liver, kidney and spleen one year old fishes were used and for ovarian and testicular tissue four to six months old fishes were used.

3.2.2.3 Preparation of tissue and mode of culture to screen the most appropriate ones

The above mentioned five tissues were independently minced in PBS over rubber cork using surgical scalpel blades into $1mm^3$ pieces after keeping covered with aliquots of PBS. The minced pieces from liver, kidney and spleen were washed repeatedly in PBS to remove blood cells, and ovary and testis were not subjected to any such washing as they were very soft and delicate. A minimum number of 10-15 pieces of each tissue were transferred into tissue culture bottles containing 0.5 ml fetal bovine serum and were subsequently supplemented with 4.5 mL growth media after 2 to 3 hours of incubation. Twenty one such growth media were tried for each tissue tested. The bottles were stoppered with rubber cork and incubated at room temperature 28 ± 0.5^0 C for three days continuously before the first observation was made. The bottles were observed under an inverted microscope and the extent of attachment, growth and monolayer formation were examined and recorded.

3.2.3. Development of an appropriate subculturing technique for the cell cultures originated from the various tissue

From the above series of experiments an appropriate medium for each tissue for the development of primary cell cultures by the explant method could be segregated. Before going further, the most important stage in the process of development of cell lines is to have an effective methods of subculturing by which the cells from one bottle can be safely dislodged with out the loss of viability to another where it will again grow and form a monolayer. The cell dissociation solution used in general were TPVG (trypsin phosphate versene glucose) with four different concentration of trypsin such as 0.2%, 0.1%, 0.05% and 0.025% trypsin. Besides two non-enzymatic solutions such as NES 1 and 2 having 50% dilutions were also employed. Two physical methods such as simple agitation and removal of cell sheets with cell scraper were also attempted. In

addition to this two cocktail solutions consisting 1. TPVG (0.025% trypsin and 50% strength non-enzymatic solution 1 and 2. Cocktail which consisted of TPVG (0.0125% trypsin and non-enzymatic solutions 1 and 2 having 25% strength each. (For more details please refer the section 2.2.5). In every case for the tissues the percentage of cells dislodged and the percentage of cells attached on to the new bottles were assessed.

3.2.4 Efficacy of tissue derived growth factors and mitogens as additives for enhanced growth and monolayer formation from various tissues

Efficacy of tissue derived growth factors and mitogen as growth enhancers were tested in the basal media segregated based on the experiment conducted as per the section 3.2.2. The basal media selected according to that experiment were MEM Earle's (AT017) and Medium 199 for liver tissue, MEM Earle's (AT017) for Spleen, MEM Earle's (AT017) and M199 for Kidney and L-15 for testicular tissue and MEM Earle's (AT017), M199 and L-15 for Ovary. The growth factors incorporated were FME, PME, Lectin 1 and Lectin 2, LPS, Glucose, Sucrose, Trehalose, Ovary extract, Prawn shell extract, Chitin, Insulin, Prawn haemolymph, Clam haemolymph, and Fish skin extract. The preparation of tissue derived growth factors and the mitogens incorporated are described under section 2.2.4.

3.2.5 Duration required for effecting subculturing the monolayer formed from various tissues

As a rule in every instance subculturing is carried out when growth gets arrested by way of contact inhibition. This duration depends upon various growth conditions of which the composition of the medium used is most important. Therefore based on the information obtained on the efficacy of tissue-derived growth factors and mitogens in enhancing the formation of a cell culture a programme for determining the duration of subculturing was formulated. Under

this category a primary cell culture was initiated in the segregated medium which was amended at varying levels and the completion of growth at each instance was recorded by visual observations. The media used at different levels of amendments were as follows:

A. Liver

1. MEM+FBS+FME+PME
2. MEM+FBS+FME+PME+lectin2
3. MEM+FBS+FME +PME+LPS
4. MEM+FBS+FME +PME+Glucose
5. MEM+FBS+FME +PME+OE
6. MEM+FBS+FME +PME+Lectin2+LPS+Glu+OE

B. Spleen

1. MEM+FBS+FME+PME;
2. MEM+FBS+FME+PME+Lectin1
3. MEM+FBS+FME+PME+LPS
4. MEM+FBS+FME+PME+OE
5. MEM+FBS+FME+PME+PHL
6. MEM+FBS+FME+PME+Lectin1+LPS+OE

C. Kidney

1. MEM+FBS+FME+PME
2. MEM+FBS+FME+PME+Lectin1
3. MEM+FBS+FME+PME+LPS
4. MEM+FBS+FME+PME+OE and
5. MEM+FBS+FME+PME+Lectin1+LPS+Glucose+OE.

D. Testis

1. L-15+FME+PME;
2. L-15+FME+PME+LPS;
3. L-15+FME+PME+OE;
4 L-15+FME+PME+LPS+OE+PHL

E. Ovary

1. L-15+FME+PME
2 L-15+FME+PME+Lectin
3 . L-15+FME+PME+LPS
4. L-15+FME+PME+Glucose
5. L-15+FME+PME+OE
6. L-15+FME+PME+PHL
6. L-15+FME+PME+Lectin2+LPS+Glucose+OE+PHL

3.2.6 Optimum temperature of growth of the cell cultures developed from various tissue

The temperatures selected were 5, 10, 15, 20, 25, 28, 30 and 35^{0}C. The diploid cell lines generated from all the five tissues were subjected for the experiments. The cell cultures at the 2nd or 3rd passage were dislodged employing the most appropriate cell dissociation solution selected individually for each cell line viz. TPVG containing 0.05% trypsin for liver and kidney, TPVG containing 0.1% trypsin for spleen, cocktail of solutions containing TPVG (0.025% trypsin) plus non-enzymatic solution 1 (50%) and non-enzymatic solution 2 (50%) for the testicular tissue and the solution containing TPVG (0.125%) trypsin, non-enzymatic solution 1 (25%) and non-enzymatic solution 2 (25%) for the ovarian tissue. The protocol of sub culturing was the same as described under section 2.2.8. In the complete growth media cells were dispersed into the Leighton tubes, plugged with rubber cork and incubated in the above mentioned temperatures. After 4 days of incubation the cells were harvested and counted and assessed the extent of growth visually.

3:2.7 Description of the cell cultures developed

The primary cell cultures and diploid cell lines developed were examined for the type of cells formed under an inverted microscope.

3.2.8 Cryopreservation of ovarian tissue for subsequent development of cell cultures

Females of *Clarias gariepinus* with developing ovary weighing 600-800gm were brought alive from a farm (Rosen Fisheries, Marathakara, Trichur) and starved in clean tap water for three days. The fish were sacrificed by giving a hard blow on the forehead region and was disinfected by immersing in diluted sodium hypochlorite containing 600ppm chlorine for 15 minutes and subsequently swabbed/rinsed with 70% ethanol and washed repeatedly with sterile tap water. The abdomen was cut open at the ventral side and the ovarian tissue was aseptically removed to PBS supplemented with 0.2ml antibiotic mixture per 100 ml. The ovary was divided in to two lot, one for preservation at varying concentration of dimethylsulfoxide (v/v) such as 10 %, 7.5%, 5 %, 2.5 % and 1% and the other at varying concentrations of glycerol (v/v) such as 10%, 7.5%, 5%, 2.5% and 1%. The tissue was minced in to 2-3mm^3 pieces in L-15 and transferred to 2 ml cryovials and one lot was added with L-15 amended with 10% (v/v) FBS, FME, PME and varying concentrations of dimethylsulfoxide and other lot with L-15 amended with 10% (v/v) FBS, FME and PME and varying concentrations of glycerin. The cryovials were maintained at 4^0C for one hour and then transferred to low temperature (at -20^0C) for one hour. From there one set of tubes were shifted to -35^0C (Lab Line, India) and another set to liquid nitrogen (liquid phase) at -195^0C. Twenty four hours after preservation and later once in a week, an ampoule each was retrieved from the low temperatures and thawed rapidly by plunging in sterile distilled water at room temperature. The ampoules were centrifuged at 6000rpm, supernatant removed and replaced with fresh growth medium and maintained at 4^0C. Centrifugation at 4^0C and replacing with fresh growth medium was repeated once more. The tissue pieces thus thoroughly washed and made free of the cryoprotectants were seeded in to tissue culture bottles and added with growth medium supplemented with 10% (v/v) FBS, FME and PME and were incubated at 28 ± 0.5^0C. Attachment of explants to the tissue culture bottle, cell spreading and proliferation were noted.

3.2.9 Application of ovary extract as substitute of fetal bovine serum in a tissue culture

Extracts of developing ovary of *C. gariepinus* was prepared in PBS, (for more details please refer section 2.2.4) and incorporated into several growth media and their efficacy was checked. The extract containing a variety of unknown growth factors was found to enhance the attachment of explants, and cell proliferation. Therefore it was thought of applying it in the established fish cell line, RTG-2 as a substitute for fetal bovine serum.

The cell line RTG-2 was procured from the National Centre for Cell Science, Pune and subcultured in MEM (Eagle's) with 10% FBS (v/v). Using the cell line the efficacy of the ovary extract as subcultured following standard procedure as follows: The spent medium was decanted and the cell line was washed twice with PBS devoid of calcium and magnesium. The cell line was subsequently rinsed twice with TPVG containing 0.2% trypsin and as the cells got rounded off, the bottle was tapped, the action of the trypsin was stopped by adding the growth medium. Cells from the bottle was divided and transferred to two tissue culture bottles and one was supplemented with foetal bovine serum to a final concentration of 10% and the second with ovary extract to a final concentration of 10% (v/v). Both the bottles were incubated under the same conditions and time required for monolayer formation and completion of growth was monitored so as to enable subculturing. The subculturing was carried out in the same fashion and continued on one hand with FBS and on the other hand with ovary extract.

3.3 Results and Discussion

3.3.1 An appropriate protocol for disinfecting the surface of the animal

Results of the experiments conducted to develop an appropriate protocol for disinfecting the surface of *Clarias gariepinus* are presented in Table 3.1. It is well evident from the data that an exposure to 600ppm chlorine and subsequent disinfection with 70% ethanol for 5 minutes was necessary to sterilize the surface of the fish. Requirement of higher concentration of chlorine indicated that the surface was with a heavy microbial load. There was excess mucous on the surface and that also might have contributed to the higher demand of the chlorine content. In literature different authors (Wolf and Ahne, 1982, Chen *et al.*, 1983, Singh *et al.*, 1995) have used different methods for disinfecting the fish surface indicating the requirement of separate standardization for each fish species. Thus the protocol finalized for the disinfection is as follows: Starve the animal for three days in clean tap water, sacrifice either by plunging in ice cold water on by giving a hand blow on the forehead. Immerse in Sodium hypochlorite containing 600 ppm chlorine for 15 minutes and wash with sterile tap water, soak with 70 % ethanol for 5 minutes and wash repeatedly with sterile tap water.

3.3.2. Screening of commercially available media to select the most suitable ones for developing cell culture systems from different internal organs

Results of the experiments conducted to screen the commercially available growth media for the development of cell culture systems from liver, spleen, kidney, testis and ovary are summarized in Table 3.2. The assessment was made based on the extent of attachment, growth and monolayer formation in the presence of each medium. Among the 21 growth media employed, the performance was as follows; Liver, spleen and kidney preferred MEM with Eagle's salt with sodium bicarbonate, L-glutamine and antibiotic mixture. Meanwhile, testis and ovary preferred L-15 (Leibovitz-15) with L-glutamine,

without antibiotics. The media were prepared as described under section 2.2.2.1. Fernandez *et al.*, (1992) on studying the comparative growth response of 13 salmonid and 14 non-salmonid fish cell lines, in three different media such as MEM, M199 and L-15 observed that Eagle's MEM had been performed as the choice of media by most fish tissue culturists, needless to say that almost all of the present cell lines had been initially propagated using this medium. This further confirms and agrees with the report of Fryer *et al.*, (1965) on the superiority of Eagle's MEM over that of other commercial preparations. In the light of these previous observations, the finding made in the present work that the tissues such as liver, spleen and kidney preferred Eagle's MEM assumes much importance. However, in the present study tissues like testis and ovary preferred L-15 than the other commercial media. Several instances can be cited from literature regarding the use of the medium L-15 as the medium of choice among the commercial media available. Nicholson (1985) on reviewing fish cell cultures observed that the most widely used media for fish cells are Eagle's Minimum Essential Medium (MEM) and Leibovitz medium (L-15) supplemented with 5-10% Foetal Bovine Serum (FBS). Other instances where L-15 was successfully used as the medium of choice are from Kocal (1988) in the hepatocyte culture from trout, Tung *et al.*, (1991) in the development of three cell lines from spleen and kidney of Black porgy and from Chen (1983) in the development of a cell line from *Tilapia* ovary.

3.3.3 Development of an appropriate subculturing technique for the cell cultures originated from the various tissue

Results of the experiments carried out to choose the right cell dissociation solution and the subculturing technique for the primary cell cultures developed from five tissue of *C. gariepinus* are summarized in Table 3.3 to 3.7. Assessment of performance of the cells to various enzymatic, non-enzymatic and cocktail preparations was based on the visual observation and determination of the percentage (in approximate) of the cells dislodged and the percentage of cells among which attached and grown into monolayer. The primary cell culture

developed from the liver could be effectively subcultured with 70% dislodgment of cells and the same percentage of attachment using TPVG containing 0.05% trypsin (Table 3.3). Meanwhile, the primary cell culture developed from spleen required TPVG containing 0.1% trypsin for the dislodgment of highest percentage of cells (70%) and the subsequent attachment of 65% (Table 3.4). The cell culture from kidney required just like that of cells originated from liver, 0.05% trypsin in TPVG. By this way 80% of the cells could be dislodged and the same percentage was found to get attached (Table 3.5). Differing from the above tissues primary cell cultures from the testicular tissue could be passaged by employing a cocktail of cell dissociation solution which contained TPVG (0.025% trypsin), non-enzymatic solution 1 (50%) and non-enzymatic solution 2 (50%) (Table 3.6). A higher percentage of 95% cell could be dislodged by this fashion. The primary cell cultures from the ovarian tissue required another cocktail dislodgment solution. For the effective and safe preparation the cocktail contained TPVG (0.0125% trypsin) plus NES 1, (25%) plus NES 2, (25%) and with this preparation not less than 90% of the cells could be dislodged and from among them 95% could get attached to the fresh tissue culture bottle and grew into monolayer.

Different authors have used a wide variety of methods for subculturing. Chen *et al.*, (1983) used 0.1% trypsin prepared in EDTA/PBS for subculturing the cell lines derived from *Tilapia* ovary. Rao *et al.*, (1997) used 0.025% trypsin EDTA for subculturing a primary cell culture developed from the heart tissue culture of Indian major carps. Lidgerding *et al.*, (1984) subcultured a confluent monolayer of cells with 0.1% trypsin in 0.02% versene in Ca and Mg free phosphate buffered saline (PBS). Driever and Rangini (1993) on working with Zebrafish embryo cell lines, observed that during initial passage, cells are very sensitive to EDTA. Hence they passaged the cells first by washing with Hank's Balanced Salt Solution (HBSS, minus Ca and Mg) and dissociation using trypsin only at 0.025% in HBSS. Wolf and Ahne (1982) on examining the problems related with fish cell culture, observed that the general method of sub- culturing included treatment with trypsin or versene or preferably with a combination of the

two. In combination, the final concentration of trypsin is usually 0.1% and that of versene is 0.02% or 200µg mL^{-1}. This is a clear departure from the procedure generally followed in the case of mammalian and avian cell lines, where 0.25% trypsin is required for the dislodgment of cells. The experience in the present work along with the observations by previous workers strongly suggest that trypsin > 0.1% is toxic to fish cell lines in general.

In the case of primary cell cultures from testicular and ovarian tissues a cocktail solution prepared out of trypsin and non-enzymatic solution was most effective. In literature the application of non-enzymatic solution for dislodging cell lines are very seldom reported. Bols *et al.*, (1994) on developing a cell line from primary cultures of rainbow trout gill attempted subculturing a primary cell culture by trypsin and two commercially available non-enzymatic cell dissociation solutions used in the present context.

Employing the cell dissociation solution standardized here, the cell cultures could be passaged repeatedly proving their efficacy. Meanwhile, Faisal (1995) when developed a continuous liver cell culture from a marine teleost, 0.025% trypsin in 0.02% EDTA solution for 10 minutes was used. For arresting the activity of trypsin medium containing 10% FBS and twice with HBSS was used.

Physical methods of cell dislodgment such as pipetting, application of policeman to scrap off the cells were very seldom employed. Agitation by pipetting is applied only in the cases where the cells grow loosely attached to the glass or plastic flasks as done by Part *et al.*, (1993). Wolf and Quimby (1962) on working with established eurythermic lines of fish cells *in vitro* could subculture the cell lines by scraping wit h policeman after 10 minute cold dispersion with sodium versinate (20µg/100ml) followed by immediate neutralization with old medium.

3.3.4. Efficacy of the tissue derived growth factors and mitogens as additives for enhanced growth and monolayer formation from various tissues.

Efficacy of tissue derived growth factors and mitogens was tested on cell cultures developed employing the basal media which had been segregated earlier by a series of experiments from 21 commercially available media. In the case of liver tissue both MEM and M199 were used as the basal medium and the medium MEM (Earle's) amended with 10% FBS, FME and PME and 0.5% OE, 0.02μg mL^{-1} lectin2, the same quantity of LPS and 0.2mg mL^{-1} glucose favored 75% attachment of explant and monolayer formation within 96 hrs (Table 3.8; Fig. 3.1 and 3.2). The spleen tissue preferred MEM amended with 10% FBS, FME, PME and 0.5% OE, 0.02μg mL^{-1} lectin 1, the same quantity of LPS, 0.2 mg mL^{-1} glucose and 2% prawn haemolymph (PHL). In this combinations of growth factors, 90% of explants could get attached with the formation a monolayer having 50% confluency (Table 3.9; Fig. 3.3 and 3.4). Explant of kidney responded positively to MEM amended with 10% PBS, FME, PME, 0.05% OE, lectin 1, 0.02μg mL^{-1} and the same quantity of LPS. In this combinations 75% of explants could get attached, initiating monolayer formation within 90 minutes attaining 50% confluency (Table 3.10; Fig. 3.5 and 3.6). Testicular tissue preferred L-15 as the growth medium and when it was amended with 10% FBS, FME, PME and 0.05% OE, 0.02μg mL^{-1} LPS and 2% PHL, 80% of the explants got attached and within 72 hours cell proliferations could begin attaining 50% confluency (Table 3.11; Fig 3.7 and 3.8). In the case of ovarian tissue, three media such as M199, MEM and L-15 were employed as the basal media with amendments. In the trial run L-15, amended with 10% FBS, FME, PME and 0.5% OE, 0.02μg mL^{-1} lectin 2 and the same quantity of LPS, 0.2mg mL^{-1} glucose and 2% PHL was found to be the most suitable combination in which 95% attachment of explants could take place and cell proliferation could begin with 24 hrs attaining 60% confluency (Table 3.12; Fig. 3.9 and 3.10).

