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CELL CULTURE TECHNIQUE AND ITS APPLICATION IN AQUACULTURE

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INTRODUCTION

Aquaculture is one of the fastest growing food sectors in the world, and it constitutes an important source of livelihood and food security in many countries. However, the development of this sector, face the serious threats from the emergence of many infectious diseases. Of the various infectious diseases, viral diseases cause severe economic losses to aquaculture industry This has increased the thrust for development of diagnostic techniques and *in vitro* models, so early preventive measures can be started to tackle the disease menace. Cell line and cell culture technique has, therefore, gained attention over a period of time for diagnosis and epidemiological study of virulent and latent viruses in aquaculture system. Moreover, there is a real need for development of *in vitro* technique of cell culture to ease pressure on wild stock and optimize growth condition for aquaculture. Cell lines are nothing but transformed cells that are adapted well to grow and multiply outside the host in *in vitro* culture system. Apart from their vital role in disease diagnosis, cell lines play an important role in many biomedical research areas like carcinogenesis, toxicology, endocrinology, immunology, cellular physiology, genetic regulation, cloning and expression of recombinant proteins and also, in many vital aspects of quality control programs. Characterized cell lines deposited in national and international repositories like ATCC cell line repository, Riken cell bank etc. are used for research and industrial purpose.

Cell line research in aquaculture

According to literature, the first fish cell line, Rainbow Trout Gonad (RTG-2), had been established in 1962 (Wolf and Quimby, 1962). The burgeoning use of cultured fish cells have resulted in the establishment of a number of cell lines from wide range of species. At present, more than 157 fish cell lines which represent 34 families of fishes have been established (Fryer and Lannon, 1994). Salmonids and cyprinids provide maximum number of cell lines reflecting their economic importance and the wide spread culture of these

families. A large number of cell lines have been established in fresh water fishes or anadromous fish species (Hong *et al.*, 2004; Zhou *et al.*, 2008). The studies of marine fish cell lines have developed recently and at least 17 cell lines from tissues of commercially important marine fish have been reported since 1980 (Sahul Hameed *et al.*, 2006).

Albeit, viral disease is one of the major bottlenecks in shrimp culture industry, tissue culture and cell lines are still in its infancy. Chen *et al.*, 1986) first reported the cell culture of *Penaeus monodon*. Then onwards, primary cell cultures have been developed from various tissues and organs of the penaeid shrimp, including the lymphoid organ, gut, nerve cord, hepatopancreas and gonads (Owens and Smith, 1999; Lang *et al.*, 2002). Many of this primary cell culture have been used for the growth and pathogenesis study of many penaeid shrimp viruses (Wang et al., 2000). Although success has been made in primary cell culture, there are only a very few described cell lines from crustaceans, two from SV-40 transformed primary cultures of the lymphoid organ of the shrimp *Penaeus stylirostris* (Tapay *et al.*, 1995), and one from a crayfish (Neumann *et al.*, 2000). However, these cell lines are not available in any cell culture repository.Attempts are being made to immortalize the shrimp cells by transfecting it with oncogene and making cancerous (Claydon and Owens, 2008).

In India, few fresh water cell lines and primary cell culture of Indian Major Carps, *Clarius batrachus* and golden mahseer, *Tor putitora* have been developed (Sathe *et al.*, 1995; Rao *et al.*, 1997; Lakra *et al.*, 2006). Sahul Hameed *et al,* (2006) have established India's first marine fish cell line, SISK from the kidney of sea bass. Recently, two new cell lines, designated RE and CB were derived from the eye of rohu, *Labeo rohita*, and the brain of catla, *Catla catla*, respectively (Ishaq Ahmed *et al.*, 2009). The observation suggests that in the scenario of virus plagued aquaculture industry, the primary cell culture and cell lines would be useful for *in vitro* assay of many deadly viral infections.

