Chapter V

DISCUSSION

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Groupers are one of the major economically important cultured fishes in Southeast Asia. Since honeycomb grouper were traded in live condition throughout the world for food and ornamental purpose, translocation to new environments has the potential to introduce exotic diseases. This increases the likelihood of disease transmission to other species in different regions of the world and threatens the balance of ecosystems. The lack of an established cell culture system for honeycomb grouper may have resulted in the limited experience with viral isolation and identification. In the absence of susceptible cell culture systems, scientists must rely on other methods and techniques, such as electron microscopy and bioassays, which are not as simple and easily reproducible as in vitro cell cultures. Therefore, there is a need to establish honeycomb grouper cell lines from various organs and tissues in order to provide an enhanced capability for viral detection and identification in this aquatic animal species.

The in vitro cell culture would address some of the key elements identified as strategies for integrated health management in aquaculture,
including the standardization and validation of diagnostic methods, development of new safe therapeutants, and the implementation of effective disease control methodologies. It is expected that any long-term rise in seafood production will depend on the future progress of aquaculture. However, the development of aquaculture faces a number of problems, of which diseases, particularly the emergence of new viral pathogens represent serious risks for the production of aquatic animals. The complex interactions underlying disease outbreak and progression may be better studied using in vitro models that use cell culture methods and experimental systems.

The present work describes the development and characterization of cell line derived from fin of a cultivable teleost - honeycomb grouper, *Epinephelus merra*. Although the attempt was aimed at establishing primary cell culture from multiple tissues and organs including caudal fin, heart, swim bladder, kidney and spleen, we were able to establish a cell line from caudal fin only. In our earlier studies we found that the fin tissue was better compared to the other tissues to develop cell line (Swaminathan *et al.*, 2010 and 2011) due to its faster regenerative capability.

With no cell line available from honeycomb grouper *E. merra*, our current research sought to establish cell lines from different tissues of this species. Specifically, we had established a new cell line, Honey comb grouper (HGF) derived from caudal fin tissue by explant method. This cell line has been continuously passed over 35 times since this establishment in 2009. This newly established cell lines grow optimally in L-15 medium at 25 to 30°C with 15% FBS.

**5.1. Development of primary culture**

There have been several continuous cell lines developed using the explant procedure (Chang *et al.*, 2001; Dewitte-orr *et al.*, 2006; Dong *et al.*,
2008; Wei et al., 2010 and Swaminathan et al., 2011) this method was also particularly successful in the present study. It was difficult to get tissue fragments to adhere while establishing cell lines through explant method (Wolf and Quimby, 1969). It was likely that the flask substrate required treatment to encourage attachment of the tissue fragments, for example with serum or other attachment factors. Freshney (2005) mentioned that plasma clots were used historically to increase attachment of explants. Here, we had used fetal bovine serum (FBS) for the adherence of tissue fragments. Confluent cell cultures are readily prepared with this simple planting of fragmented fish tissues, but the cell sheets are less uniform and develop somewhat slower than those started from trypsinised tissues. Minced tissue cultures are suitable for virological applications and for initiating fish cell lines. Because of the simplicity in establishing cultures, some researchers prefer this method over trypsinization. (Wolf and Quimby, 1976). Cell lines established by means of the explant technique, which has many advantages over the use of cell suspensions: speed, ease, maintenance of cell interactions and the avoidance of enzymatic digestion which can damage the cell surface (Avella et al., 1994).

In this study, unfortunately, the trypsinised cells that did adhere and exhibited outgrowth did not produce a culture. These cells did not survive for a substantial amount of time and hence were never subcultured. It may be that the conditions for growth were simply unsuitable, or that the cells were not in a favourable environment for survival, therefore cell death occurred relatively quickly. Another possible explanation may be that there were too few cells. To an extent cell proliferation is regulated by signals from the environment and optimal cell densities. Low cell densities leave cells with free edges and allow them to spread but the cells will not divide. High cell densities inhibit growth through cell to cell contact and the resultant change in shape of the cell (Freshney, 2005). However this does not guarantee that a
low density of cells would promote growth and high density inhibit it. Low cell densities in culture can prove to be inhibiting to growth rather than stimulatory.