Other than the requirement of serum growth factors there is difference in opinion among researchers on the need for mitogens and other tissue derived growth in developing new cell cultures. Kocal *et al.*, (1988) studied the influence of trout serum to prepare primary cell cultures of hepatocytes from rainbow trout. They obtained that in the absence of serum, less than 10% hepatocytes attached and none of them spread on the collagen substrate. Trout serum at concentrations as 1.25% in the medium resulted in a pronounced concentration dependent increase in hepatocytes attachment. According to their observation foetal bovine serum had little influence on the attachment on spreading of trout hepatocytes. In the present study instead of fish serum, prawn haemolymph was added at 0.2% level of 50% diluted prawn haemolymph, which resulted in tremendous improvement in the attachment of explant and spreading of cells. This was especially observed in spleen tissue, testicular and ovarian tissues. Prawn haemolymph greater in quantity than this was toxic. Use of fish and prawn muscle extracts in the development of fish cell lines is not commonly accepted except the reports of Singh *et al.*, (1995) by Lakra and Bhondae (1996) who used fish muscle extract as additive in the growth media. However Tong (1996) attempted to initiate cell culture from *Penaeus chinensis* tissue used as growth media L-15 supplemented with FBS, shrimp muscle extract, and haemolymph of the mantis shrimp *Squilla oratonia*. Recently Kumar *et al.*, (1998) reported the growth of the primary cell culture developed from the embryonic tissue of *Poecilia reticulata*. All these earlier works along with the one described here support the view that tissue derived growth factors added in small quantity to the growth medium can enhance the attachment of explants, spreading of cells and monolayer formation.

The amended media with all the growth factors and mitogens together had a synergistic effect on the cell cultures which was demonstrated starting from the per cent attachment of growth factors to the formation of monolayer. In the context it is worth mentioning the difficulty experienced by Klauning *et al.*, (1985); Lipsky *et al.*, (1986) and the achievement of Blair *et al.*, (1990) in the development of a system of primary, non replicating culture of trout liver cells on

dishes coated with fish skin extract in defined medium. However, a similar addition of fish skin extract in the process of developing primary cell culture from the ovarian tissues did not evoke any response at all.

Segner *et al.*, (1994) observed that incubation of trout liver cells with fructose, lactose, glucose, dihydroxyacetone or glycerol resulted in enhanced rate of cellular glucose and lactate production suggesting that the cultured cells not only could be maintained but ever enhanced their gluconeogenic capacity. Application of glucose in medium in the present study was effective and has been accepted as one of the ingredients in the case of liver tissue, spleen and ovary.

The usefulness of ovary extract in enhancing the extent of attachment of explants and shortening the time required for initiating cell spreading and monolayer formation has to be highlighted. This lead to incorporating these extracts as the additives in the final formulation of the media. Virtually for all tissues, ovary extract has been incorporated as one of the additives.

Faisal (1995) observed that concanavalin A and bacterial lipopolysaccharides augment DNA synthesis in cultured liver cells. This observation has been indirectly confirmed in the present study by way of demonstrating enhanced growth of the primary cell cultures.

3.3.5 Duration required for effecting subculturing the monolayer formed from various tissues.

The duration required for effecting subculturing each cell culture system derived from different tissues was determined employing the segregated media amended at varying levels. Subculturing was carried out when the growth got arrested, assessed by observing under microscope. The duration required for effecting subculturing, the monolayer formed at different levels of amendments for the cell culture developed from the liver tissue is presented in Table 3.13. The

shortest duration of 6-9 days for subculturing was observed with 9 passages in the amended MEM with FBS, FME, PME, Lectin2, LPS, Glucose and OE. In all other combinations, the period required for the completion of growth was prolonged and only up to 5 passages the culture could go. Altogether 80 trials could be made employing the media described above, and, by delaying subculturing the cell line (CGL-1) could be maintained more than one year.

Table 3.14 summarizes the duration required for effecting passages of the cell cultures derived from spleen in growth media MEM amended at various levels. Among the cell cultures in the media mentioned in the Table, the one in MEM amended with the FBS, FME, PME, Lectin 1, LPS and OE required at the initial stage 12 days for subculturing and it subsequently got reduced to 8 to 9 days and a maximum of 8 passages could be obtained. The cell line developed is named as CGS-1.

The duration required for effecting subculturing the monolayer formed from the kidney in the media at various levels of amendments is presented in Table 3.15. The shortest duration of 8-9 days was obtained in MEM amended with FBS, FME, PME, Lectin1, LPS, glucose and OE. In this medium the cell line could be subcultured eight times. In all other media it required a prolonged period for effecting subculturing and the number of times it could be subcultured also was very low. The cell line is named as CGK-1.

A monolayer formed from the testicular tissue in L-15 amended with FME, PME, LPS, OE and PHL could be subcultured 6 times once in 5-9 days. By delaying subculturing the cell lines could be maintained for 60 days and altogether 11 trials could be made (Table 3.16). The cell line is named as CGT-1.

Duration required for effecting subculturing in days the cell culture developed from ovarian tissue in L-15 under different levels of amendments is summarized in Table 3. 17. The cell line generated from developing ovary on L-

15 amended with FME, PME, Lectin 2, LPS, Glucose, OE and PHL was found to grow very fastly attaining completion of growth with in 2 to 3 days in the initial period of growth and after 11 passages there was slight reduction in growth to 4 to 6 days. Altogether 16 passages could be attained. In total 80 trials could be made and without fluid change and by delaying subculturing the cell lines could be maintained for more than one year (Table 3.17; Fig. 3.11 to 3.16) The cell line is named as CGO-1.

From the liver tissue of both temperate and tropical fishes 4 cell lines have so far been developed, but out of which no certified cell line could be generated. Watanabe and Moritomo (1987) developed a cell line YEL-13 from the embryonal liver cells. The cell line was composed of epithelial cells with a round nucleus and abundant granular intracytoplamic organelles. Faisal (1995) developed a continuous liver cell culture from the marine teleost (*Leiostomus xanthurus)* and subculturing was possible and three continuous cultures (designated as SLW, SLN and SLF with the potential to become immortal cell lines were developed.

Development of liver cell lines has always been a very difficult task and attempts to culture fish hepatocytes have been tried (Reviewed in Baksi and Frasier, 1990). The loose attachment to substrate, the absence of active proliferation, and the rapid degradation of enzymes have limited their routine application as a research tool. (Klauning, 1994; Klauning *et al.*, 1985; Lipsky *et al.*, 1986). Recently a procedure was developed to isolate and maintain hepatocytes of same marine teleost from the Chesapeake (Faisal *et al.*, 1991). It is apparent that the species of the donor fish play the most important role in the success of the cell cultures (Faisal, 1995) and some reasons for this species difference may be the difference in basal metabolic rates and varying abilities to adapt to a wide range of osmolarities. From tropical fish species no permanent cell line has ever been developed. With the developments achieved here it can be

expected that CGL-1 may get established with the addition of an appropriate mutagen for effecting *in vitro* transformation.

Nicholson *et al.*, (1987) established a cell line from perch liver (PL) in Eagle's Minimum Essential Medium with 10% Fetal bovine serum and have been subcultured over 120 times. Ostnander *et al.*, (1993) developed a long term primary culture of epithelial cells from rainbow trout liver. In addition to hepatocytes, proliferative biliary cells persisted in the cultures for atleast 30 days. Finally a third type epithelial cells, which have been termed spindle cells consistently appeared and proliferated to confluence in three cultures.

Alike the liver, very limited success stories could be seen in literature with regard to the development of primary cell cultures and cell lines from spleen. Ellender *et al.*, (1979) established a spleen cell line (SP-2) from *Bairdiella chrysuna* (Silver perch). For growth L-15 medium supplemented with foetal bovine serum and sodium chloride (to 0.150M) was employed. Cells replicated best at 25^0C to 28^0C. Tung *et al.*, (1991) reported the development of two cell lines from spleen (BDS-1 and BPS-4) of the black porgy (*Acanthopagnus schlegeli*). The BPS-1 cell lines and BPS-4 cell line consisted of epithelial like and fibroblastic like cells respectively. These cell lines could be developed in L-15 medium supplemented with 10% FBS and 0.15M NaCl at 28^0C and could be passaged to 105 and 104 times respectively. But as per the available information these cell lines are also not available commercially.

As per the listing of teleost fish cell lines published in 1980 by Wolf and Mann, only one cell lines such as KIK from *Clarias batrachus* was documented, composed of fibroblastic cells with a temperature optimum of 25^0C. Tung *et al.*, (1991) developed a cell line (BPK) from the kidney of Black porgy, which is composed of fibroblast-like and epithelia like cells. Using L-15 medium supplemented with 10% fetal calf serum 87 times the cell line could be passaged. Singh *et al.*, (1995) could develop a primary cell culture from the kidney of the

fish *Heteropneustus fossilis* by using M199 supplemented with 20% FME and FBS. Chen *et al.*, (1987) listed out 14 cell lines from warm water fishes and observed the establishment of 4 kidney cell lines such as EK-1 from *Anguilla japonicus*, TK-1 (*Tilapia* hybrid) OK (Snakehead-Chana), and BKG (Banded grouper) and the optimum temperature of the cell line ranged from 24^0 to 32^0C.

No report has ever been obtained with regard to a cell line from the testicular tissue of fish. The present work can be considered as the first one under this category. However, there are several reports on the development of cell culture from the ovarian tissue of fishes. Wolf and Mann (1980), reported the cell lines from gonadal and ovarian tissue of fishes. They are 1. GDII (*Clarias batrachus*) with 25^0C; 2. TG (*Tinea tinia*) with 22-27^0C as optimum temperature; 3. PG (*Essox lucius*) 22^0C as optimum temperature; 4. CCO (*Ectalurus punctatus*) 30^0C as optimum temperature; 5. RTO *Salmon gairdneri*; 6. ASO (*Salmo salar*) optimum temperature 20^0C. Meanwhile, Chen *et al.*, (1987) reported the existence of 1.EO-2 (*Anguilla japonica*) and TO-2 Hybrid *Tilapia*. These reports indicate that it is comparatively easier to get cell lines from gonad especially from the ovarian tissue. In the present study also among all the five tissues tested the ovarian tissue was the most promising one with which a cell culture could be developed with in three days.

3.3.6 Optimum temperature of growth of the cell cultures developed from various tissue

Optimum temperature of growth of the cell culture developed from all the five tissues are summarized in Table 3.18. In the case of cell cultures developed from the liver, spleen, kidney and testis the assessment was made based on the visual observation and in the case of cell lines from ovary it was by both visual observation and by the cell count. The optimum temperature of the cell cultures developed from liver, kidney and spleen was 28^0C and those from testis and ovary grew at 25^0C. Generally the cell lines derived from homeothermic animals require

37^{0}C for maximum growth and temperature optima of 25 to 28^{0}C is the property of cell lines derived from poikilothermic animals. In general the optimum temperature of growth of temperate fishes lies at around 20^{0}C and those from warm water fishes between 25-30^{0}C (Yoshimizu *et al.*, 1987). Chen and Kou (1987) pointed out that a temperature preference of 28-31^{0}C exist between the 14 permanent cell lines established from normal tissue of seven species of warm water fishes cultured in Taiwan. A cell line developed from the liver cells of the marine teleost spot required a temperature ranging from 21- 27^{0}C. Fernandez *et al.*, (1993) is of the view that in general fish cell lines can be grown at lower temperatures compared to mammalian and avian cells. However, according to Wolf and Ahne (1982) fish cell lines could be adapted for growth at slightly higher temperature than growth with reasonable limits. But the present experience suggests that temperatures above 28^{0}C is lethal, better always to incubate between 25-28^{0}C (Fig 3.17).

3.3.7 Cryopreservation of ovarian tissue for subsequent development of cell culture

The practice of cryopreservation of immortalized cell lines at ultra low temperature using classical procedures and controlled cryopreserving equipments have long been in vogue (Saxena *et al.*, 1995, Mazul *et al.*, 1971; Merryman *et al.*, 1962, Smith *et al.*, 1987). Cryopreservation technology is endowed with several advantages 1. It's heterogenity in various cell preparations, b) It minimizes genetic variations due to multiple passaging, c) It avoids cellular ageing-associated biochemical alterations due to the continuous cultures, d) It serves as a standby in case of contamination in tissues, e) It is an easily transportable supply sources of cells. There is no report so far on the long term preservation of tissues which are useful for cell culture development. But so much materials are available for the cryopreservation cell lines from non-transformed mammalian cell lines.

Fish cell cultures may be preserved by freezing and holding at –80^{0}C or lower, by the same procedure used routinely with cells from homeotherm

vertebrates. In addition, fish cells (particularly those from cold water species) have advantage of being available to low temperature incubation and consequently cells can be stored for considerable periods of time without being frozen. This feature eases the task of maintaining fish cell lines during periods when they are not needed.

When fish cells are to be frozen, Wolf and Ahne (1982) recommended that the culture medium contained 10% or more serum. Either of two cryoprotectants – glycerol or dimethylsulfoxide (DMSO) is added to the freeze medium. The compounds should be reagent grade and added to a final concentration of 5-10%.

Significance of the cryopreservation of the ovarian tissue of *Clarias gariepinus* is very much, as the tissue preserved during breeding season can be retrieved at any moment and a cell culture can be developed with two to three days. As the entire protocol for the generation of primary cell culture and diploid cell lines is available the technique of cryopreservation assures the continuous availability of cell cultures from the ovarian tissue of the fish. It has to be recalled that the cell culture developed can be passaged for >15 times.

Response of the ovarian tissue to cryopreservation at -35^0C and -195^0C (liquid phase) in the presence of DMSO and glycerine in L-15 supplemented with tissue derived growth factors is presented in table 3.19. Attachment of explant, cell spreading and cell multiplication were the parameters used to measure the response of the ovarian tissue to the cryopreservation under varying conditions. Explant attachment could be observed under variety of conditions with different concentration of cryoprotectants and the tissue could remain viable only for around 10 days. Among all the conditions given preservation of the tissue at -35^0C with 7.5% DMSO as the cryoprotectant was the most promising as in this conditions the explants remained viable throughout the experimental period of 60 days. The tissue retrieved on the 30th day could lead to the formation of monolayer (Fig.3.19). This primary cell culture could not be passaged. It is hoped

that by fine turning of the technique a viable protocol for the cryopreservation of the ovarian tissue will be able to be developed.

For fish cells to be maintained frozen, Wolf and Ahne (1982) recommended that the culture medium contained 10% or more serum. Either of the two cryoprotectant – glycerol or dimethylsulfoxide is added to a final concentration of 5-10%. In the present experiment for cryopreserving the ovarian tissue, dimethylsulfoxide was found to be superior to glycerol and 7.5% was better than 10% or lower levels. With regard to the temperature of preservation – 35^0C was found to be promising than liquid nitrogen (liquid phase). The data generated suggest that it would be possible to preserve the ovarian tissue under sub zero temperatures to retrieve them later as and when required to develop cell culture as per demand. This sort of preservation can be done during breeding season, (June to October) and the ovarian tissue from one fish is sufficient enough to prepare not less than 100 tissue culture bottles. Suitability of -35^0C than the liquid nitrogen storage make the process economically viable. It is not known why storage in liquid nitrogen did not give the expected positive result. Suitability of -35^0C for cell / tissue storage has not been reported elsewhere. Generally -80^0C, -90^0C, -120^0C and -195^0C are the temperatures of choice which give good results for cell lines. (Ostrader *et al.*, 1993, Meguro *et al.*, 1991) and the result obtained here may be cited as an unique situation with regard to the ovarian tissue of this species of fish.

3.3.8. Application of ovary extract as substitute of fetal bovine serum in fish tissue culture

Serum is the most important and widely the most expensive component of fish cell culture media. Much of the earlier development of fish cell line was done with fetal bovine serum, the most expensive of the commonly used animal sera. That pattern of using fetal bovine serum continues to the present, largely because

investigators are reluctant to change developing cell lines to other sera. Fish cell lines (especially established cell lines) do not take kindly to a medium in which a foreign serum is used. If a cell line is initiated or developed on fetal bovine serum it will generally not accept, without significant reduced growth response, any other sera. At worst, the cells may simply refuse to grow (Wolf and Ahne 1982).

The usual level of medium supplementation with serum is 10%. Some cases cell lines grow satisfactorily with only 5% serum, but growth rates are slightly reduced. Still, further reduction in serum levels is typically accompanied by reduced rates of growth and economy is at the expense of time needed by the cultures to grow to confluency.

The use of serum at levels above 10% has been recommended for the primary cultures and only a very few cell lines, e.g., a 15% level is suggested for the CAR (Gold fish) cell line higher quantity is required.

Serum is an extremely complex mixture of many small and large biomolecules with different, physiologically balanced growth promoting and growth inhibiting activities. Some of the serum components that have been found to support survival and growth of many mammalian cells in culture are 1. Hormonal factors stimulating cell growth and 2. Attachment and spreading factors (biomatrix) 3. Transport protein carrying hormones, minerals, lipids etc.

Results of the experiments in terms of the days required for effecting subculturing RTG-2 when OE was used as the substitute of FBS are presented in Fig 3.18. Altogether four subculturing could be carried out and the first subculturing could be done 26 days after the substitution, against 10-12 days when FBS was supplemented. In the experimental setup with OE, as the subculturing was continued there was prolongation of days required for the completion of growth from 26-33. However, there was no loss of viability of cells or reduction in the percentage of cells attach to the new tissue culture bottle. Only

variation observed was in the progressive extension of time required for completion of growth. Therefore it can be concluded that OE can be considered a substitute of FBS for cell maintenance and based on the results it can be postulated that reduction in the rate of growth experienced can be compensated by supplementing with 1% FBS. In this instance also substantial savings could be achieved in the routine cultivation of RTG-2 cells.

Numerous formulations for serum-free media have been developed in recent years, the majority of them have been used for mammalian cell cultures (Sato and Reid, 1978; Rizzino *et al.,* 1979; Iscone and Melchers, 1978; Muzik, 1982; Huchings, 1978; Rizzino and Sato, 1978) with some developed for use in invertebrate cell culture (Brooks and Tsang, 1979). The removal or reduction of serum in cell culture media may eliminate a number of potential problems associated with serum use such as differences between lots of serum, which may alter the reproducibility of experiments performed in cell culture (Temin *et al.,* 1972). Sato (1975) proposed that one of the major role of serum is to provide groups of hormones for cell growth; if differences exist in batches of serum the supplementation of hormones would also be variable, leading to alteration of cellular function. Serum may be possible sources of contamination with virus (Nuttal *et al.,* 1977) or mycoplasma (Barile and Kern 1971). But ovary extract as a substitute for FBS does not eliminate any of the problems discussed above which are associated with the utilization FBS. The only advantage is that it can lead to considerable cost reduction as, the most expensive component of animal tissue culture medium is still the sera used.

3.4 Summary

An effective protocol for disinfecting the surface of *Clarias gariepinus* was developed which facilitated the aseptic removal of the internal organs. By screening 21 commercially available growth media the most appropriate one for

each tissues was segregated. For developing cell culture from liver, kidney and spleen, MEM (Earle's Salts) was most suitable for that of testis and ovary L-15 was the medium of choice. An appropriate protocol for subculturing the monolayer formed was generated. To have enhanced growth and monolayer formation the growth media were amended drastically with tissue derived growth factors and mitogens. The diploid cell cultures developed were named as CGL-1, CGS-1, CGK-1, CGT-1 and CGO-1. They demonstrated different rate of growth and longevity. Among these cell lines CGO-1 was the most promising one likely to get established. Optimum temperature of growth of above cell lines was determined, for those from liver and spleen it was 28^0C and those generated from testis and ovary it was 25^0C. The developing ovarian tissue could be preserved at -35^0C in the presence of 75% DMSO and tissue could be retrieved and a primary cell culture could be developed. Aqueous extract of the ovarian tissue may be useful as substitute of FBS especially in the maintenance of cell lines.