Examples of widely used fish cell lines

Fish species	Designation	Cell type/ morphology	Tissue source
Oncorhynchus mykiss, rainbow trout	RTG-2	Fibroblast	Fry testis and ovary
Carassius auratus goldfish	CAR	Fibroblast	Normal fin
Danio rerio, zebrafish	ZF4	Fibroblast	Embryo fibroblasts
Fugu rubripes Tora fugu	Fugu eye	Epithelial	Eye
Ictalurus nebulosus brown bullhead	BB	Fibroblast	CT and muscle
Ictalurus punctatus, channel catfish	CCO	Fibroblast	Ovary
Lepomis macrochirus bluegill	BF-2	Fibroblast	Caudal trunk
I. punctatus channel catfish	1G8	Lymphoblast	Blood cells

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Fish species	Designation	Cell type/ morphology	Tissue source
O. tshawytscha (chinook salmon)	CHSE-214	Mixed	Embryo
Pimephales promelas (fathead minnow)	FHM	Epithelial	Caudal Trunk and muscle
Poeciliopsis lucida (topminnow)	PLHC-1	Hepatocyte	Hepatocellular cacinoma
Oncorhynchus mykiss	RTgill-W1	Epithelial	Gill
Clarias batrachus	G1B	Pleomorphic	Gill
Salmo salar (Atlantic salmon)	ASK	Epithelial	Kidney
Carrassius auratius	*GAKS*	epithelial-like	fish scale fibroblast
Cyprinus carpio	EPC	Epithelioid	Epidermis

Source: ATCC, USA and RINKEN Cell Bank, Japan.

Types of cell culture

Primary cell culture: Cell culture can be divided into primary cell culture and culture of established cell lines. Primary cell culture can be defined as *in vitro* proliferation of single cells which is no longer organized as tissues and isolated directly from the donor tissue or the organism. Usually it survives for a limited period of time and is heterogeneous in population. Primary cell cultures have an advantage in that they are recently removed from the *in vivo* situation and therefore expected to more closely resemble the function of that cell or tissue *in vivo*. The main disadvantage of primary cell culture is that they react rapidly to a constantly changing environment over the first few days or weeks *in vitro*, results in poor recovery of cells from mixed cell culture.

Established or Continuous Cell Lines: These are transformed and immortal cells derived either from repeated sub culturing of primary cell culture of normal cells or from tumorogenic and cancerous tissue. Most of the fish cells lines are derived from normal tissue as only 14 out of 159 fish cell lines are from cancerous cells or tumorogenic tissue (Fryer and Lannan, 1994). This contrasts with mammalian cell lines where over 50% of listed cell lines at the ATCC were derived from cancerous tissue or transformed cells. Most notable fish cell lines derived from cancerous tissue are EPC and RTH-149 which are derived from epithilioma and Hepatoma respectively.

Fundamental techniques in cell culture

Basically, the aim of any cell culture technique is to provide an environment that mimics, to the greatest extent possible, the *in vivo* environment of the specific cell type. As cultured cells are biological entities with specific physiological needs like adequate nutrients, optimal environment and regular checkups, the cells can be unacceptable for further diagnostic purpose if suitable environment is not provided.

Laboratory set up

A successful cell culture demands a sophisticated laboratory with essential equipment and infrastructure facility which determine in part, how well the cultures can be maintained free of contamination. Ideally, there should be separate areas for maintenance of cell line stock, viral stocks and actual cell culture work. This minimizes the chance of contamination and false positive reaction.

The essential equipment required for a cell culture laboratory include Incubator, laminar air flow hood, an autoclave for sterilization and an inverted microscope for the observation of cultured cells. The incubator should run at 25^0C-32^0C with 5% carbon dioxide to keep the medium at the correct pH. The pH is important for maintaining the appropriate ion balance, optimal function of cellular enzymes and for binding of growth factors to cell surface receptors. Most cells tolerate a pH in the range of 6.5 to 7.8. Different cells have somewhat different iso-osmotic points, and therefore a different osmolality requirement which can be regularly checked by osmometer.