5.2. Cell culture from different tissues

Cultures derived from swim bladder, kidney and spleen tissues, depending on treatments, demonstrated variable degrees of cell attachment and spreading. Some of the cultures were subcultured once but then failed to show any evidence of cellular replication and were eventually discarded. While cultures derived from splenic tissue of *E. merra* appeared to have cells attached and survived in culture for 55 d. Whether this inability to thrive was due to the relatively small amounts of tissue initially cultured or to the use of unsuitable culture medium, was not clear. The spleen cells appeared to be ‘healthy’ throughout the incubation period and thus it would seem worthwhile to attempt initiation of further cell cultures using larger amounts of splenic tissue. The cells isolated from the spleen appear to be mainly leukocytes. This is because spleen plays an important role in the immune system of a fish. It traps antigens and houses proliferating lymphocytes (Horton and Ratcliffe, 1993). It also holds large numbers of red blood cells which is evident from the large numbers found in culture after isolation (Roberts and Ellis, 2001). Parameswaran et al. (2006c) demonstrated that the cells migrated from the spleen tissue fragments formed monolayer within 15 days. Flano et al. (1998) have established spleen cell cultures from rainbow trout by explant culture technique and further they also observed that the area of tissue with a frame work of reticular cells, leukocytes, extracellular matrix and sinusoids containing blood cells.

Contamination was the most significant problem when attempting to culture kidney tissue. Despite extensive washing of the excised tissue, using antibiotic-supplemented media, cultures were overcome did not survive due
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to bacterial contamination. Wang et al. (2011) used antibiotics (1000 U per ml penicillin, 1000 U per ml streptomycin) for the transport of kidney tissue to avoid the microbial contamination of cell culture of southern flounder, *Paralichthys lethostigma*. Dannevig et al. (1995) established SHK-1 cell line from head kidney of Atlantic salmon for the isolation of Infectious Salmon Anaemia virus. Head kidney is a haematopoietic organ in several fish species, analogous to bone marrow in mammals and harbour number of microbial pathogens (Ciba et al., 2008). They found that along with the first subcultures and accompanied removal of remaining tissue pieces the fibroblastoid cells of head kidney primary culture were increasingly replaced by cells with different morphologies like polyonal-shaped cells and giant cells.

Cell cultures derived from explanted heart tissue appeared to have sufficient numbers of cells but these did not attach to the substrate and all cultures were discarded after 13 d incubation. It is unclear why the cellular morphology in the heart cultures ‘changed’ after the tenth passage from an epithelial-like morphology to a fibroblastic-like appearance. Whether a subpopulation of fibroblast-like cells outgrew the epithelial-like cells or whether a morphological transformation occurred could not be determined. The spontaneously contracting heart cells were not anticipated. There has been no report of this phenomenon occurring in fish primary culture previously. Wolf and Quimby (1969) did report a swim bladder culture exhibited peristaltic–like movements in rainbow trout. It does not appear to be an unusual occurrence for mammalian heart cells to exhibit a contractile ability in culture (Satin et al., 2004). Heart muscle cells are myogenic meaning that they stimulate their own contraction without the need of any neuronal input. A single heart muscle if left without input will contract at a steady rate as well as stimulating any other cells in contact to contract. The contraction of cardiac muscle in vertebrates is activated by an increase in
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intracellular Ca\textsuperscript{2+} either from the sarcoplasmic reticulum or extracellular fluid (Randall et al., 1997). This may explain how cells within culture may exhibit spontaneous contraction. The media contained enough Ca\textsuperscript{2+} ions to cross the plasma membrane and increase intracellular levels hence stimulating contraction.

There was success with the fin explants. The fin fragments attached and quick outgrowth of cells was observed. However contamination was so severe, even after treatment with antibiotics, that the cells were overwhelmed. As a result the cultures had to be discarded. The cells of fin will regenerate quickly when compared with other tissues and therefore, when isolated from tissue, are much more likely to proliferate. This was seen within the cultures before contamination overwhelmed the cells. Cells outgrew quickly and new growth was visible twenty-four hours after isolation.

We used FBS as an attachment factor for the tissue explant to the substrate of the culture vessel. There was past evidence of basic improvements to the culture vessel substrate in an attempt to increase attachment of cells. Serum was used to coat a tissue culture vessel to enhance attachment of tissue fragments (Lakra et al., 2006a; Lakra et al., 2010a; Swaminathan et al., 2010 and Swaminathan et al., 2011). Butler and Nowak (2004) also demonstrated that some attachment factors were inhibiting the cell growth. Bols et al. (1994) investigated the use of several attachment factors finding that only collagen IV improved cell adherence. The success of attachment factors appears to vary from organ to organ and investigation may be required for every fish species studied. As the attachment of cells is one of the first obstacles to overcome when developing a culture it can prove important to primary isolation.
Most continuous fish cell lines have been developed in trypsinization method (Bejar et al., 1997). But in this study, trypsin dissociation of the tissues yielded individual cells with poor attachment. However on incubation of the harvested cells, cell attachment to the culture flasks was very poor and monolayer formation was not observed. This could be due to the fact that as a result of trypsin treatment, the cell surface receptors facilitating attachment might have been destroyed (Hashimoto et al., 1997). There are numerous variations to the basic method of trypsinization and each attempt at isolating cells from a new species and/or organ will require a certain amount of optimization (Wolf and Quimby, 1976).