Table 3.1 Efficacy of the protocol developed for disinfecting surface of *Clarias gariepinus*

Growth on Nutrient Agar Plates				Growth on Sabouraud Dextrose Agar Plates				Blood Agar				Thioglycollate Broth			
A	**B**		**C**	**A**	**B**		**C**	**A**	**B**		**C**	**A**	**B**		**C**
++	100	++	++	++	100	++	+	++	100	+	+	++	100	+	+
++	200	+	+	+	200	+	+	++	200	+	+	+	200	+	+
++	300	+	+	+	300	+	+	++	300	+	+	+	300	+	+
+	400	++	+	+	400	+	+	+	400	+	+	+	400	+	+
+	500	+	+	+	500	+	+	+	500	+	-	+	500	+	-
+	600	+	-	+	600	-	-	+	600	+	-	+	600	+	-
+	700	-	-	+	700	-	-	+	700	+	-	+	700	+	-
+	800	-	-	+	800	-	-	+	800	-	-	+	800	-	-
+	900	-	-	+	900	-	-	+	900	-	-	+	900	-	-
+	1000	-	-	+	1000	-	-	+	1000	-	-	+	1000	-	-

+ : Growth
++: Heavy growth
- : No growth

100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 : Sodium hypochlorite concentrations in ppm.

A : Before disinfection
B : After treatment with sodium hypochlorite
C : After treatment with 70% ethanol

Table 3.2 Screening of commercially available media for developing cell culture systems from *Clarias gariepinus*

Sl.no	Name of Media	Type of Tissues				
		Liver	Spleen	Kidney	Testis	Ovary
1	Dulbecco's Modified Eagle Medium (AT006)	+++	++	+++	++	+
2	Nutrient Mixture F-10 (HAM) modified (AT084)	-	-	-	-	-
3	BME (AT040)	-	-	+	+	+
4	Medium 199 (AT 014)	+	+	+	+	++
5	Earle's balanced Salt Solution (TS 1002)	+	+	-	-	-
6	RPMI-1640 (AT 028)	+	+	++	+	+
7	BME with NEAA (AT010)	-	-	-	-	-
8	MEM Eagle (Modified) (AT018)	++++	+++	+++	+	++
9	Hank's Balanced Salt Solution (TS 1003)	+	+	+	+	+
10	Glassgow's Modified Eagle Medium (AT 058)	+	+	+	+	+
11	LY Medium (AT 012)	-	-	-	-	-
12	Lactalbumin Hydrolysate Medium (ELH) (AT052)	-	-	-	-	-
13	MEM Eagle (With Phenol Red) (AT045)	++	++	+++	++	++
14	Nutrient Mixture (F12 HAM) (AT086)	-	-	-	+	+
15	MaCoy's 5a Medium Modified AT071)	-	+	-	+	+
16	Lactalbumin Hydrolysate Medium (ELH) (AT053)	-	-	-	-	-
17	MaCoy's 5a Medium (AT057)	-	-	+	-	-
18	Way Mouth Medium MB (AT 091)	-	-	-	-	-
19	L-15 (Leibovitz) Medium (AT 011)	++	++	++	++++	++++
20	MEM Eagle (AT017)	++++	++++	++++	+++	++
21	Medium 199(AT015)	++	++	++	+++	++

+ : attachment, growth and monolayer formation
- : no attachment and growth

Table 3.3 Application of different cell dislodgment solutions and techniques in subculturing the primary cell culture systems developed from the liver of *C. gariepinus*

Sl.No	Dissociation Solution	Dislodged cells in %	Attached cells in %
1	TPVG (0.2% trypsin)	100	30
2	TPVG (0.1% trypsin)	80	65
3	TPVG (0.05% trypsin)	70**	70**
4	TPVG (0.025% trypsin)	45	45
5	NES1	50	50
6	NES 2	60	45
7	NES 1 (50%)	35	65
8	NES 2 (50%)	60	65
9	Simple Agitation	40	20
10	With Cell Scraper	70	10
11	TPVG(0.025% trypsin)+NES1 50%+ NES2 50%	50	60
12	TPVG(0.0125% trypsin)+NES1 25%+ NES2 25%	50	50

NES 1 : Non Enzymatic Solution 1, NES 2 : Non Enzymatic Solution 2

** : Most suitable preparation

Table 3.4 Application of different cell dislodgment solutions and techniques in subculturing the primary cell culture systems developed from spleen of *C. gariepinus*

Sl.No	Dissociation Solution	Dislodged cells in %	Attached cells in %
1	TPVG (0.2% trypsin)	100	20
2	TPVG (0.1% trypsin)	70**	65**
3	TPVG (0.05% trypsin)	50	70
4	TPVG (0.025% trypsin)	45	45
5	NES1	50	50
6	NES 2	60	45
7	NES 1 (50%)	35	65
8	NES 2 (50%)	60	65
9	Simple Agitation	40	20
10	With Cell Scraper	70	10
11	TPVG(0.025% trypsin)+NES1 50%+ NES2 50%	50	70
12	TPVG(0.0125%trypsin)+NES1 25%+ NES2 25%	50	50

NES1 : Non Enzymatic Solution 1, NES2 : Non Enzymatic Solution 2

** : Most suitable preparation

Table 3.5 Application of different cell dislodgment solutions and techniques in subculturing the primary cell culture systems developed from the kidney of *C. gariepinus*

Sl.No	Dissociation Solution	Dislodged cells in %	Attached cells in %
1	TPVG (0.2% trypsin)	100	30
2	TPVG (0.1% trypsin)	80	50
3	TPVG (0.05% trypsin)	80**	80**
4	TPVG (0.025% trypsin)	45	45
5	NES1	50	50
6	NES 2	60	45
7	NES 1 (50%)	35	65
8	NES 2 (50%)	60	65
9	Simple Agitation	40	20
10	With Cell Scraper	70	20
11	TPVG(0.025% trypsin)+NES1 50%+ NES2 50%	50	85
12	TPVG(0.0125%trypsin)+NES1 25%+ NES2 25%	60	90

NES1 : Non Enzymatic Solution 1, NES2 : Non Enzymatic Solution 2

** : Most suitable preparation

Table 3.6 Application of different cell dislodgment solutions and techniques in subculturing the primary cell culture systems developed from the testicular tissue of *C. gariepinus*

Sl.No	Dissociation Solution	Dislodged cells in %	Attached cells in %
1	TPVG (0.2% trypsin)	100	0
2	TPVG (0.1% trypsin)	100	5
3	TPVG (0.05% trypsin)	70	10
4	TPVG (0.025% trypsin)	65	45
5	NES1	50	50
6	NES 2	60	45
7	NES 1 (50%)	55	65
8	NES 2 (50%)	60	65
9	Simple Agitation	30	10
10	With Cell Scraper	70	20
11	TPVG(0.025% trypsin)+NES1 50%+ NES2 50%	95**	90**
12	TPVG(0.0125%trypsin)+NES1 25%+ NES2 25%	80	85

NES1 : Non Enzymatic Solution 1, NES2 : Non Enzymatic Solution 2

** : Most suitable preparation

Table 3.7 Application of different cell dislodgment solutions and techniques in subculturing the primary cell culture systems developed from the ovarian tissue of *C. gariepinus*

Sl.No	Dissociation Solution	Dislodged cells in %	Attached cells in %
1	TPVG (0.2% trypsin)	100	0
2	TPVG (0.1% trypsin)	100	5
3	TPVG (0.05% trypsin)	70	10
4	TPVG (0.025% trypsin)	65	45
5	NES1	50	50
6	NES 2	40	35
7	NES 1 (50%)	55	65
8	NES 2 (50%)	50	60
9	Simple Agitation	25	20
10	With Cell Scraper	55	28
11	TPVG(0.025% trypsin)+NES1 50%+ NES2 50%	80	80
12	TPVG(0.0125%trypsin)+NES1 25%+ NES2 25%	90**	95**

NES1 : Non Enzymatic Solution 1, NES2 : Non Enzymatic Solution 2

** : Most suitable preparation

Table 3.8 Response of the Liver of *C. gariepinus* to varying combinations of media, growth factors and mitogens.

Sl. No	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in Hrs	Growth and monolayer formation @
1	M199	10%FBS	20	144	+
2	MEM	10%FBS	25	144	+
3	M199	10%FBS+10%FME	25	144	+
4	MEM	10%FBS+10%FME	45	144	+
5	M199	10%FBS+10%PME	40	144	+
6	MEM	10%FBS+10%PME	35	144	+
7	M199	10%FBS+10%FME+10%PME	40	144	+
8	MEM	10%FBS+10%FME+10%PME	60	144	+++
9	M199	10%FBS+10%FME+10%PME+Lec1*	50	120	+
10	MEM	10%FBS+10%FME+10%PME+Lec1*	45	144	+
11	M199	10%FBS+10%FME+10%PME+Lec2*	40	144	+
12	MEM	10%FBS+10%FME+10%PME+Lec2*	45	96	+++
13	M199	10%FBS+10%FME+10%PME+LPS*	45	96	+
14	MEM	10%FBS+10%FME+10%PME+LPS*	45	96	+++
15	M199	10%FBS+10%FME+10%PME+Glu•	45	144	++
16	MEM	10%FBS+10%FME+10%PME+Glu•	48	120	++
17	M199	10%FBS+10%FME+10%PME+Suc•	48	144	++
18	MEM	10%FBS+10%FME+10%PME+Suc•	45	144	++
19	M199	10%FBS+10%FME+10%PME+Tre•	35	144	++
20	MEM	10%FBS+10%FME+10%PME+Tre•	40	144	++
21	M199	10%FBS+10%FME+10%PME+0.5% OE	70	96	++
22	MEM	10%FBS+10%FME+10%PME+0.5% OE	80	96	+++
23	M199	10%FBS+10%FME+10%PME+0.5% PSE	20	160	+
24	MEM	10%FBS+10%FME+10%PME+0.5% PSE	20	160	+
25	M199	10%FBS+10%FME+10%PME+0.5% Chit	20	160	+
26	MEM	10%FBS+10%FME+10%PME+0.5% Chit	40	160	+
27	M199	10%FBS+10%FME+10%PME+insulin*	25	144	+
28	MEM	10%FBS+10%FME+10%PME+insulin*	25	144	+
29	M199	10%FBS+10%FME+10%PME+0.5% PHL	60	96	++
30	MEM	10%FBS+10%FME+10%PME+0.5% PHL	65	96	++
31	M199	10%FBS+10%FME+10%PME+0.5% CHL	25	144	-
32	MEM	10%FBS+10%FME+10%PME+0.5% CHL	30	144	-
33	M199	10%FBS+10%FME+10%PME+0.5% FSE	60	120	+
34	MEM	10%FBS+10%FME+10%PME+0.5% FSE	45	120	+
35	M199	10%FBS+10%FME+10%PME+0.5% OE+ Lec2*+LPS*+Glu•	60	120	+++
36	MEM	10%FBS+10%FME+10%PME+0.5% OE *+ Lec 2+LPS*+Glu•	75	96	+++++

FBS=Fetal Bovine Serum, FME=Fish Muscle Extract, PME= Prawn Muscle Extract, LPS= Lipopolysaccharides, PHL=Prawn haemolymph, CHL=Clam haemolymph, FSE= Fish Skin Extract

Lec1=Lectin1, Lec 2= Lectin2, Glu=Glucose D, Suc=Sucrose, Tre= Trehalose, OE= Ovary Extract, Chit= Chitin,

+: 10% confluency, ++: 20% confluency, +++: 30% confluency, ++++: 40% confluency, +++++: 50% confluency,
*($0.02\mu g.mL^{-1}$), • ($0.2 mg.mL^{-1}$)

Table 3.9 Response of the spleen of *C. gariepinus* to varying combinations of media, growth factors and mitogens

Sl. No	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in Hrs	Growth and monolayer formation @
1	MEM	10%FBS	25	168	+
2	MEM	10%FBS+10%FME	45	168	+
3	MEM	10%FBS+10%PME	35	168	+
4	MEM	10%FBS+10%FME+10%PME	70	144	+++
5	MEM	10%FBS+10%FME+10%PME+Lec1*	45	168	+
6	MEM	10%FBS+10%FME+10%PME+Lec2*	45	120	+++
7	MEM	10%FBS+10%FME+10%PME+LPS*	75	120	+++
8	MEM	10%FBS+10%FME+10%PME+Glu•	48	168	++
9	MEM	10%FBS+10%FME+10%PME+Suc•	45	168	++
10	MEM	10%FBS+10%FME+10%PME+Tre•	40	168	+++
11	MEM	10%FBS+10%FME+10%PME+0.5% OE	80	144	+++
12	MEM	10%FBS+10%FME+10%PME+0.5% PSE	20	168	+
13	MEM	10%FBS+10%FME+10%PME+0.5% Chit	40	168	+
14	MEM	10%FBS+10%FME+10%PME+insulin*	25	144	+
15	MEM	10%FBS+10%FME+10%PME+0.5% PHL	85	120	++++
16	MEM	10%FBS+10%FME+10%PME+0.5% CHL	25	144	-
17	MEM	10%FBS+10%FME+10%PME+0.5% FSE	60	120	+
18	MEM	10%FBS+10%FME+10%PME+0.5% OE+ Lec1*+LPS*+Glu•+PHL	90	120	+++++

FBS=Fetal Bovine Serum, FME=Fish Muscle Extract, PME= Prawn Muscle Extract, LPS= Lipopolysaccharides, PHL=Prawn haemolymph, CHL=Clam haemolymph, FSE= Fish Skin Extract

Lec1=Lectin1, Lec2= Lectin2, Glu=GlucoseD, Suc=Sucrose, Tre= Trehalose, OE= Ovary Extract, Chit= Chitin

+: 10% confluency, ++: 20% confluency, +++: 30% confluency, ++++: 40% confluency, +++++:50% confluency

*(0.02μg.mL^{-1}), •(0.2mg.mL^{-1})

Table 3.10 Response of the Kidney of *C. gariepinus* to varying combinations of media, growth factors and mitogens.

Sl. No	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in Hrs	Growth and monolayer formation @
1	M199	10%FBS	20	120	+
2	MEM	10%FBS	25	120	+
3	M199	10%FBS+10%FME	25	120	+
4	MEM	10%FBS+10%FME	35	120	+
5	M199	10%FBS+10%PME	35	120	+
6	MEM	10%FBS+10%PME	35	120	+
7	M199	10%FBS+10%FME+10%PME	30	120	+
8	MEM	10%FBS+10%FME+10%PME	40	120	+++
9	M199	10%FBS+10%FME+10%PME+Lec1*	50	96	+
10	MEM	10%FBS+10%FME+10%PME+Lec1*	45	120	++
11	M199	10%FBS+10%FME+10%PME+Lec2*	40	120	+
12	MEM	10%FBS+10%FME+10%PME+Lec2*	45	72	+
13	M199	10%FBS+10%FME+10%PME+LPS*	43	72	+
14	MEM	10%FBS+10%FME+10%PME+LPS*	45	72	+++
15	M199	10%FBS+10%FME+10%PME+Glu•	45	72	++
16	MEM	10%FBS+10%FME+10%PME+Glu•	48	120	++
17	M199	10%FBS+10%FME+10%PME+Suc•	48	72	++
18	MEM	10%FBS+10%FME+10%PME+Suc•	45	72	++
19	M199	10%FBS+10%FME+10%PME+Tre•	35	72	++
20	MEM	10%FBS+10%FME+10%PME+Tre•	40	72	++
21	M199	10%FBS+10%FME+10%PME+0.5% OE	70	72	++
22	MEM	10%FBS+10%FME+10%PME+0.5% OE	65	72	+++
23	M199	10%FBS+10%FME+10%PME+0.5% PSE	10	72	+
24	MEM	10%FBS+10%FME+10%PME+0.5% PSE	10	72	+
25	M199	10%FBS+10%FME+10%PME+0.5% Chit	10	120	+
26	MEM	10%FBS+10%FME+10%PME+0.5% Chit	12	120	+
27	M199	10%FBS+10%FME+10%PME+insulin*	30	144	+
28	MEM	10%FBS+10%FME+10%PME+insulin*	30	144	+
29	M199	10%FBS+10%FME+10%PME+0.5% PHL	60	72	++
30	MEM	10%FBS+10%FME+10%PME+0.5% PHL	65	72	++
31	M199	10%FBS+10%FME+10%PME+0.5% CHL	25	72	-
32	MEM	10%FBS+10%FME+10%PME+0.5% CHL	30	72	-
33	M199	10%FBS+10%FME+10%PME+0.5% FSE	60	48	+
34	MEM	10%FBS+10%FME+10%PME+0.5% FSE	45	72	+
35	M199	10%FBS+10%FME+10%PME+0.5% OE+ Lec1*+LPS*	60	120	+++
36	MEM	10%FBS+10%FME+10%PME+0.5% OE +Lec1*+LPS*	75	90	+++++

FBS= Fetal Bovine Serum, FME=Fish Muscle Extract, PME= Prawn Muscle Extract, LPS= Lipopolysaccharides, PHL=Prawn haemolymph, CHL=Clam haemolymph, FSE= Fish Skin Extract

Lec1=Lectin1, Lec2= Lectin2, Glu=GlucoseD, Suc=Sucrose, Tre= Trehalose, OE= Ovary Extract, Chit= Chitin,

+: 10% confluency , ++: 20% confluency, +++:30% confluency, ++++: 40% confluency, +++++:50% confluency,

*(0.02 $\mu g.mL^{-1}$), •(0.2$mg.mL^{-1}$)

Table 3.11 Response of the testicular tissue of *C. gariepinus* to varying combinations of media, growth factors and mitogens.

Sl. No	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in Hrs	Growth and monolayer formation @
1	L-15	10%FBS	20	120	+
2	L-15	10%FBS+10%FME	20	120	+
3	L-15	10%FBS+10%PME	40	72	+
4	L-15	10%FBS+10%FME+10%PME	45	72	++
5	L-15	10%FBS+10%FME+10%PME+Lec1*	35	72	++
6	L-15	10%FBS+10%FME+10%PME+Lec2*	40	72	++
7	L-15	10%FBS+10%FME+10%PME+LPS*	70	72	+++
8	L-15	10%FBS+10%FME+10%PME+Glu•	30	120	+
9	L-15	10%FBS+10%FME+10%PME+Suc•	30	120	+
10	L-15	10%FBS+10%FME+10%PME+Tre•	50	120	++
11	L-15	10%FBS+10%FME+10%PME+0.5% OE	80	48	+++
12	L-15	10%FBS+10%FME+10%PME+0.5% PSE	30	120	++
13	L-15	10%FBS+10%FME+10%PME+0.5% Chit	30	120	+
14	L-15	10%FBS+10%FME+10%PME+insulin*	20	120	+
15	L-15	10%FBS+10%FME+10%PME+0.5% PHL	60	48	+++
16	L-15	10%FBS+10%FME+10%PME+0.5% CHL	20	72	-
17	L-15	10%FBS+10%FME+10%PME+0.5% FSE	45	48	+
18	L-15	10%FBS+10%FME+10%PME+0.5% OE +LPS*+0.5%PHL	80	72	+++++

FBS= Fetal Bovine Serum, FME=Fish Muscle Extract, PME= Prawn Muscle Extract, LPS= Lipopolysaccharides, PHL=Prawn haemolymph, CHL=Clam haemolymph, FSE= Fish Skin Extract

Lec1=Lectin1, Lec2= Lectin2, Glu=GlucoseD, Suc=Sucrose, Tre= Trehalose, OE= Ovary Extract, Chit= Chitin

+: 10% confluency , ++: 20% confluency, +++: 30% confluency, ++++: 40% confluency, +++++:50% confluency,

*($0.02\mu g.mL^{-1}$), •($0.2 mg.mL^{-1}$)

Table 3.12 Response of the ovarian tissue of *C. gariepinus* to varying combinations of media, growth factors and mitogens.