Cell culture media

The essential idea of maintaining the ideal environment for cell culture is to provide the nutritional and hormonal requirement of the cell *in vivo*. The commonly used culture media include Leibovitz (L-15), Minimum Essential Medium (MEM), Dulbecco basic medium / Nutrient Mixture Ham's F12 (F12) etc. Nutritional supplement like aminoacids (L-glutamine-10 mM), Hanks Balanced salt solution, 1% of an antibiotic/ antimycotic solution (100 IU/ml penicillin and 10pg /ml streptomycin, 25 pg ml-' amphotericin), buffers(0.15 mM Hepes) and 5- 20 % Foetal Calf Serum (FCS) must be added to improve the growth of the cells. Antibiotic's cytotoxicity, stability and its spectrum of antimicrobial activity should be well considered before its use. Culture media over cells must be optically clear and free of any microbial contamination as single incidence of any contamination can affect the entire stock.

Basic Methods

Good aseptic technique is the first and foremost factor that determines the success of any cell culture. For this, working area, glass wares and associated equipment must be disinfected with 70% isopropanol. The animal should be anesthetized, dipped in 5% suitable antibiotics and wiped with 70% alcohol. The concerned organ or tissue should be dissected aseptically and washed three times in antibiotic containing culture media.

Cell culture techniques should be designed to maximize the yield of functionally viable cells isolated from cultured tissues or organs. Cell culture can be initiated from a variety of tissues, viz. embryonic, normal or tumour. Embryonic tissues forms best and simplest source. Variations in methodologies are due to complex and dynamic nature of the extra cellular matrix which differ tissue to tissue and with developmental stage. The commonly used cell culture techniques for processing of tissue include explant method and enzymatic method.

Explant method: Explant method involves cutting tissue into small size of 1 mm^3 and allowing it to grow in suitable culture medium. For this, tissue pieces should be washed to make it completely free of blood clots, plasma and other unwanted biological material. Also, the tissues must be dissected to small size to facilitate the gas exchange between explants and for proper attachment of explant to substratum. Explant methods are generally performed in the case where cells are protease sensitive (smooth muscle cells) or in case of small amount of tissue. This type of technique is easier to initiate, but cell growth may not continue for more than a few days.

Enzymatic method: This technique involves the disaggregation of tissue into its component cell by enzymatic digestion (trypsin or collaginase or Hyaluronidase). Mechanical separation of tissues is necessary to perform prior to the use of enzymes as it helps to maximize the surface area for enzymatic digestion. This can be achieved by finely cutting up the tissues in to 1 mm^3 or smaller pieces. For epithelial cells which are highly packed with very little intercellular material, addition of chelating agents such as EDTA helps in dissociation of intercellular tight bonds by chelating Ca/Mg ions. In this method, cells are inoculated in to the culture flask at a concentration of 5 x 10^5 cells per ml. This technique yields more representative sample and is comparatively faster than explant method.

Subculture or passages

Subcultures of cells or transplantation of cells from one vessel to another is important once cell culture attains confluence. Sub culturing helps to periodically provide fresh nutrients and growing space for continuously growing cell lines. Subculture can be done using 0.1% trypsin and 0.02% EDTA as detaching agents. All procedures must be done under strict sterile condition. Cell morphology should be periodically examined with inverted microscope. Good quality cell culture must be confluent and should not have foci or areas of necrosis.

Cell Quantification

To measure performance or reproducibility, quantification of the cultured cell population is imperative. During routine propagation, viable cells can be determined using haemocytometer with the help of chromogenic substance Trypan Blue, a vital stain which distinguish between viable and nonviable cells in a cell count. Other quantification methods are Flow cytometry, Mitotic index analysis, assessing DNA synthesis rate by estimating its reactivity to Brd U (5-bromo-2deoxyuridine), Tritiated thymidine, and Ag NOR (argyrophilic nucleolar organizer region).

Cryopreservation

Once a confluent cell layer is formed, the culture flask can be transferred to further lower temperature until use. Cells at logarithmic growth phase can be harvested and stored in

liquid nitrogen. By changing the growth medium and return to appropriate temperature, the cell replication can be resumed.

Application of cell lines in aquaculture

Cell lines and cell culture play a major role in many areas such as disease diagnosis, genetic analysis, cloning and expression of recombinant proteins, the production of transgenic organisms, drug sensitivity and cytotoxicity assays. In many of these areas cell culture has largely replaced the use of living animals as it mimic the characteristics of whole living organisms.

1. **Disease diagnostics and prophylactics:** Cell cultures become an essential primary tool for the detection and surveillance of pathogenic fish viruses and thus forms an important instrument in fish health assurance program.

    a. **Isolation and characterization of virus:** Unlike other microorganisms which can be readily grown in artificial nutrient medium, viruses are obligatory intracellular pathogens and their isolation and propagation are totally dependent on the availability of a live host and permissive cell culture. In the *in vitro* methods of cell culture viral identification is based upon the microscopic demonstration of cytopathic (due to cell lysis) or syncytial formation (produced by cell-cell fusion), or detection of fish antibodies against the pathogenic viruses by means of the viral neutralization test (LaPatra, 1996; Chi *et al.*, 2005). Study of obligate intracellular bacterial and protozoan parasites also been reported with the help of fish cell lines (Lee *et al.*, 2006).

    b. **Quarantine programme:** Cell culture plays a pivotal role in many quality control programmes involving fish health management aspects. Developed cell lines can be used in national and international quarantine and certification programme for procuring virus free stock.

    c. **Vaccinations and Immunostimulants:** *In vitro* studies help to standardize the immunization challenge protocols in fishes unlike *in vivo* experiments which show difficulty in obtaining reproducible results due to the interferences between immunocompetence, stress, and other environmental factors. Cell lines are used for the production of killed and live viral vaccines. With regards to DNA vaccine, several fish cell lines have been used for the selection of appropriate DNA vectors and reporter genes, to facilitate the transfection of the fish cells (Anderson *et al.*, 1996). Recently, Kumar *et al.*, 2008 have reported the use of cell line for the development of DNA vaccine.

    Cell culture based techniques are also used to investigate the cellular mechanisms such as proliferation of lymphocytes, activation of macrophages and granulocytes etc. of various immunostimulants used in aquaculture. Role probiotics in suppressing inflammation and reducing cancer incidence were recently studied using a fibroblast cell line (SAF-1) and an epithelioma cell line (EPC) (Salinas *et al.*, 2008).

2. **Fish immunology:** The development of clonal leukocyte cell lines from channel catfish, *Ictalurus punctatus*, have provided the body of knowledge necessary to establish the existence of T, B, natural killer, and immune accessory cell in teleosts (Warr, 1997). In addition, it has wide application in fish immunology which includes testing of antibody secreting cells, large scale production of monoclonal antibodies, lymphocyte proliferation, secretion of cytokines and study of immunomodulatory effects of many antibiotic drugs using leukocyte culture etc., (Bols *et al.*, 2001). Likewise, cell culture has been an indispensable tool to study presence and functions of fish cytokines. Interferon activity was the first cytokine demonstrated in fish using viral-infected cultures of the RTG-2 cell line (de Sena and Rio, 1975).

3. **Cytogenetics and Biotechnology:** Fish cell culture has wide application for determination of karyotypes and other aspects of cytogenetics. Cell culture method helps to select the appropriate genes and methods for the production of transgenic organisms, optimization of new vectors for expression & cloning of recombinant proteins.

   Similar to other vertebrates, piscine pluipotent embryonic stem (ES) cell cultures provide an *in vitro* system for the investigations of extracellular factors affecting survival, growth and differentiation of neuronal cells. Embryonic Stem like cells from Zebrafish (Collidi *et al.*, 1992; Ghosh *et al.*, 1997), Medaka (Wakamutsu and Ozato, 1994), Red sea bream (Bejar and Hong, 2002), Flatfish (Holen and Hamre, 2003) etc. have been developed for the *in vitro* study of early stages of cell development.