Sl.No	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in Hrs	Growth and monolayer formation @
1	M199	10%FBS	30	72	+
2	MEM	10%FBS	30	72	+
3	L-15	10%FBS	35	72	+
4	M199	10%FBS+10%FME	30	72	+
5	MEM	10%FBS+10%FME	30	72	+
6	L-15	10%FBS+10%FME	40	72	++
7	M199	10%FBS+10%PME	30	72	+
8	MEM	10%FBS+10%PME	35	72	+
9	L-15	10%FBS+10%PME	40	48	++
10	M199	10%FBS+10%FME+10%PME	40	72	+
11	MEM	10%FBS+10%FME+10%PME	44	72	+
12	L-15	10%FBS+10%FME+10%PME	65	48	+++
13	M199	10%FBS+10%FME+10%PME+Lec1*	40	72	+
14	MEM	10%FBS+10%FME+10%PME+Lec1*	45	72	+
15	L-15	10%FBS+10%FME+10%PME+Lec1*	65	48	+++
16	M199	10%FBS+10%FME+10%PME+Lec2*	40	72	+
17	MEM	10%FBS+10%FME+10%PME+Lec2*	45	72	+
18	L-15	10%FBS+10%FME+10%PME+Lec2*	75	48	++++
19	M199	10%FBS+10%FME+10%PME+LPS*	43	72	+
20	MEM	10%FBS+10%FME+10%PME+LPS*	45	72	++
21	L-15	10%FBS+10%FME+10%PME+LPS*	70	48	++++
22	M199	10%FBS+10%FME+10%PME+Glu•	45	72	++
23	MEM	10%FBS+10%FME+10%PME+Glu•	48	72	++
24	L-15	10%FBS+10%FME+10%PME+Glu•	80	36	++++
25	M199	10%FBS+10%FME+10%PME+Suc•	48	72	++
26	MEM	10%FBS+10%FME+10%PME+Suc•	45	72	++
27	L-15	10%FBS+10%FME+10%PME+Suc•	70	48	+++
28	M199	10%FBS+10%FME+10%PME+Tre•	35	72	++
29	MEM	10%FBS+10%FME+10%PME+Tre•	40	72	++
30	L-15	10%FBS+10%FME+10%PME+Tre•	65	48	++
31	M199	10%FBS+10%FME+10%PME+0.5% OE	70	72	+++
32	MEM	10%FBS+10%FME+10%PME+0.5% OE	65	72	+++
33	L-15	10%FBS+10%FME+10%PME+0.5% OE	90	24	+++++
34	M199	10%FBS+10%FME+10%PME+0.5% PSE	10	72	+
35	MEM	10%FBS+10%FME+10%PME+0.5% PSE	10	72	+
36	L-15	10%FBS+10%FME+10%PME+0.5% PSE	30	48	++
37	M199	10%FBS+10%FME+10%PME+0.5% Chit	10	72	+
38	MEM	10%FBS+10%FME+10%PME+0.5% Chit	12	72	+
39	L-15	10%FBS+10%FME+10%PME+0.5% Chit	30	48	++
40	M199	10%FBS+10%FME+10%PME+insulin*	30	72	+
41	MEM	10%FBS+10%FME+10%PME+insulin*	30	72	+
42	L-15	10%FBS+10%FME+10%PME+insulin*	20	72	+
43	M199	10%FBS+10%FME+10%PME+0.5% PHL	60	48	++
44	MEM	10%FBS+10%FME+10%PME+0.5% PHL	65	48	++
45	L-15	10%FBS+10%FME+10%PME+0.5% PHL	70	48	+++
46	M199	10%FBS+10%FME+10%PME+0.5% CHL	25	72	-
47	MEM	10%FBS+10%FME+10%PME+0.5% CHL	30	72	-
48	L-15	10%FBS+10%FME+10%PME+0.5% CHL	25	72	-
49	M199	10%FBS+10%FME+10%PME+0.5% FSE	60	48	+
50	MEM	10%FBS+10%FME+10%PME+0.5% FSE	45	72	+
51	L-15	10%FBS+10%FME+10%PME+0.5% FSE	70	48	+
52	M199	10%FBS+10%FME+10%PME+0.5% OE+ Lec2*+LPS*+Glu•+0.5%PHL	45	72	+++
53	MEM	10%FBS+10%FME+10%PME+0.5% OE +Lec2*+LPS*+Glu•+0.5%PHL	40	72	+++
54	L-15	10%FBS+10%FME+10% PME+0.5% OE +Lec2*+LPS*+Glu•+0.5%PHL	95	24	++++++

FBS= Fetal Bovine Serum, FME=Fish Muscle Extract, PME= Prawn Muscle Extract, LPS= Lipopolysaccharides, PHL=Prawn haemolymph, CHL=Clam haemolymph, FSE= Fish Skin Extract Lec1=Lectin1, Lec2= Lectin2, Glu=Glucose D, Suc=Sucrose, Tre= Trehalose, OE= Ovary Extract, Chit= Chitin

+: 10% confluency, ++: 20% confluency, +++:30% confluency, ++++: 40% confluency, +++++: 50% confluency, ++++++ 60% confluency; *(0.02μ g.mL^{-1}), •(0.2mg.mL^{-1})

Table 3.13 Duration required for effecting subculturing the monolayer formed at different levels of amendments for liver of *C. gariepinus*

Passage No.	Days required					
	MEM+ FBS+ FME+ PME	MEM+ FBS+ FME+ PME+ Lec 2	MEM+ FBS+ FME+ PME+ LPS	MEM+ FBS+ FME+ PME+ Glu	MEM+ FBS+ FME+ PME+ OE	MEM+ FBS+ FME+ PME+ Lec 2 LPS+Glu+ OE
1	12	13	10	12	10	9
2	15	12	9	14	11	9
3	17	11	9	15	9	7
4	14	12	7	9	10	6
5	13		9	6	10	7
6						7
7						8
8						9
9						(Going on)

MEM : Minimum Essential Medium (Eagle, Modified)
FBS : Fetal Bovine Serum
FME : Fish Muscle Extract
PME : Prawn Muscle Extract
LPS : Lipopolysaccharides
Lec 2 : Lectin 2
Glu : Glucose D
OE : Ovary Extract

Table 3.14 Duration required for effecting subculturing the monolayer formed at different levels of amendments for spleen of *C. gariepinus*

Passage No.	Days required					
	MEM+ FBS+ FME+ PME	**MEM+ FBS+ FME+ PME+ Lec 1**	**MEM+ FBS+ FME+ PME+ LPS**	**MEM+ FBS+ FME+ PME+ OE**	**MEM+ FBS+ FME+ PME+ PHL**	**MEM+ FBS+ FME+ PME+ Lec 1 LPS+ OE**
1	15	14	13	14	10	12
2	15	15	13	15	10	12
3	14		13	15	11	11
4	14				11	10
5					11	9
6						9
7						8
8						9
9						(Going on)

MEM : Minimum Essential Medium (Eagle, Modified)
FBS : Fetal Bovine Serum
FME : Fish Muscle Extract
PME : Prawn Muscle Extract
LPS : Lipopolysaccharides
Lec 1 : Lectin 1
OE : Ovary Extract

Table 3.15 Duration required for effecting subculturing the monolayer formed at different levels of amendments for kidney of *C. gariepinus*

Passage No	Days required				
	MEM+ FBS+ FME+ PME	MEM+ FBS+ FME+ PME+ Lec 1	MEM+ FBS+ FME+ PME+ LPS	MEM+ FBS+ FME+ PME+ OE	MEM+ FBS+ FME+ PME+ Lec 1+ LPS+Glu+OE
1	13	13	9	10	9
2	14	12	9	10	9
3	14	11	9	9	8
4	14	12	10	11	9
5		13		11	9
6					9
7					8
8					9
9					(Going on)

MEM : Minimum Essential Medium (Eagle, Modified)
FBS : Fetal Bovine Serum
FME : Fish Muscle Extract
PME : Prawn Muscle Extract
LPS : Lipopolysaccharides
Lec 1 : Lectin 1
Glu : Glucose D
OE : Ovary Extract

Table 3.16 Duration required for effecting subculturing the monolayer formed at different levels of amendments for testicular tissues of *C. gariepinus*

Passage No.	Days required			
	L-15+ FME+ PME	L-15+ FME+ PME+ LPS	L-15+ FME+ PME+ OE	L-15+ FME+ PME+ LPS+ OE+ PHL
1	15	15	6	5
2	13	13	7	6
3		15	9	7
4			11	8
5				7
6				9

L-15 : Leibovitz medium
FME : Fish Muscle Extract
PME : Prawn Muscle Extract
LPS : Lipopolysaccharides
OE : Ovary Extract
PHL : Prawn Heamolymph

Table 3.17 Duration required for effecting subculturing the monolayer formed at different levels of amendments for ovarian tissues of *C. gariepinus*

Passage No	Days required						
	L-15+ FME+ PME	L-15+ FME+ PME+ Lec 2	L-15+ FME+ PME+ LPS	L-15+ FME+ PME+ Glu	L-15+ FME+ PME+ OE	L-15+ FME+ PME+ PHL	L-15+FME+ PME+Lect 2+ LPS+Glu+ OE+PHL
1	5	4	5	4	3	3	2
2	6	6	5	4	4	3	3
3	7	9	7	10	4	5	3
4	6	10	7	11	4	5	3
5	7	9	8	13	4	4	3
6	5				4	4	3
7					4	4	3
8					4	5	3
9					4	5	3
10							3
11							4
12							4
13							4
14							4
15							5
16							6

L-15 : Leibovitz medium
FME : Fish Muscle Extract
PME : Prawn Muscle Extract
Lec 2 : Lectin 2
LPS : Lipopolysaccharides
Glu : Glucose D
OE : Ovary Extract
PHL : Prawn Heamolymph

Table 3.18 Preference for varying temperature for the cell culture derived from the embryonic tissues of *Clarias gariepinus*

a) Liver

Temp. in ^{0}C	Time in Hours			
	24	48	72	96
5	-	-	+	+
10	-	-	+	+
15	-	+	+	++
20	++	++	+++	+
25	++	+++	+++	++
28	++++	+++++	+++++	+++++
30	+	+	-	-
35	-	-	-	-

b) Spleen

Temp. in ^{0}C	Time in Hours			
	24	48	72	96
5	-	-	+	-
10	-	-	+	+
15	-	+	+	++
20	++	++	+++	++
25	++	+++	+++	+++
28	++++	+++++	+++++	+++++
30	+	+	-	-
35	-	-	-	-

c) Kidney

Temp. in ^{0}C	Time in Hours			
	24	48	72	96
5	-	-	++	++
10	-	-	+	+
15	-	+	+	++
20	++	++	+++	++
25	++	+++	+++	+++
28	++++	+++++	+++++	+++++
30	+	+	+	+
35	-	-	-	-

Cont...

Cont..

d) Testis

Temp. in ^{0}C	Time in Hours			
	24	**48**	**72**	**96**
5	-	-	++	++
10	-	-	+	+
15	-	++	++	++
20	++	++	+++	+++
25	+	++++	++++	++++
30	+	-	-	++
35	-	-	-	-

e) Ovary

Temp. in ^{0}C	Time in Hours			
	24	**48**	**72**	**96**
5	-	-	++	++
10	-	-	+	+
15	-	+	+	++
20	++	++	+++	++
25	++	+++	+++	+++
30	+	+	-	-
35	-	-	-	-

+ : Attachment
++ : Growth
+++ : Monolayer formation

Table 3.19 Response of ovarian tissue to cryopreservation at –35^{0}C and –195^{0}C in the presence of DMSO and glycerol in L-15 supplemented with tissue derived growth factors.

Sl no	CP	Temp ^{0}C	% CP	Medium. L-15 mL	FBS mL	PME mL	FME mL	Duration in days						
								1	10	20	30	40	50	60
1	DM	-35	10	1.4	0.2	0.2	0.2	+	+	-	-	-	-	-
2	DM	-165	10	1.4	0.2	0.2	0.2	-	-	-	-	-	-	-
3	DM	-35	7.5	1.4	0.2	0.2	0.2	++	++	++	+++	++	++	++
4	DM	-165	7.5	1.4	0.2	0.2	0.2	+	+	+	+	-	-	-
5	DM	-35	5	1.4	0.2	0.2	0.2	+	+	+	++	-	-	-
6	DM	-165	5	1.4	0.2	0.2	0.2	+	++	+	-	-	-	-
7	DM	-35	2.5	1.4	0.2	0.2	0.2	+	+	-	-	-	-	-
8	DM	-165	2.5	1.4	0.2	0.2	0.2	+	-	-	-	-	-	-
9	DM	-35	1.0	1.4	0.2	0.2	0.2	+	-	-	-	-	-	
10	DM	-165	1.0	1.4	0.2	0.2	0.2	+	-	-	-	-	-	-
11	GL	-165	10	1.4	0.2	0.2	0.2	++	-	-	-	-	-	-
12	GL	-35	10	1.4	0.2	0.2	0.2	++	+	+	-	-	-	-
13	GL	-165	7.5	1.4	0.2	0.2	0.2	++	+	-	-	-	-	-
14	GL	-35	7.5	1.4	0.2	0.2	0.2	+	-	-	-	-	-	-
15	GL	-165	5.0	1.4	0.2	0.2	0.2	-	-	-	-	-	-	-
16	GL	-35	5.0	1.4	0.2	0.2	0.2	-	-	-	-	-	-	-
17	GL	-165	2.5	1.4	0.2	0.2	0.2	+	+	-	-	-	-	-
18	GL	-35	2.5	1.4	0.2	0.2	0.2	-	-	-				
19	GL	-165	1.0	1.4	0.2	0.2	0.2	-	-	-				
20	GL	-35	1.0	1.4	0.2	0.2	0.2	+	+	-	-	-	-	-

FBS - Fetal Bovine Serum
PME - Prawn Muscle Extract
FME - Fish Muscle Extract
CP - Cryoprotectant
DM - DMSO
GL - Glycerol
+: Attachment; ++: Attachment and cell proliferation; -: No attachment

1. Fig.3.1 Explant of liver tissue of *Clarias gariepinus* with proliferating cells (10X)

2. Fig.3.2 Second passage of the cell culture developed from the liver tissue of *C.gariepinus* (10X). Cells are mostly fibroblastic (CGL-1)

3. Fig.3.3 Cell culture derived from the spleen tissue of *C.gariepinus* ,1st passage (10X). Cells are mostly fibroblastic

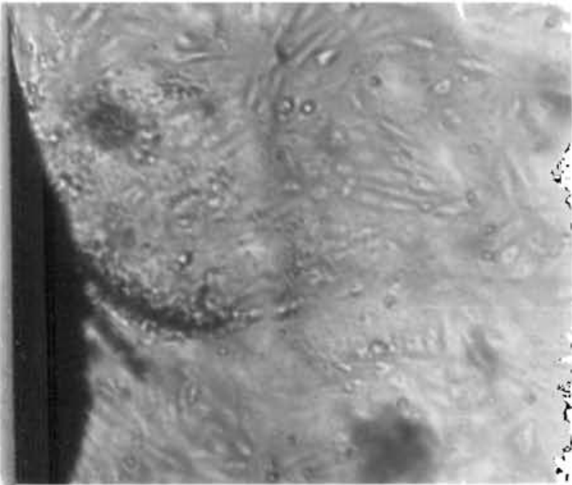
4. Fig. 3.4 Cell line CGS-1, 2nd passage, derived from the spleen tissue of *C.gariepinus* (10X). Cells are mostly fibroblastic

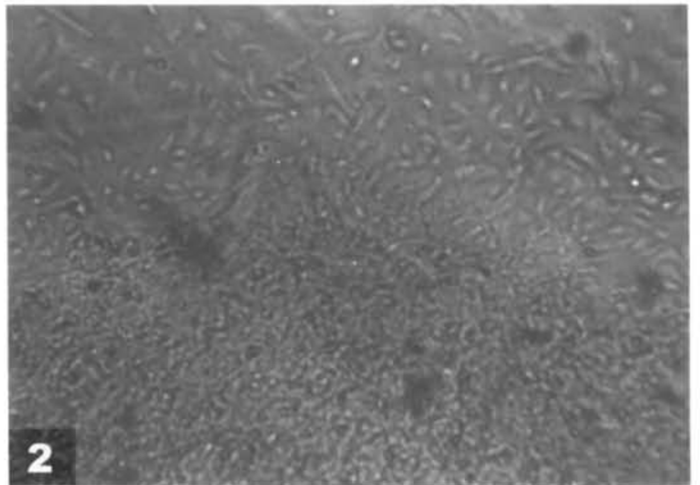
5. Fig. 3.5 Primary cell culture derived from kidney of *C.gariepinus* by explant method. Cells are fibroblastic (10X)

6. Fig 3.6 Third passage of the cell culture derived from the kidney of *C.gariepinus* (CGK-1) (10X)

7. Fig. 3.7 Primary cell culture derived from the testis of *C.gariepinus* . Cells are slender, thin and mostly fibroblastic .(10X)

8. Fig. 3.8 Primary cell culture derived from the testis of *C.gariepinus* . Dividing cells can be seen (20X)

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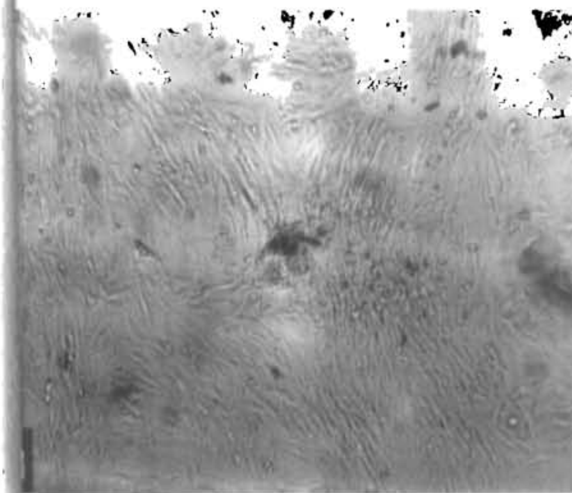

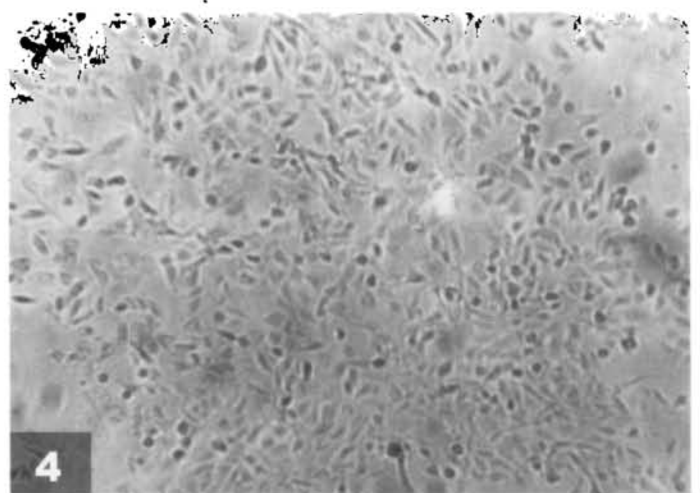
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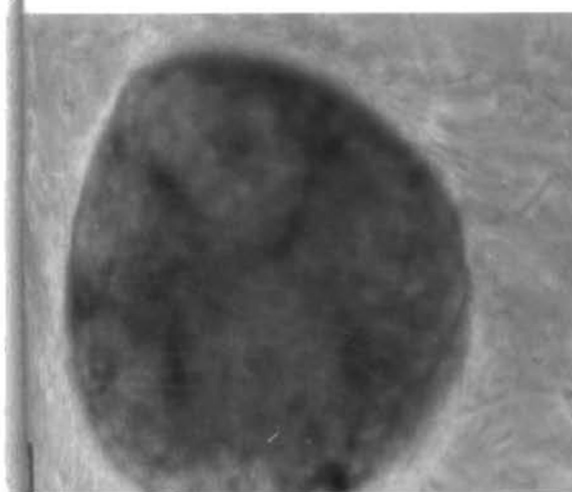