   Another growing application of cell culture in an industrial setting is the production of large quantities of recombinant proteins for pharmaceutical or other industrial uses. Fish cell lines have been widely used for rapid screening of many recombinant proteins for its biological action rather than going directly for *in vivo* testing (Gagne and Blaise, 2000; Morita *et al.*, 2003). In this way, the cells expressing a recombinant protein become a valuable asset for the production of the commercial products.

4. **Cellular and physiological mechanism:** Cell lines forms an *in vitro* model for investigating several aspects of hypothalamo- hypophysial systems and the factors involved in their regulation. For example, organ culture of pituitary glands and cell lines derived from tilapia, eel, carps and rain bow trout have been used for the biosynthesis of growth hormones (Bols *et al.*, 1995) and gonodotropic hormones (Ge *et.al.*, 2003). Nutritional and biochemical studies of lipids, metabolism of Poly Unsaturated Fatty Acids and other biomolecules are also being carried out with the help of established cell lines (Tocher and Dick, 2000; Ghioni *et al.*, 2002). Recently, Hirayama *et al.*, (2008) has reported a micro array analysis on Medaka cell line elucidating transcriptional responses associated with temperature dependent changes in fish metabolism.

5. **Toxicological studies:** Aquatic animals have provided models for many toxicological studies, and are good bioindicators of environmental pollution. Cell lines derived from aquatic organisms can be used as a better alternative to whole living organisms in

eco-toxicological studies. Rapid and short term *in vitro* bioassay helps to screen vast number of chemicals, pesticides, heavy metals and radioactive compounds for their potential eco-toxicity. Rachilin and Pearl Mutter (1968) first suggested the use of cultured fish cells for the acute *in vitro* cytotoxic assays of aquatic pollutants. *In virto* assays have been further developed and applied by using various cell lines like RTG-2, rainbow trout gonad cell line (Riva *et al.*, 2005), the gill cell line (FG-9307) of the marine fish *P. olivaceus* (Li and Zhang, 2001), the hepatoma cells (PHLC-1) of the fish *Poeciliopsis lucidas* (Zurita *et al.*, 2007; Louiz *et al.*, 2008). Recently, potential risk of manufactured engineered nanoparticles, its intrinsic genotoxic and cytotoxic potential have been studied in rainbow trout gonad (RTG-2) cell lines (Vevers and Jha, 2008). This cell line has also been found to be more sensitive than standard mammalian cells (i.e. Chinese hamster ovary or CHO cell line) for different genotoxic endpoints (Raisuddin and Jha, 2004). Thus, the use of fish cell lines in toxicology and ecotoxicology is relatively broad and several reviews have been published on the topic; Bols *et al.* (2005) and Schirmer (2006). Fish cell lines provide best live medium to evaluate cellular level changes and morphological alteration due to potential enviornmental pollutants.

CONCLUSION

Fish cell lines are as amenable to the modern cell culture techniques as mammalian cell lines. However, this vast resource, comprising thousands of vertebrate species, has not been used to its full potential. Similar to other vertebrate cell culture, they can be propagated for a limited number of times, as primary cell culture or indefinitely as immortal cell lines. Cell culture generates a continuous source of experimental material with well reproducible results due to its homogeneity. Initially, fish cell lines were used to study piscine pathogens and disease diagnosis. By now, research relying on fish cell culture, ranges from cellular and molecular understanding of fish cells to the development of a suitable model for eco-toxicological risk assessment of heavy metals, hazardous chemicals and radioactive compounds. In 21st centaury fish cell culture hold the great potential to serve as living factories for large scale production of viral vaccines, antibodies, recombinant proteins and pharmaceutical compounds. Finally, it provides the best live medium for much valuable research and avoids the legal, moral and ethical problems of animal experimentation.

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