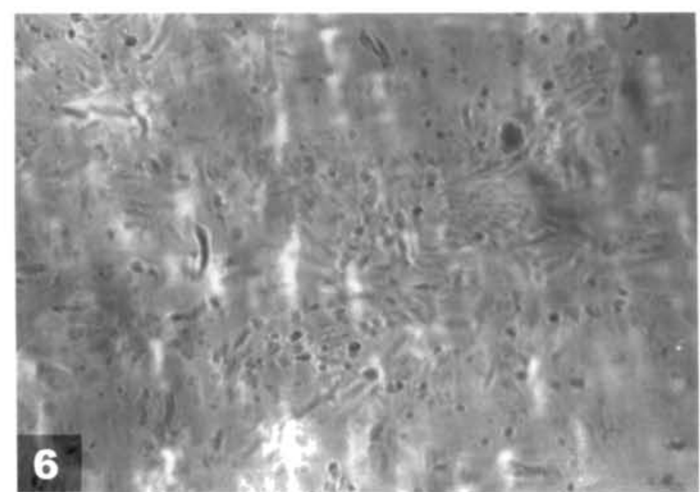
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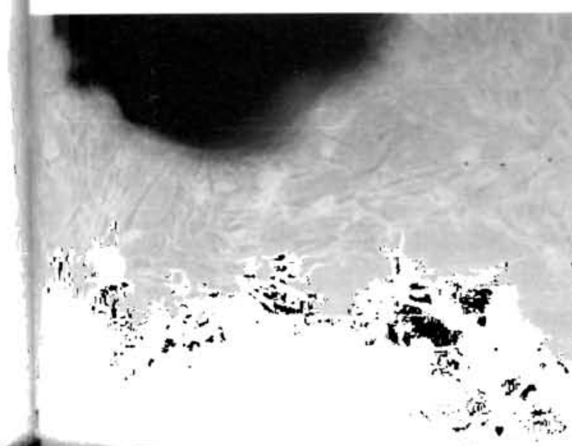

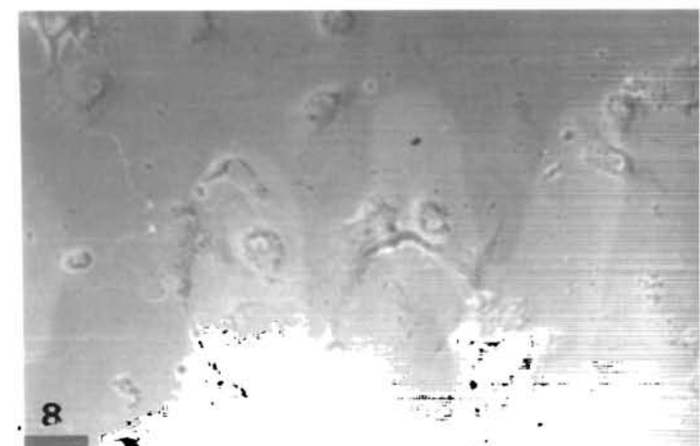
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1. Fig. 3.9 Primary cell culture from the ovarian tissue of *C. gariepinus* (10 X)

2. Fig. 3.10 Cell culture derived from the ovarian tissue of *C. gariepinus* composed of both fibroblastic and epithelioid cells – 2nd passage (10X). Composed of both fibroblastic and epithelioid cells

3. Fig 3.11 Cell culture derived from the ovarian tissue of *C. gariepinus* composed of both fibroblastic and epithelioid cells – 3rd passage (10X). Composed of both fibroblastic and epithelioid cells

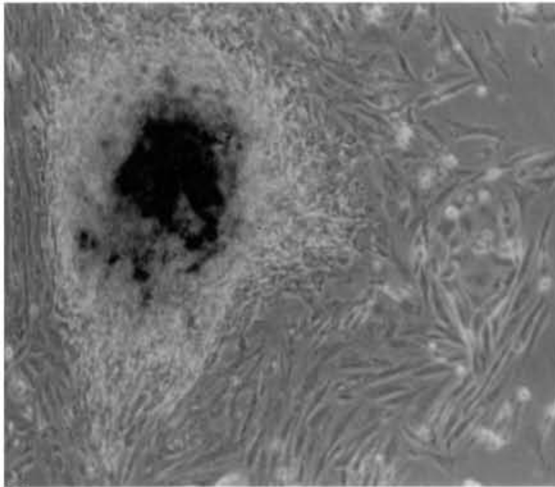
4. Fig 3.12 Cell culture derived from the ovarian tissue of *C. gariepinus* composed of both fibroblastic and epithelioid cells – 4th passage (10X). Composed of both fibroblastic and epithelioid cells

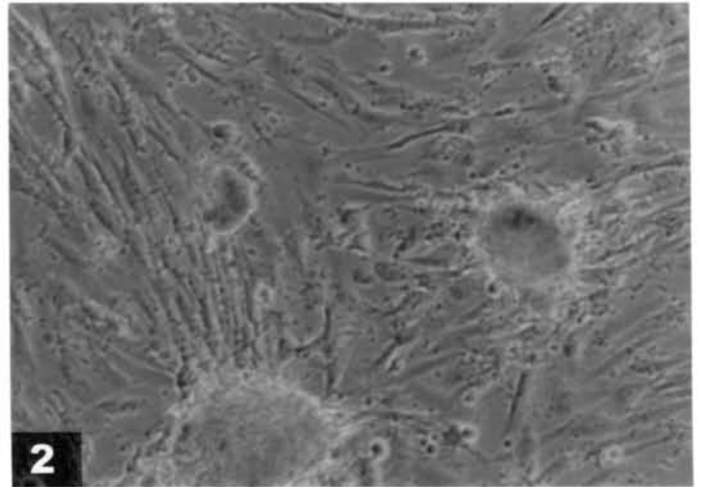
5. Fig 3.13 Cell culture derived from the ovarian tissue of *C. gariepinus* composed of both fibroblastic and epithelioid cells – 6th passage (10X). Composed of both fibroblastic and epithelioid cells

6. Fig 3.14 Cell culture derived from the ovarian tissue of *C. gariepinus* composed of both fibroblastic and epithelioid cells – 10th passage (10X). Composed of both fibroblastic and epithelioid cells

7. Fig 3.15 Cell culture designated as (CGO-1) derived from the ovarian tissue of *C. gariepinus* . 15th passage (10X).

8. Fig 3.16 Specialized fibroblastic cells derived from the ovarian tissue of *C. gariepinus*

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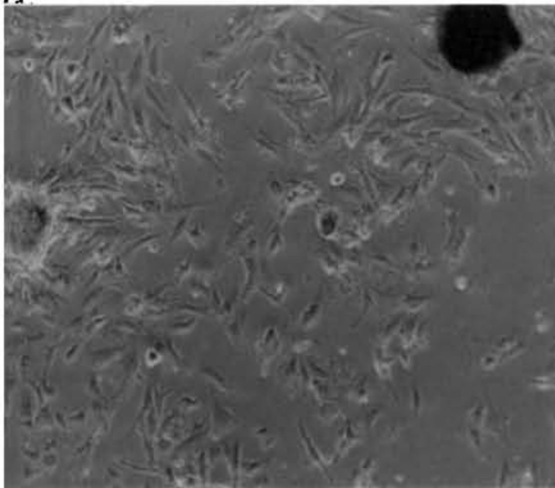

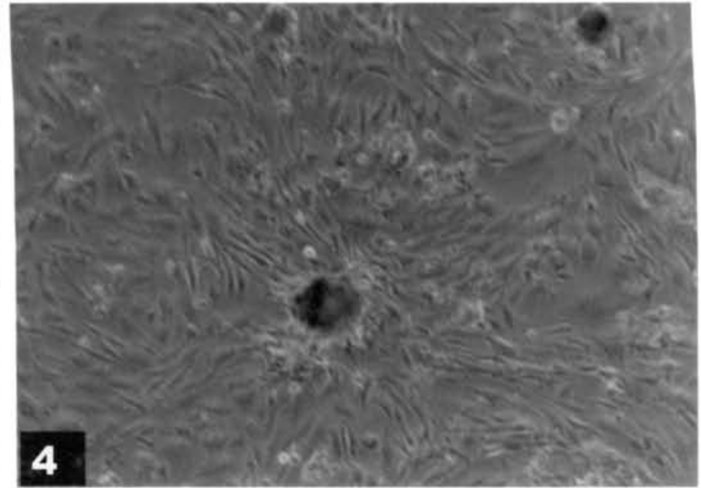
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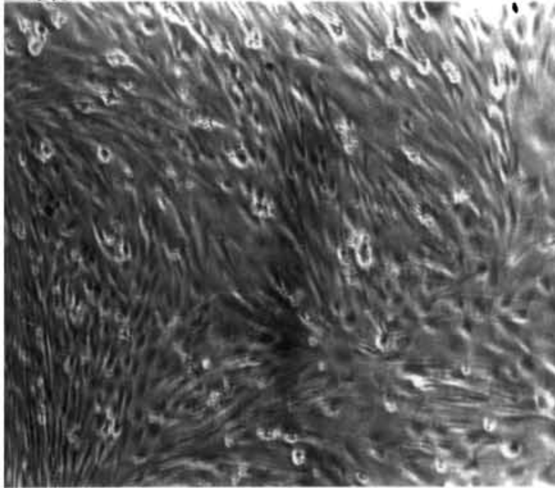

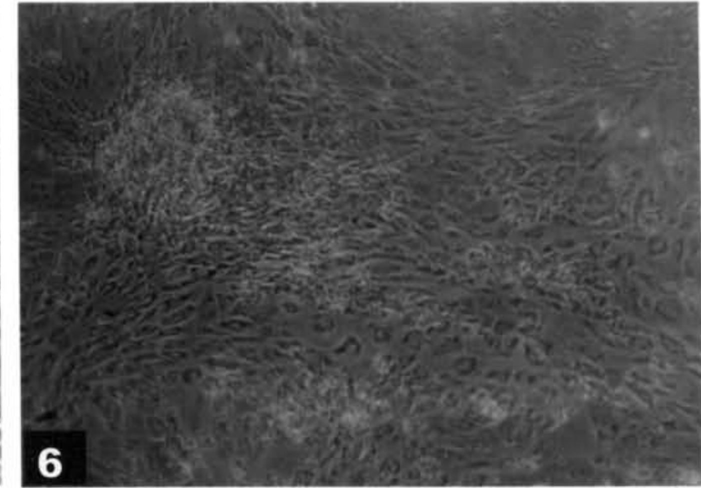
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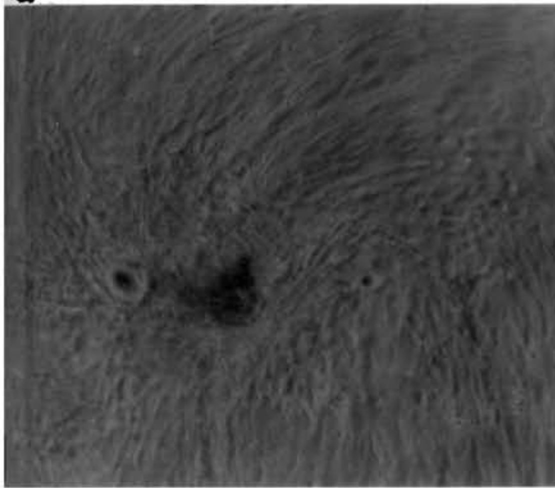

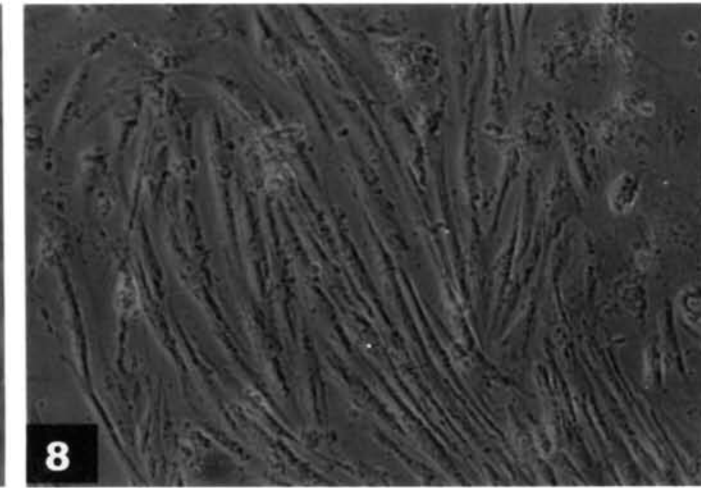
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Fig 3.17 Optimum temperature of growth of cell culture developed from *C.gareipinus*

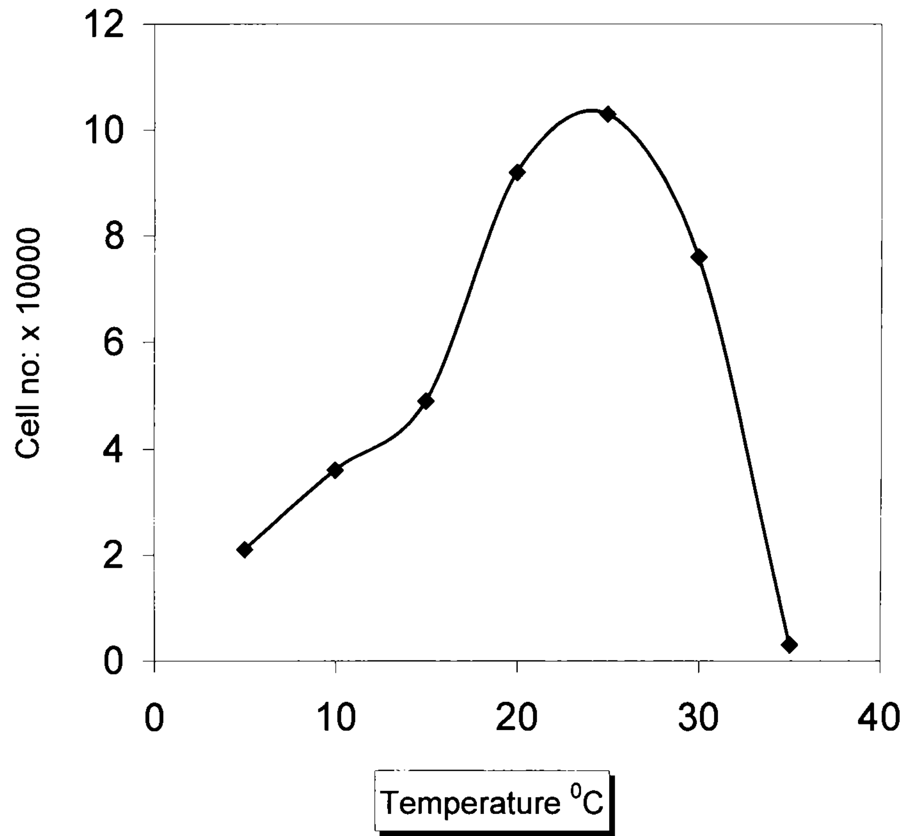
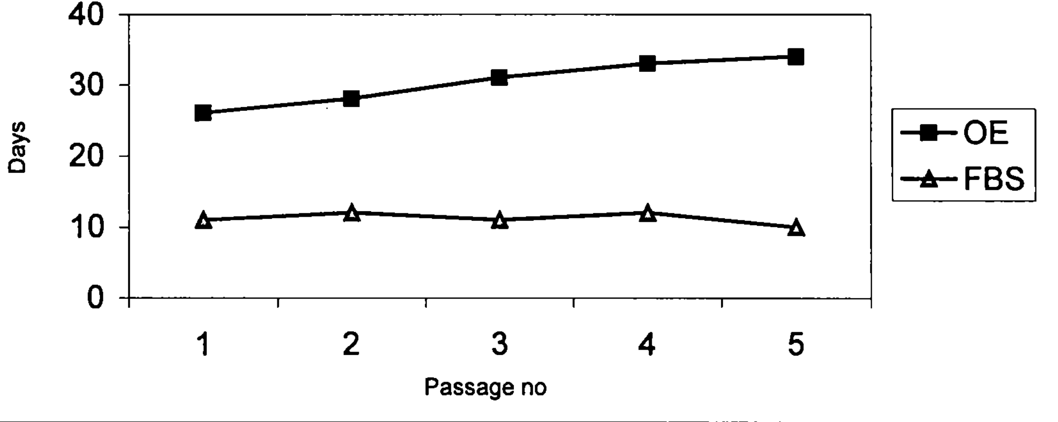


Figure 3.18 Days required for effecting subculturing RTG-2 on using ovary extract (OE) as substitute of FBS



CHAPTER 4

CELL CULTURE SYSTEMS FROM THE HEPATOPANCREAS OF PENAEUS INDICUS

CHAPTER 4

CELL CULTURE SYSTEM FROM THE HEPATOPANCREAS OF *PENAEUS INDICUS*

4.1 Introduction

a. Biology, reproduction, ecology and distribution of *Penaeus indicus*

There are atleast 60 species of commercially important penaeid shrimps. The genus *Penaeus* occurs in tropical and subtropical waters around the world and it is by far the most commercially important genus. *Penaeus indicus* commonly known, as Indian white prawn in trade circles constitutes the major portion of the frozen shrimp exported from India every year. This is a very important prawn species occurring along both East Coast and West of India, relatively abundant in coastal zones of Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa and West Bengal. They have a preference for sandy substratum. They are hardy and grow well at high salinities (20 to 30ppt) while they cannot tolerate salinities outside the range of 5 to 40ppt. The temperature above 34^0C is lethal to them. They are omnivorous, feeding mainly on detritus, small crustaceans, polycheates etc. They have relatively short larval (7 days) and post larval periods (10 days) and the survival rates are relatively good. They reach a maximum recorded size of 230mm; attain marketable size of 80-120mm in 90-100 days under culture conditions.

There is marked sexual dimorphism in *P. indicus*. They attain maturity in six months in natural condition. At maturity, females are much larger than males. The adults spawn in offshore waters at depth of 18-28 meters generally from March to September, with a bimodal peak in the premonsoon periods. The

darkness, low temperature and high pressure in the deep sea stimulate the onset of sexual maturation and result in breeding of *P. indicus*. The fully mature female gravid ovary undergoes a pre-spawning moult period with the male that deposits the spermatophore in the thelycum of the female. Spawning usually takes 24hrs mostly during nights. The spawned eggs are fertilized by the spermatozoa, discharged simultaneously from the spermatophore deposited by the male during copulation. The number of eggs released by each prawn during spawning depends on the size of the animal. The eggs are temporarily planktonic and become demersal after the formulation of the fertilization membrane. The eggs hatch out into nauplii in 16 - 17 hours in 28^{0}C. The larval development is much more elaborate and complex process, the hatchlings having to pass through several stages in nauplii, zoea, mysis stages, before they attain post larval stage. The planktonic larvae remain offshore for about 3 weeks and develop through 6 nauplii, 3 zoea, and 3 mysis stages .The changeover from one stage to another takes place after a moult. Following the mysis are several post mysis or post-larval stages. Only the post larvae that reach estuarine nursery grounds become juveniles and continue to grow. A combination of current patterns and behavioral responses make this migration to estuary possible. Temperature may trigger a return to offshore spawning grounds at the onset of sexual maturity, when they are approximately 10-12 cm in length (Neal, 1985; Jinghran, 1988 and Pillay, 1990).

b. Significance of using *P. indicus* as donor animal

The uncontrolled and unregulated expansion of aquaculture farms and their intensive operations with unscientific approach led to the development and transmission of many diseases within cultured and as well as final populations. Among the various types of microbial diseases of cultured penaeids, viral diseases have been documented to cause the most extensive mortalities in captive populations leading to serious economic loss during the recent past (Nunan *et al.*, 1998). One of the impediments in the management of prawn viruses is the

absence of an established cell line with which the pathogen can be isolated and studied *in vitro*. Since such a system is unavailable as on today the study of prawn viruses is restricted to the application of histological and electron microscopic method. Studies relating to the pathological effects of the virus at cellular level, quantification of the virus by plaque assay, LD_{50}, latency of virus, production of antigens for the development of antisera and preparation of vaccines etc., can be accomplished only if a right cell culture system or cell line is available and the techniques relating to its isolation and propagation are standardized. (Lightner and Redman, 1998).

Penaeus indicus and *Penaeus monodon* are the most popular species of prawns cultured in India. There is so much uniformity in the range of viral pathogens, which these two species are susceptible to. Among the two species *P. indicus* is easily available and less expensive and can be easily maintained in laboratory. These qualities made it a good donor animal in the development of a cell culture system.

c. Cell cultures developed from Penaeids

The first ever report of an *in vitro* primary cell culture system from the penaeid shrimp was from Chen et al., (1989) who reported the development of a cell culture system from *Penaeus penicillatus* lymphoid tissue and ovary employing L-15. The confluent sheet of cells developed could be subcultured once. Luedman and Lightner (1992), developed a primary culture from the ovarian tissue of prawn using 1X Leibovitz's L-15 supplemented with several growth factors. The osmolority of the holding medium was adjusted to 750 mmol kg^{-1}. Ghosh *et al.*, (1995) developed a primary culture from the hepatopancreas of prawns employing M199. Meanwhile, Hsu *et al.*, (1995) developed a cell culture system from the lymphoid organ of *Penaeus monodon* employing L-15 supplemented with various growth factors. Kasornchandra and Boonyaratpalin

(1998) developed a primary cell culture from the lymphoid organ of *Penaeus monodon* with which they could titrate the white spot virus. However, no cell culture could be established so far from prawns.

4.2 Materials and Methods

4.2.1 Development of an appropriate protocol for disinfecting the surface of *Penaeus indicus* to remove hepatopancreas aseptically

Hepatopancreas is the largest gland / organ in prawns, which is very much vulnerable to the physico-chemical changes of the aquatic environment. Maintenance of the animals at room temperature 15 minutes after death leads to liquefaction of hepatopancreas. Therefore all process of preparation of animal and tissue removal have been programmed to be carried out at 4^0C.

P. indicus juveniles weighing 6 to 8g were brought alive from wild and maintained for a couple of days in seawater having 20ppt salinity, fed on a specially prepared diet. Prior to the standardization of disinfection protocol, the animals were starved for a day in seawater having 20ppt with continuous removal of faecal matter. The animals were sacrificed by plunging in ice cubes for 3 minutes and disinfected in a series of cold 4^0C sodium hypochlorite for 10 minutes prepared in seawater (20ppt). The strength of available chlorine in these solutions ranged from 100 to 1000. Subsequently the animals were washed with sterile cold (4^0C) 20ppt seawater and dipped in cold (4^0C) 70% ethanol for 2-3 minutes. The animals were thoroughly washed with sterile seawater (20ppt). At every stage of disinfection, first after exposing to chlorine and subsequent washing and later after disinfecting with 70% ethanol and subsequent washing, swabs were taken and streaked on Sabouraud Dextrose Agar, Nutrient agar, Blood agar and inoculated in to Thioglycollate broth. The media were incubated at room temperature (28 ± 0.5^0C) for 3 days and observed for growth. The general

composition of the media was described under the section 2.2.1. The only difference was, that all media were prepared in seawater (20 ppt).

4.2.2. Screening of commercially available media for developing primary cell cultures from hepatopancreas

Three commercially available media widely used for cell culturing were used for selecting the best one. They were L-15 (Leibovitz) Medium (AT011), MEM Eagle's (AT017) and Medium 199 (A015) (HiMedia Laboratory Ltd., Bombay). Details of preparation of the media can be read from section 2.2.2.1. The only difference was, all the media were prepared in filter sterilized 20ppt seawater. The media after completion were once again filter sterilized and never autoclaved. Sterility of the media was tested by inoculating an aliquot to nutrient agar, Sabouraud dextrose agar slants and thioglycollate broth.

Animals were collected from Cochin backwaters alive and brought to the laboratory under well-aerated condition without giving much stress to the animal. They were starved in 20ppt seawater and sacrificed by plunging in ice cubes, disinfected by immersing in cold sodium hypochlorite prepared in 20ppt seawater, for 10 minutes, washed thoroughly with sterile (autoclaved 20ppt seawater) and dipped in cold 70% ethanol for 3 minutes and washed repeatedly with sterile 20ppt seawater. The animals pinned to a board was quickly dissected and the entire stretch of alimentary canal along with the hepatopancreas was removed from the body aseptically and placed in a petridish kept on an ice bath inside laminar flow hood. Using a scalpel blade lobes of hepatopancreas from both sides of intestine were sliced and transferred to autoclaved cold (4^0C) seawater supplemented with 0.2ml antibiotic mixture per 100ml placed on an ice bath. The tissue was minced in to small pieces of $1mm^3$ and transferred to tissue culture bottles, added with 0.5ml foetal bovine serum. The media mentioned above were added at 4.5 ml aliquots and the bottle stoppered with rubber cork and incubated

at room temperature (28±0.5^{0}C) and observed for attachment, cell spreading and cell proliferation.

4.2.3 Appropriate sodium chloride concentration/salinity in growth media for providing the right osmolarity

Osmolarity is one of the most important factors, which determines the success of cell culture and this is especially true with regard to penaeids. Since they are of marine origin, their cells require sodium chloride or equivalent in the medium to support growth. To find out at what percentage of sodium chloride or at what salinity the right osmolarity shall be made available, the present experiment was carried out. The concept was at the right salinity or at the right sodium chloride content explants of hepatopancreas may attach to tissue culture bottle, and cells start proliferating.

All the three media such as L-15, MEM and M199 were used for the purpose. They were on one hand supplemented with 1.5, 2.0, 2.0, 2.5, 3.0 and 3.5% sodium chloride and on the other hand were prepared in 15, 20, 25, 30 and 35 ppt seawater collected from an offshore area. The mode of preparation was the same as described under section 4.2.2. The hepatopancreas was prepared as described under section 4.2.3 and seeded tissue culture bottles were provided with 0.5 ml FBS. The media with different sodium chloride content and salinity were added to the bottles at 4.5ml aliquots. The bottles were incubated closed at room temperature (28±0.5^{0}C) and observed for the attachment of explants and cell proliferation.

4.2.4 Response of hepatopancreas of *Penaeus indicus* to varying combinations of media growth factors and mitogens.

To know how hepatopancreas responded to the growth media prepared in seawater having two salinities (25ppt and 30ppt) and how did the addition of 10% (v/v) prawn muscle extract (PME), prawn haemolymph (PHL) and 0.02μg mL^{-1}

lectin 1, the following experiment was conducted. The growth media L-15, MEM and M199 prepared in seawater were amended as described above. The preparations of animal and tissue removal were done as described under section 4.2.1. The tissues removed were minced in cold condition and seeded the tissue culture bottles and were supplemented with amended growth media. The bottles were incubated at room temperature (28 ± 0.5^{0}C) and observed for the attachment of explants and proliferation of cells.

4.3 Results and Discussion

4.3.1 An appropriate protocol for disinfecting the surface of *Penaeus indicus*

Results of the experiments conducted to standardize an appropriate protocol for disinfecting the surface for safe removal of hepatopancreas is presented in Table 4.1. Sodium hypochlorite diluted in seawater (20ppt) with the available chlorine level of 400ppm was found to be suitable, provided there was an associated disinfection in 70% ethanol. All processes have to be carried out at cold (4^{0}C) to avoid any liquefaction of hepatopancreas. Only apparently healthy animals collected from wild have to be transported to the laboratory under well aerated conditions. They have to be starved for a day in filtered seawater (20ppt). Animals have to be sacrificed by plunging in ice cubes and disinfected in sodium hypochlorite prepared in 20ppt seawater in cold (4^{0}C) having 400ppm available chlorine for 10 minutes. The animals are then thoroughly washed in autoclaved seawater (20ppt) and immersed in cold 70% ethanol for 3 to 4 minutes, washed subsequently with sterile seawater.

The external surface of most invertebrates are heavily contaminated with bacteria which if care is not taken will contaminate the resulting tissue culture (Vago, 1971). To avoid such contamination, surface sterilization of the tissues are

carried with disinfectants viz. 70% ethanol, $HgCl_2$ in alcohol, $NaClO_2$ and quaternary ammonium compounds. Chen *et al.*, (1986) in order to develop a cell culture from tissues of grass prawn *Penaeus monodon* the animal was surface sterilized by immersing in freshly prepared 10% sodium hypochlorite for 10 minutes and then wiped with 70% ethanol for 5 minutes at intervals of three minutes. Chen *et al.*, (1989) suggested wipe drying the surface with 70% alcohol saturated cotton wool and then placing at 4^0C refrigerator for 1 hour. They again suggested immersion of prawn in freshly prepared 5% (v/v) Clorox containing 5.25% sodium hypochlorite for 10 minutes.

Considering the previous experience and the results obtained in the present study, exposure to 400ppm chlorine for 10 minutes and subsequent disinfection with 70% ethanol for 3 minutes sounds reasonable. But the temperature at which the disinfection has to be carried out is of utmost importance and considering the viability of the cells and tissues, the temperature of disinfection maintained should be around 4^0C. To avoid the possibility of bacterial invasions and for the best results, the animals should not be subjected to much stress prior to sacrifice.

4.3.2. An appropriate commercially available medium for developing primary cell cultures from hepatopancreas

Response of the tissue to the three media tested in terms of attachment of explants and cell proliferation are presented in Table 4.2. The best results were obtained in MEM (Eagles) (AT017) – prepared in seawater and filter sterilized.

For getting best results the mode of preparation of tissue for seeding the tissue culture bottle has to be regulated with great care. Ghosh *et al.*, (1995) executed a programme of tissue perfusion in which prawns were injected with heparin (5000U) in to the periarthroidal space. After 10 – 15 minutes the animals were placed on a surgical board and the carapace removed and the hepatopancreas was subjected to perfusion with 50ml media containing $0.2gL^{-1}$ streptomycin

sulphate at a flow rate of $2mL.min^{-1}$. The organ was then carefully removed and transferred to a beaker containing the perfusion fluid, minced subsequently and seeded the bottle after cell counting and were then supplemented with M 199 containing 3.75 mM HEPES, 2.1 mM sodium bicarbonate, $0.2gL^{-1}$ glutamine and antibiotic mixture.

Chen *et al.*, (1987) used L-15 for preparing a cell culture from the hematopoietic tissue and ovary of *Penaeus monodon* containing 1000 $units.mL^{-1}$ streptomycin, 1000 $units.mL^{-1}$ penicillin and 100 $\mu g\ mL^{-1}$ fungizone. Hsu *et al.*, (1995) developed an *in vitro* culture system from the lymphoid organ of *P. monodon*. They used Medium 199, Leibovitz L-15 and Dulbecco's modified Eagle Medium supplemented with penicillin, streptomycin, amphotericin B, gentamycin, foetal bovine serum, and a variety of growth factors. For generating a primary culture of lymphoid nerve and ovary Nadala *et al.*, (1993), used media such as L-15, M 199, RPMI 1640, TC 100, Grace's and M and M. Leudeman and Lightner (1991) used L-15 as the growth medium for developing an *in vitro* primary cell culture system from the penaeid shrimp. It was based on these reports the three media such as MEM, L-15 and M 199 which were tried here to segregate the most appropriate one for hepatopancreas of *P. indicus* were employed. Meanwhile, it has to be highlighted that the processing of tissue at 4^0C adopted here is hitherto unreported. This protocol gave better results than the one done at room temperature.

4.3.3. Appropriate sodium chloride concentration/salinity in growth media for providing the right osmolarity required

Results of the experiments conducted to reveal the percentage of NaCl/salinity, which would give the right osmolarity to the growth media, are presented in Table 4.3. Attachment of explants could be obtained in MEM supplemented with 2.5, 3.0 and 3.5% NaCl and the same prepared in seawater having 25 and 35ppt. However the best results were obtained with MEM prepared

in 30ppt seawater, where, there was attachment of explants and cell proliferation (Fig. 4.1). It has to be emphasized here that the seawater used was the one collected from offshore area and as per the requirement it was diluted with double distlilled water. It was never autoclaved instead always filter sterilized using membrane having 0.22μ porosity.

Leudeman and Lightner (1991) determined the optimum osmolarity of the culture medium (L-15) they used for a primary cell culture from prawn as approximately 750 $mmol.kg^{-1}$. Hsu *et al.*, (1995) used L-15, supplemented with $5gL^{-1}$ NaCl with a final osmolarity of 470 – 500 $mmol.kg^{-1}$. Chen *et al.*, (1987) recommended a final osmolarity of 720 ± 10 $mmol.kg^{-1}$ for penaeids. In the present study 3 % NaCl or 30ppt seawater was found to be optimum for initiating growth of cells from the explants.

4.3.4. Response of hepatopancreas of *Penaeus indicus* to varying combinations of media growth factors and mitogens.

Results of the experiment to determine the response of hepatopancreas to varying combinations of media growth factors and mitogens are presented in Table 4.4. Among several combinations tried, MEM (Eagle's) prepared in 30ppt filter sterilized seawater supplemented with 10% (v/v) FBS, FME, PHL and 0.02μg mL^{-1} lectin 1 was found to be most acceptable where there was explant attachment, cell spreading and cell proliferation.

In literature, for the development of primary cell culture from *penaeids* more growth factors have been recommended than that of fishes. Prawn haemolymph, prawn muscle extract, lobster haemolymph (Chen *et al.*, 1986). Hsu *et al.*, (1995) tested several growth factors for developing a subculture system of shrimp cells, such as epidermal growth factors (EGF), transforming growth factors β (TGF β), insulin-like growth factor, fibroblast growth factor (b FGF) etc. It is obvious that growth factors are essential for the development of cell

cultures from prawns. The growth factors added in the present work were of two categories, one, tissue derived such as prawn muscle extract, prawn haemolymph along with FBS and the other is a mitogen, Lectin 1. However, the results indicate that more improvement is required in the development and modification of an appropriate medium with which a cell line could be developed and established.

4.4 Summary

A primary cell culture from the hepatopancreas of *Penaeus indicus* has been developed. To attain this objective an appropriate protocol to disinfect the animal surface without the loss of viability of cells of hepatopancreas has been developed. A protocol for the removal of hepatopancreas by maintaining at 4^0C has also been standardized. The growth medium (MEM Eagle's), was segregated from the three media tested and was prepared in 30ppt filter sterilized seawater without autoclaving which gave better results. For better performance the medium was further supplemented with 10% (v/v) FBS, PME and PHL and 0.02μg mL^{-1} Lectin 1. The primary cell culture developed in this way was with majority of fibroblastic cells.

Table 4.1 Efficacy of the protocol developed for disinfecting both surface of *Penaeus indicus*

Growth on Nutrient Agar Plates				Growth on Sabouraud Dextrose Agar Plates				Blood Agar				Thioglycollate Broth			
A	B		C	A	B		C	A	B		C	A	B		C
+	100	+	+	+	100	+	+	+	100	+	+	+	100	+	+
+	200	+	+	+	200	+	+	+	200	+	+	+	200	+	+
+	300	+	+	+	300	+	+	+	300	+	-	+	300	+	-
+	400	+	-	+	400	+	-	+	400	-	-	+	400	+	-
+	500	+	-	+	500	-	-	+	500	-	-	+	500	-	-
+	600	+	-	+	600	-	-	+	600	-	-	+	600	-	-
+	700	-	-	+	700	-	-	+	700	-	-	+	700	-	-
+	800	-	-	+	800	-	-	+	800	-	-	+	800	-	-
+	900	-	-	+	900	-	-	+	900	-	-	+	900	-	-
+	1000	-	-	+	1000	-	-	+	1000	-	-	+	1000	-	-

+ : Growth
++: Heavy growth
- : No growth

100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 : Sodium hypochlorite concentrations in ppm.

A: Before disinfection
B: After treatment with sodium hypochlorite
C: After treatment with 70% ethanol

Table 4.2 Screening of commercially available media for developing primary cell culture system from the hepatopancreas of *P. indicus*

Sl.no	Name of Media	Response of the tissue
1	L-15 (Leibovitz) Medium (AT 011)	+
2	MEM Eagle (AT017)	+++
3	Medium 199(AT015)	+

+ : Attachment

+++ : Attachment, growth and monolayer formation

5

Table 4.3 Determination of appropriate NaCl concentration/salinity in growth media for providing the right osmolarity required for the developing cell culture systems from the penaeid prawns

Media	NaCl (%)	Response	Media	Sea water‰	Response
L-15	1.5	-	L-15	15	-
L-15	2.0	-	L-15	20	-
L-15	2.5	-	L-15	25	-
L-15	3.0	-	L-15	30	-
L-15	3.5	-	L-15	35	-
MEM	1.5	-	MEM	15	-
MEM	2.0	-	MEM	20	-
MEM	2.5	-	MEM	25	+
MEM	3.0	-	MEM	30	+++
MEM	3.5	-	MEM	35	+
M199	1.5	-	M199	15	-
M199	2.0	-	M199	20	-
M199	2.5	-	M199	25	-
M199	3.0	-	M199	30	-
M199	3.5	-	M199	35	-

+ : Attachment and growth and monolayer formation
- : No attachment and growth
MEM : Minimum Essential Medium
L-15 : Leibovitz Medium
M199 : Medium 199

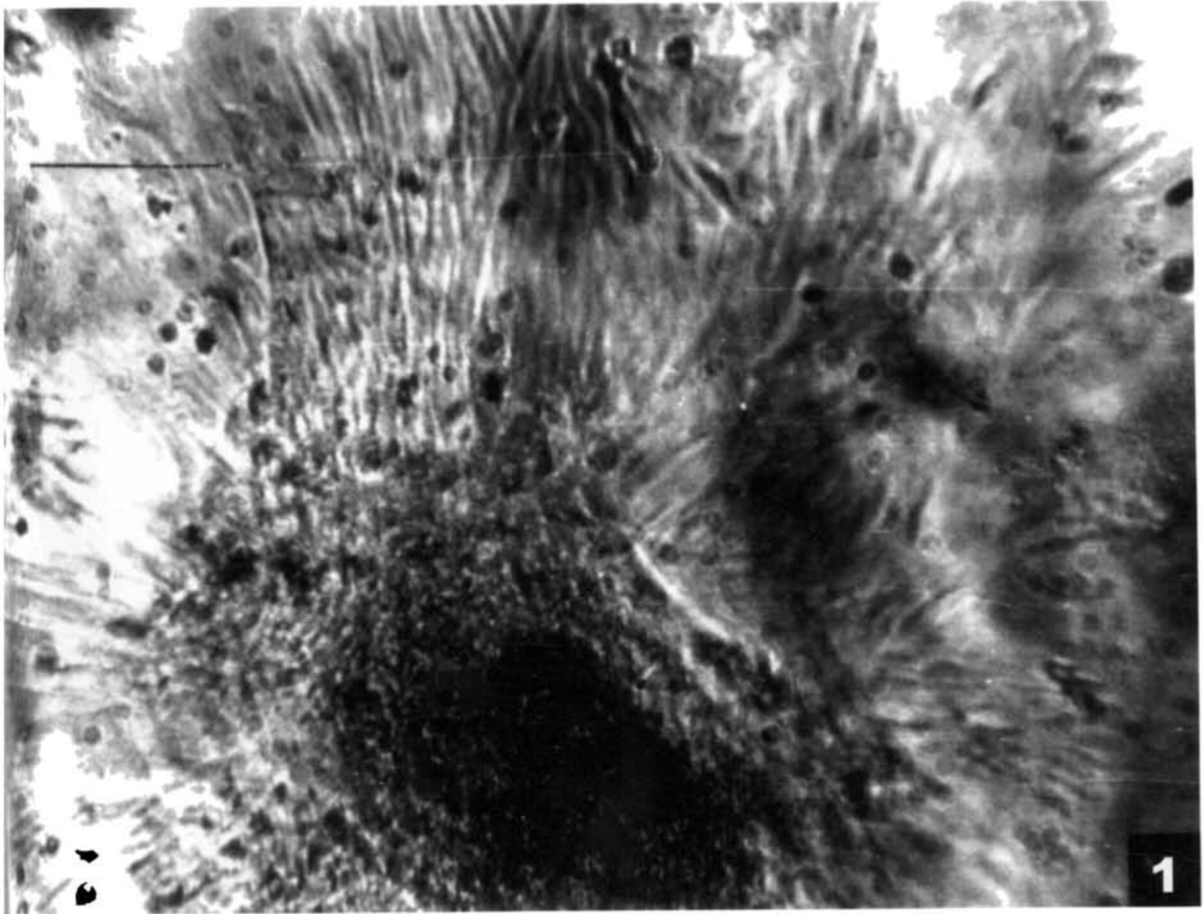
Table 4.4 **Response of the hepatopancreas of *Penaeus indicus* to varying combinations of media, growth factors and mitogens.**

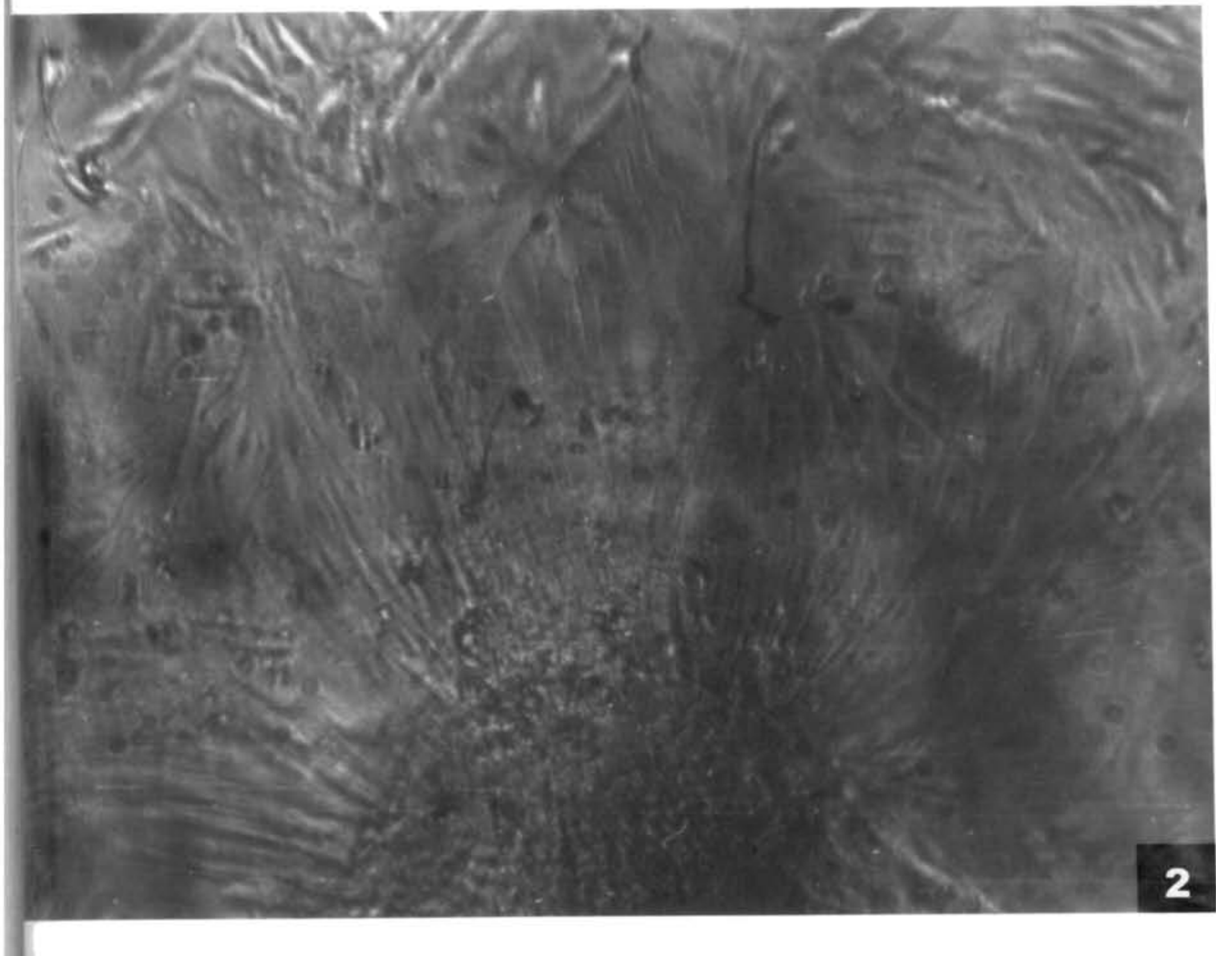
Phase No	Sl. No.	Medium	Supplements	% attachment of the explants	Time taken for attachment & beginning of cell proliferation in Hrs	Growth and monolayer formation @
1	1	M199 in 25ppt Seawater	10%FBS+10%PME+ 0.5%PHL+ Lectin 1	30	72	-
	2	MEM in 25 ppt Seawater	10%FBS+10%PME+ 0.5%PHL+ Lectin 1	30	72	+
	3	L-15 in 25 ppt Seawater	10%FBS+10%PME+ 0.5%PHL+ Lectin 1	35	72	-
2	1	M199 in 30ppt Seawater	10%FBS+10%PME+ 0.5%PHL+ Lectin 1	45	120	-
	2	MEM in 30 ppt Seawater	10%FBS+10%PME+ 0.5%PHL+ Lectin 1	30	120	+++
	3	L-15 in 30 ppt Seawater	10%FBS+10%PME+ 0.5%PHL+ Lectin 1	35	120	-

FBS - Fetal Bovine Serum
PME - Prawn Muscle Extract
PHL - Prawn Haemolymph
Lec1 - Lectin1*($0.02\mu g.mL^{-1}$)
+++ : Cell proliferation and attachment

1. Fig. 4.1 Primary cell culture developed from the hepatopancreas of *Penaeus indicus*, explant method (10X)

2. Fig. 4.2 A monolayer formed from the explant hepatopancreas of *Penaeus indicus* (10X)





CHAPTER 5

APPLICATION OF NEWLY DEVELOPED CELL CULTURES FOR ISOLATION OF VIRUSES

CHAPTER 5

APPLICATION OF NEWLY DEVELOPED CELL CULTURES FOR ISOLATION OF VIRUSES

5.1 Introduction

Cell cultures are the host systems most frequently used for virus cultivation. The type of cells used for virus isolation depends upon the specimen submitted. Three types of cell cultures are recommended for virus isolation studies, such as primary cell cultures, diploid cell and heteroploid cell lines. For instance, one virus may replicate in one type of cells whereas another may replicate in all the three types of cell cultures (Ballew *et al.*, 1975).

Many viruses produce morphological changes in cell cultures known as cytopathic effects (CPE). Certain viruses exhibit a characteristic CPE in cell cultures and thus give the experienced observer with a clue as to which major group the virus belongs.

Cytopathic changes in cell cultures may be characterized by cell rounding, refractile cells, cell clumping, vacuolation, granulation, giant cells, syncytial formation, and complete cellular destruction. For example, adenovirus replicates in certain cell cultures, producing swollen cells in clumps, measles virus produces giant cells or syncytial formation and poliovirus causes generalized cell rounding. Certain viruses replicate in cell cultures without causing a cytopathic effect.

The major field of application of fish cell culture - particularly fish cell lines - is virology. Cell cultures are widely used in fish health inspection and certification operations, in research, and in diagnostics. Ahne (1981), reviewed

serological techniques currently used for identification and detection of fish viruses and noted that serum neutralization tests and immunofluorescent technique typically employ cell cultures. Fish cell lines are used to produce the quantities of virus needed for biophysical research, for preparation of antisera and for the production of live and killed fish virus vaccines (Wolf and Ahne, 1982).

Use of fish cell cultures in toxicology studies has been ever growing. The cultures no doubt offer great convenience and uniformity than intact fish (Wolf and Ahne 1982).

Primary cultures of fish cells are widely used in studies of chromosome numbers and morphology. Dividing cell can be obtained from cultures of leucocytes (Wolf and Quimby, 1976; Sigel and Beasley, 1973) or from scale and fin epithelium.

Cultures of endocrine tissues or cells have been found to be of use in studies of fish hormones. The general methods employed are essentially those used for routine culture of tissues on cells from other organs or systems. Hightower and Renfro (1988) made a review on the recent application of fish cell culture to biomedical research. In this, several of the successful models along with the recent developments in fish cell culture in biomedical research are described. They have dealt with topics such as the application of cell cultures in the study of epithelial ion transport, endocrinological studies, the cellular stress (heat shock) response, thermotolerence, cancer biology and environmental toxicology.

In the present work, six fish cultures designed as PRL-1, CGL-1, CGS-1, CGK-1, CGT-1 and CGO-1 were developed from two fishes such as *Poecilia reticulata* and *Clarias gariepinus*.

Among them PRL-1 and CGO-1 were found to be very much promising under optimum growth conditions in several respects. Primarily they can be

generated at any moment of requirement with in a period of three to five days and can be subcultured for 10 times in the case of PRL-1 and 16 times in the case of CGO-1. Both the cell lines were employed here for reisolating the viruses, which were primarily cultured in RTG-2 for a long period as had been demonstrated by the cytopathic effects formed.

5.2 Materials and Methods

5.2.1 Selection of cell cultures

Altogether seven cell line such as PRL-1 (Embryonic/larval tissue of *Poecilia reticulata,* CGL-1, CGS-1, CGK-1, CGT-1, CGO-1 –Liver, spleen, kidney, testis and ovary of *Clarias gariepinus* and PIH-1 (Hepatopancreas of *Penaeus indicus*) were developed which could be subcultured for 4 to 16 times. Among them PRL-1 and CGO-1 were the most promising which could be maintained for a prolonged period and could be subcultured several times. The cell line were mixed type and composed of both epitheloid and fibroblastic cells. The procedures for developing primary cell cultures and their passage to form diploid cell lines have now been standardized in such a way that a primary cell culture could be developed from the embryonic/larval tissue of *Poecilia reticulata* and ovarian tissue of *C. gariepinus* within three to four days and can be passaged several times. After the third passage, the cell lines were found to have >70% confluency and were to be applied for either viral isolation or for any toxicological studies.

5.2.2 Description of two diseased condition

a. Mass mortality of blue gourami *(Trichogaster trichopterus)*

During the year 1998 there happened a mass mortality of blue gourami in a private commercial fish production centre at Trichur. External manifestation of the disease were loss of appetite, lethargy, loss of scales as patches, accumulation of ascetic fluid, yellowing of the liver and gall bladder enlargement. Moribund animals had been placed at -20^0C and it was decided to examine the possibilities of the involvement of virus in the disease process.

The infected animals were retrieved from deep freezer and placed on ice bath. Using a sterile scalpel blade dissected out the liver, kidney, and spleen from the fish individually. Using a sterile pestle and mortar these tissues were macerated in sterile phosphate buffered saline PBS (1X, pH 7.0) with sterile glass wool (HiMedia laboratory, Bombay). The macerations were carried out in ice-bath. The resultant tissue homogenate was transferred into sterile 20mL centrifuge buckets. The buckets were loaded in a fixed angle rotor centrifuge (Remi Instruments Ltd.) and spun down at 1000g for 10 minutes. The clarified supernatant was transferred to sterile centrifuge tubes and centrifuged at 10000g for 30 minutes. The resultant supernatant was removed carefully (only the upper two third position) using a sterile Pasteur pipette aseptically and filter sterilized by passing through a membrane (Sartorius India Pvt. Ltd.) having 0.22μ pore size. The filtrate was considered as the tissue homogenate containing virus suspension. Tubes were sealed with parafilm, labeled with permanent marker pen and transferred into Eppendorf storage containers which was then stored at -20^0C in deep freezer. This method was followed for the isolation of prawn viruses also.

b. White spot disease of penaeids

The white spot disease is caused by a rod shaped bacilliform virus generally termed as white spot virus (Takahashi *et al.*, 1996 and Wongteerasupaya, 1995). Since no established prawn cell line has been made available, genuine virus isolation in to a cell line could not be possible. Therefore it was thought of employing the RTG-2 cell line first to isolate the virus and then to passage it to the newly developed cell cultures.

Moribund *Penaeus indicus* with the clinical manifestation of the white spot on the inner side of the carapace, reddening of the body, empty intestine were selected for the extraction of virus. Gill tissue was dissected out and homogenized in PBS(1X) (NaCl, 8g; KCl, 0.2 g; Na_2HPO_4, 1.15g; KH_2PO_4, 0.2gm; Double distl. water 1000ml) in a mortar and pestle. The homogenate was centrifuged at 10000rpm at 4^0C and the supernatant passed through a membrane of 0.22μ porosity. The filtrate was maintained at 4^0C till used. The inoculation in to RTG-2 cell line was done within 2 hrs.

5.2.3 Isolation of the virus on to RTG-2 cell line

RTG-2 cell line was obtained from the National Centre for Cell Science, Pune, and was under continuous subculturing in the laboratory for a long time. Six days after subculturing when 100% confluency was obtained, 0.1ml of the filtrate was inoculated into the medium (5ml) overlying the cell line and incubated at room temperature (28 ± 0.5^0C) and monitored for the appearance of cytopathic effect. After 16 days of incubation, in the case of cell line which received the inoculum from gourami and 15 days in the case of that of prawn a limited extent of rounding of cells, cell dislodgment and aggregation of cells were seen and at this stage passages were made into fresh cell lines. The process was repeated till remarkable CPE could be seen in both the cases.

For effecting passage of the viruses supposed to have caused the cytopathic effect, the cell line was subjected to three times freeze thaw cycle and the cells were further disrupted by agitation by pippeting. The fluid along with the cells were drained off and centrifuged at 10000rpm for 10 minutes. The supernatant was filtered through membrane of 0.22μ porosity and was used for infecting the cell lines PRL-1 and CGO-1.

5.2.4 Passage of virus on to the newly developed cell cultures

The cytopathic effect, caused by inoculating the tissue homogenate from liver on to RTG-2 and passaged several times was considered to be due to a virus and it was named as 'gourami virus' (GV). In the same way the cytopathic effect, which could be generated in RTG-2 from the gill tissue of the infected prawn, was considered to be due to the white spot virus (WSV). The GV was passaged on to PRL-1 and WSV on to CGO-1.

For the virus inoculation PRL-1 and CGO-1 at the third passage was used. The medium was aspirated and 0.5ml of 'virus suspension' was spread on the cell sheet and inoculated for one hour. The growth medium (M199) prepared in FME was later added and supplemented with growth factors such as 10% (v/v), PME 0.02μg mL^{-1} Lectin 1, LPS, and 0.2mg mL^{-1} Glucose D and 0.5% (v/v) OE in the case of PRL-1 and in the case of CGO-1 the medium added consisted of L-15 supplemented with 10% (v/v) each FBS, FME, PME and 0.5% (v/v) OE, 0.02 μg mL^{-1} Lectin 2 and the same quantity of LPS, 0.2mg mL^{-1} and 2% PHL. The bottles were incubated at 28^0C and observed for cytopathic effects such as rounding of cells, vacuolization and cell dislodgment and lysis.

5.3 Results and Discussion

5.3.1 Description of the newly developed cell cultures

The cell cultures subjected for virus isolation through RTG-2 are presented in Fig. 5.3 (PRL-1) and 5.4(CGO-1). Both the cultures used were at 3rd passage and were composed of both fibroblastic and epitheloid. There was more than 60% confluency in each bottle at the time of inoculation. One of the advantages of employing a primary cell culture/diploid cell line is its susceptibility to a variety of viruses.

5.3.2 Observation of cytopathic effect in RTG-2 and its successive passages

The primary isolation of the virus from gourami and prawn was done in RTG-2, as this was the only cell line available in this laboratory when the disease broke out. The cytopathic effect, which got developed from both the disease situations, was characteristics of a virus involvement (Fig 5.1 and 5.2). The CPE developed on RTG-2 from gourami could be passaged 12 times, each time, the duration required for total dislodgment of cells varied from 4 to 13 days. In a similar fashion, the CPE developed on RTG-2 from the white spot virus infected prawn could be passaged for 10 times and the number of days required for the completion of cell dislodgment varied from 9 to 19 days. The reason for this sort of variation can not be explained at this stage. But, it was almost apparent that in both the cases the cytopathic effect was due to viral involvement and not due to any tissue toxicity. In this context, it was decided to passage the viruses to the new cell cultures and to look for their susceptibility to the virus already presumably isolated in RTG-2.

5.3.3 Observation of cytopathic effect in the newly developed cell cultures

The cytopathic effects in PRL-1 due to the gaurami virus and in CGO-1 due to white spot virus are presented in Fig 5.5 and 5.6. In PRL-1 the CPE is characterized by plaque formation, granulation and rounding of cells. One of the characteristics of the CPE persistence of a layer of unaffected cells beneath the plaques formed. This may be due to the situation of having more than one type of cells in the cell culture developed. As mentioned earlier the PRL-1 was mixed with both epitheloid and fibroblastic cells together. In CGO-1 the CPE was characterized by acute granulation and dislodgment of cells and the dislodged cells were found to get disintegrated. Efforts are under way to passage the CPE to a fresh batch of cell cultures and to demonstrate the virus by electron microscopy. This experiment forms just a demonstration on how the newly developed cell lines such as PRL-1 and CGO-1 can be employed for virus isolation and it may be noted that the cell lines can very well be used directly for virus isolation and it need not be through an established cell line.

5.4 Summary

Even though six diploid cell lines could be developed PRL-1 and CGO-1 alone were found to be promising for virus isolation because of the rapidity of growth and the confluency of the monolayer formed. A couple of years back when there happened a mass mortality of gourami in an ornamental fish farm a virus named GV was isolated employing the cell line RTG-2. Similarly a persistent CPE could be generated in RTG-2 when a gill extract from an infected prawn was inoculated into the cell line and passaged repeatedly. Extracts from these infected cell lines were passaged to PRL-1 and CGO-1 on doing characteristic CPE could be observed in both the cases.

1. Fig.5.1 Cytopathic effect on RTG-2 on inoculating with liver extract from a moribund specimen of blue gourami. Cell aggregation and cell dislodgment can be seen (10X)

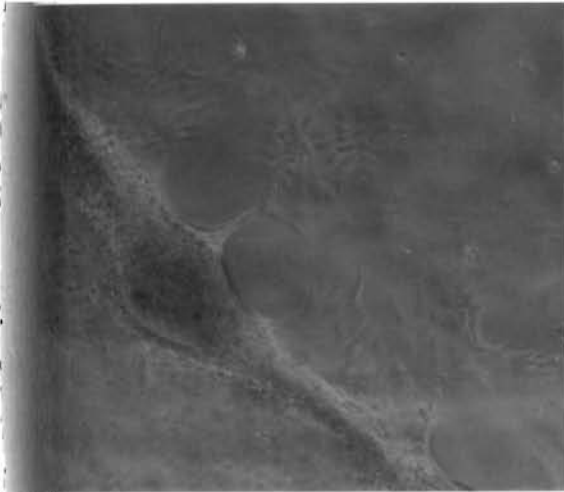
2. Fig.5.2 Cytopathic effect on RTG-2 on inoculating with gill extract of *Penaeus indicus* infected with white spot virus . Cell dislodgment and cell aggregation can be seen

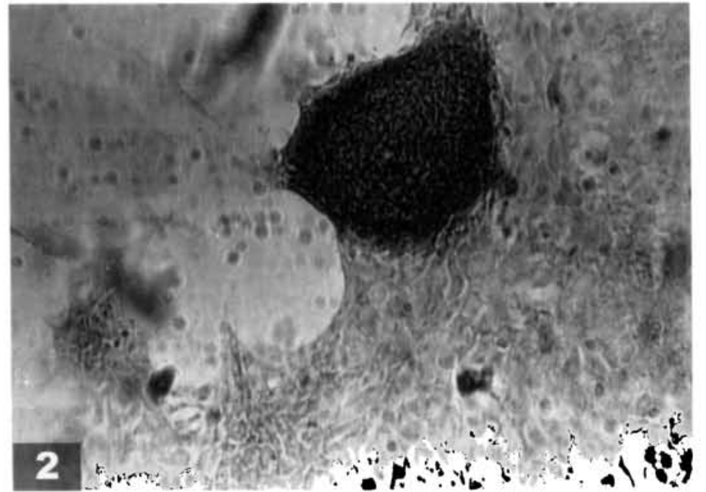
3. Fig.5.3 Normal diploid cell line PRL-1

4. Fig 5.4 Normal diploid cell line CGO-1

5. Fig 5.5 Diploid cell line PRL-1 showing CPE on inoculating from RTG-2 infected with "gourami virus". Plaque formation can be seen (10X)

6. Fig 5.6 Diploid cell line CGO-1 showing CPE on inoculating from RTG-2 infected with white spot virus- Granulation and cell dislodgment can be seen (10X)

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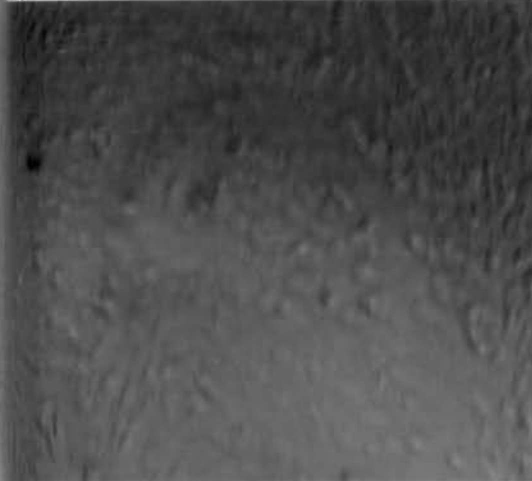

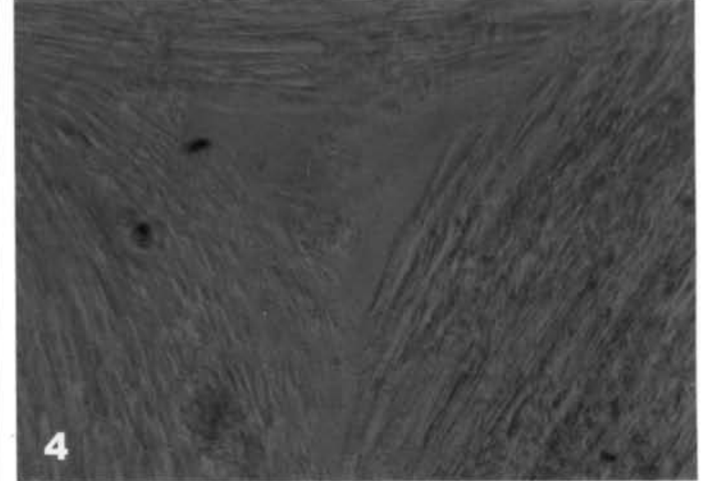
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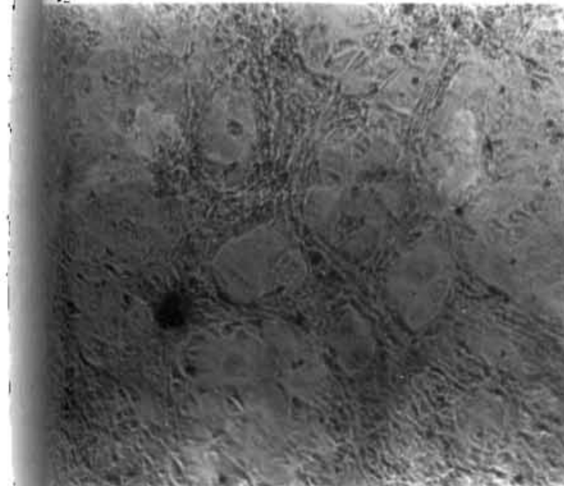

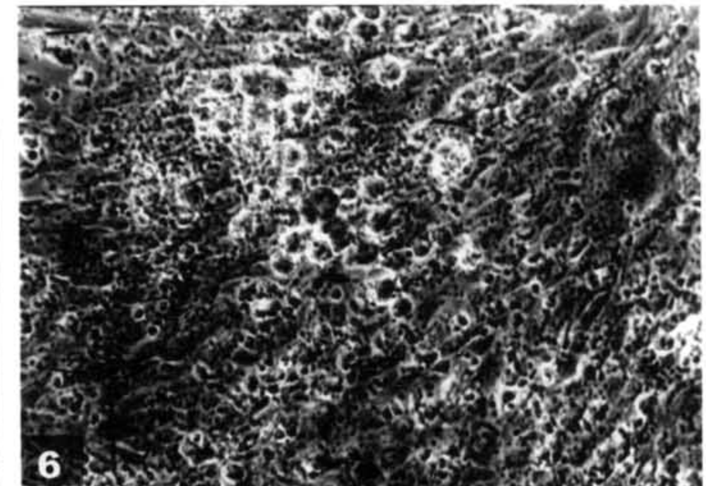
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CHAPTER 6

SCREENING, SELECTION AND STANDARDIZATION OF AN APPROPRIATE ANTIFUNGAL COMPOUND FOR TISSUE CULTURE APPLICATION

CHAPTER 6

SCREENING, SELECTION AND STANDARDIZATION OF AN APPROPRIATE ANTIFUNGAL COMPOUND FOR TISSUE CULTURE APPLICATION

6.1 Introduction

One of the biggest problems in animal tissue culture is the contamination by bacteria and fungi due to the fact that media used for the cultivation of cells and tissues are highly nutritious, supporting growth of a variety of organisms. Majority of these microorganisms have a much more rapid growth rate than cells are frequently produce toxins, which are lethal to them. Hence, the most important part of tissue culture technique comprises the avoidance of contamination and the growth of tissues in aseptic conditions. Principally, the contaminations can be prevented in two ways, one by sterilization of glass wares, media, samples, work area and by observing aseptic conditions, which implies the prevention of contamination of the material already sterile. Besides, to keep the media protected from any such contamination, an effective concentration of antibiotics also has to be incorporated.

Very many antibiotics have been used in tissue culture work. At the same time it is a bad practice to become completely dependent on antibiotics for three reasons. In the first place there is danger of concealing sloppy techniques. Secondly, when contaminations arise the organisms are already resistant. Thirdly, cryptic contaminations and development of L-forms may be fostered. At the same time total avoidance of antibiotics from tissue culture work also cannot be advocated, as it may not be successful. However many antibiotics are quite toxic

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to cells and in some cases the levels of toxicity approach the effective levels (Paul, 1975).

By far the most useful general antibiotic is Penicillin. It is usually added as sodium penicillin G to media to give a final concentration of 20 to 50 units per mL (Hu *et al.*, 1953 and Cruikshank and Lowbury, 1952). At these concentrations it is completely harmless to all cell types and is inhibitory to the vast majority of bacteria. Along with penicillin, streptomycin sulphate also can be used to give a final concentration of 50 μg mL^{-1} (Paul, 1975).

Kanamycin is effective in eliminating contamination by mycoplasma at concentrations ranging from 100-200 μg mL^{-1}. Overnight treatment with higher concentrations (600 μg mL^{-1}) will sometime eradicate the infection (Perlman and Brindle, 1963). Aureomycin at 50 μg mL^{-1} has been found to be still more effective.

For inhibiting fungal growth in tissue culture media Mycostatin (nystatin) has been recommended at a final concentration of 20 μg mL^{-1}. It should be noted that mycostatin is unsuitable and usually deteriorate almost completely after 2-3 days in tissue culture medium at 37^0C. Amphotericin B (fungizone) is also effective against many fungi at a concentration of 2.5 μg mL^{-1} (Perlman and Brindle, 1963). Paul (1975) is of opinion that it is better to retain these antibiotics for use in an emergency if a contamination should arise in a valuable strain. But the practical experience in this laboratory is that the tissue culture grade antifungal compounds such as mycostatin and amphotericin B were not effective during survival occasions. In this situation it was essential to search for other antifungal compounds available commercially and standardize them for applying in tissue culture in the right proportion. Accordingly commercially available antifungal compounds such as fluconazole, miconazole, clotrimazole, gentamycin, kanamycin and griseofulvin available commercially were screened against an *Aspergillus* sp. which was found to contaminate tissue culture

frequently. The highest concentration, which can be added to a tissue culture, by using RTG-2 as the model, was also standardized.

6.2 Materials and methods

6.2.1 Isolation of the fungus

The fungus to be tested against the antifungal compounds was isolated from contaminated tissue culture bottles. A loopful of the broth was inoculated in to Sabouraud dextrose agar plates and incubated for 72 hours at room temperature (28 ± 0.5^0C). The fungal colonies developed were isolated on to Sabouraud dextrose agar slants and based on the nature of fruiting bodies and hyphal structure they were identified to *Aspergillus* sp.

6.2.2 Determination of the minimal inhibitory concentration of the antifungal compounds.

The antifungal compounds screened against the fungus were,

1. Gentamycin (Fullford, India Limited, Hyderabad)
2. Kanamycin (Macbods Laboratories Private Limited, Bombay)
3. Clotrimazole (Glenmark Pharmaceuticals Ltd., Nasik)
4. Micanozole nitrate (Gulfic Pharma, Gujarat)
5. Flucanozole (Torrent Laboratories, India)
6. Griseofulvin (Glaxo Laboratories, Bombay)

These compounds were incorporated into Sabouraud dextrose broth at 10, 50, 100, 200 and 400 µg mL^{-1} concentrations aseptically and dispensed into culture tubes. After incorporating the compound the media were not autoclaved. The antifungal compounds were having different levels of solubility in varying

solutions as Gentamycin, Kanamycin and Griseofulvin were water soluble and Clotrimazole and Miconazole nitrate, soluble in ethanol and Fluconozole in methanol. The compounds were first chemically sterilized by using solvent ether and either dissolved in water or methanol or ethanol as per the solubility and incorporated in Sabouraud dextrose broth. As controls Sabouraud dextrose broth without the antifungal compound and in cases where organic solvents were used they were incorporated in the corresponding level to the medium. The tubes were inoculated with fungal spores and incubated at room temperature (28 ± 0.5^0C) for 72 hours. Absence of germination of spores and hyphal development was counted as the inhibitory property of the antifungal compounds used.

6.2.3 Toxicity of the segregated antifungal compounds on RTG-2 cell lines.

From the six antifungal compounds tested, Clotrimazole, Flucanazole and Miconazole were segregated and tested *in vitro* their toxicity in cell lines. For this the established cell line, RTG-2 was employed. The compounds were prepared aseptically as described above and sterility tests were performed by inoculating in to nutrient agar slants, Sabouraud dextrose agar slants and thioglycollate broth. The *in vitro* toxicity tests were performed in Leighton tubes and the compounds dissolved in the appropriate solvents was incorporated in to tubes having full grown sheets of RTG-2 cell lines. The tubes were observed for rounding off of cells, dislodgment and granulation and finally the viable cells were counted by trypan blue exclusion method.

6.3 Results and discussion

6.3.1 Minimum inhibitory concentration of antifungal compounds

Response of spores of *Aspergillus* sp to different concentrations of the antifungal compounds is presented in Table 6.1. As can be read from the table,

only Clotrimazole, Flucanazole and Miconozole nitrate were segregated for further *in vitro* toxicity tests.

6.3.2 Toxicity of the segregated antifungal compounds on RTG-2 cell lines

Extent of toxicity of the antifungal compounds on RTG-2 in terms of reduction in the number of viable cells is presented in Fig. 6.1. It is well evident that Clotrimazole did not have obvious toxicity till a concentration 200 $\mu g\ mL^{-1}$ was incorporated. At the same time, Flucanazole started exerting the influence from 65 $\mu g\ mL^{-1}$ onwards and Miconozole from 150 $\mu g\ mL^{-1}$ onwards. The data precisely indicates the suitability of Clotrimazole for *in vitro* applications. It has to be remembered that Clotrimazole can inhibit *Aspergillus* at 10 $\mu g\ mL^{-1}$ concentrations and accordingly Clotrimazole was finally selected for applying in tissue cultures.

The toxicity of Clotrimazole. on RTG-2 cell line was ascertained only based on the loss of viability of cells in its presence. However, according to Kunze and Todd (1983) opined based on a study conducted to evaluate Econazole on cell line that the toxicity study would be complete only if the colony forming efficiency, growth rate, cell cycle distribution and attachment efficiency in the presence of the chemical compound. These studies have yet to be taken up.

6.4 Summary

On examining six commercially available antifungal compounds clotrimazole alone was found to be suitable for tissue culture application as it has got high order of inhibition at low levels and less tissue toxicity.

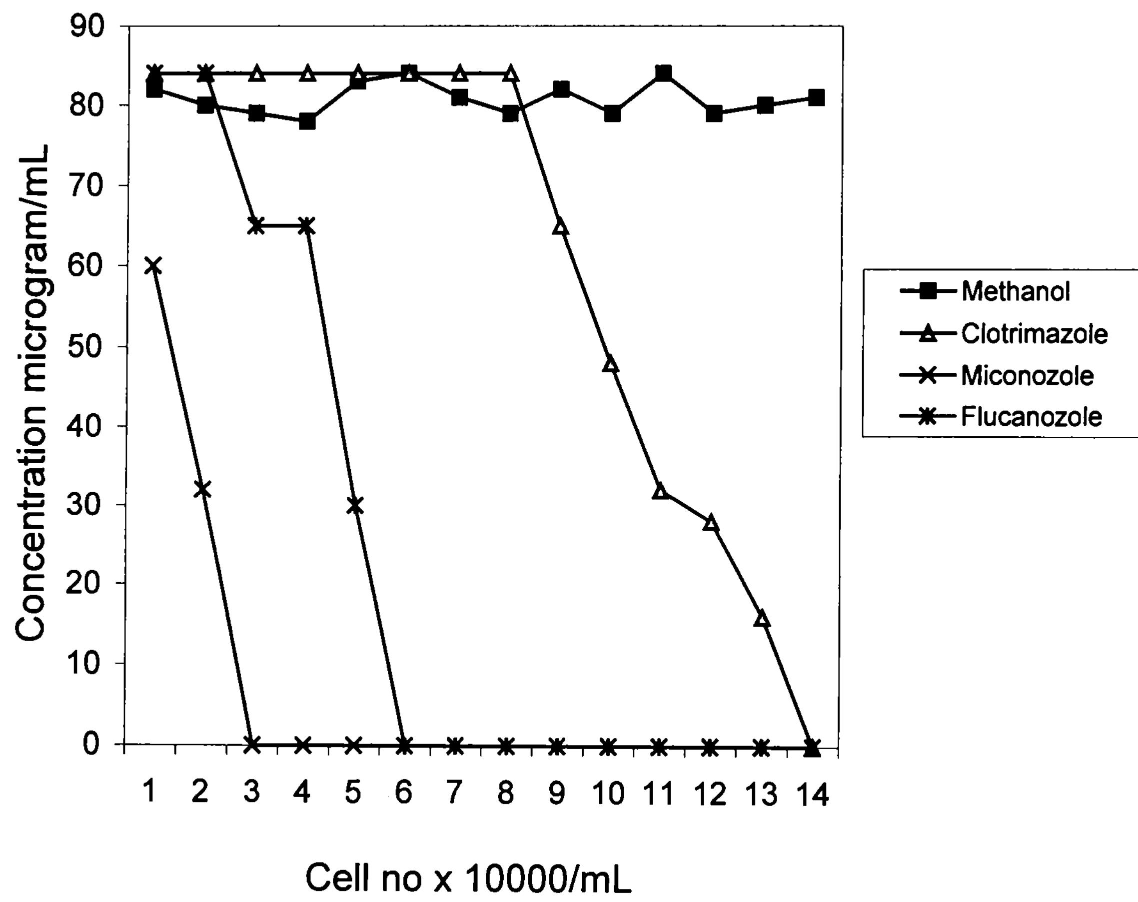
Table 6.1 Efficacy of commercially available antifungal compounds on the fungus *Aspergillus* sp grown in Sabouraud dextrose broth

Sl.no	Compound name	Concentrations (µg mL^{-1})						
		10	50	100	150	200	250	300
1	Gentamycin	+	+	+	+	+	+	+
2	Kanamycin	+	+	+	+	+	+	+
3	Clotrimazole	-	-	-	-	-	-	-
4	Flucanazole	+	+	+	+	+	+	-
5	Miconazole	-	+	+	+	+	+	+
6	Griseofulvin	+	+	+	+	+	+	+

+ : Growth of fungus

- : No growth of fungus

Fig. 6.1 Sensitivity of RTG-2 cell line to varying concentrations of antifungal compounds



CHAPTER 7

CONCLUSION

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CONCLUSION

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 *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 -35^0C 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 *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 trichoptrus*) (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 $\mu g\ mL^{-1}$. In tissue culture this can be added up to a level of 200 $\mu g\ mL^{-1}$ 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 *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.

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