5.3. Contamination of cell culture

In this study, nearly 62% of the explants were contaminated due to microbial pathogens and it was noticed by turbidity and colour change of the medium, floating of fungal colonies growth, etc. Cell line contamination is a major drawback of main cell banks of the world and it has cost of losing important biological products or valuable research. The causative agents are different chemicals, invertebrates, bacteria, fungi, parasites, viral species and even other cell lines. Contamination is not unusual, as it is difficult to decontaminate external tissue of fish unless very strict procedures are followed before isolation is attempted. Although the tissue was thoroughly washed in ethanol and antibiotics before disaggregation, it was not enough to fully eliminate the bacteria which caused the contamination. Further attempts like good laboratory practices may require removing or reducing contamination to a manageable level. Mirjalili et al. (2005) recorded that the major contaminating agents were mycoplasmas (19%) followed by mixed infection (8%), fungi (8%) and bacteria (4%) and also further found that among various bacterial species such as (except mycoplasmas) Bacillus sp., Enterococcus sp. and Staphylococcus sp. are main bacterial agents and
among various fungi Aspergillus sp., Penicillium sp., Sepedonium sp. and Botrytis sp. were main fungal causative agents of CGBRI cell line contamination.

The bacterial and fungal (including molds and yeast) contamination of cell lines (except mycoplasmas) can be readily detected, as these organisms cause increased turbidity, shift in media pH (change in medium colour) and cell destruction, but in the case of mycoplasmas their cell line contamination is always undetected for many passages (Ryan, 1998). They can proliferate within the cell, tolerate antibiotics and their growth always does not have any obvious microbial evidence like turbidity and pH changes or cytopathic effect. Mycoplasma contamination also affects function, growth, metabolism, morphology, attachment, membranes, chromosomal aberrations and so many other properties of cell lines. Their contamination also spreads quickly to the other cell lines.

5.4. Subculture

Primary cultures were subcultivated when they attained the confluency, in order to develop a cell line and also to achieve reproducible behaviour of the cells; and to monitor the cells, routine subculture was performed in regular intervals. A confluent culture was considered to be that in which all cells are in contact with other cells around their periphery and no available substrate is left uncovered (Freshney, 2005). The seeding density was increased when the cells have not reached a high-enough density by the appropriate time. The intervals between medium change and subculturing varied from one cell line to another, depending on the rate of growth and metabolism; rapidly growing transformed cells lines were usually subcultured once per wk and the medium was changed after four d (Freshney, 2005).
To keep the cells in a healthy environment, there was a medium change whether it was a primary cell culture or a subculture of a cell line. The replacement of the medium was indicated by the drop in pH of the medium in the flask, high cell concentration, cell type and morphological deterioration such as granularity around the nucleus, cytoplasmic vacuolation and rounding up of the cells with the detachment from the substrate.

It has been demonstrated that cell survival and growth can be improved by the presence of feeder layers or conditioned media which contain substrate-modifying constituents, growth factors as well as other metabolites (Freshney, 2005). It provides the missing components that may encourage growth and sustain cultures. This might explain why when the explanted tissue was removed or detached after washing, cell death occurred shortly after. The use of a feeder layer of cells or conditioned media may have increased the survival time and/or encouraged the cells to grow further into a confluent culture.

5.5. Growth conditions

After isolation cell survival and growth is influenced by environmental factors such as media, temperature, etc. The investigations within this project into temperature did demonstrate that temperatures above 20°C are not suitable for cold water fish. Most established cell lines developed from cold water species are grown at 15-20°C (Fernandez et al., 1993). Cells and tissues should be cultured at a temperature similar to the environmental temperature preferred by the donor species. Cells from coldwater species such as salmonids can grow at temperatures as low as 4°C their optimum is near 20°C but prolonged incubation at much above 25°C is apt to be somewhat inhibitory or even lethal. Cells from warm-water species can be grown at higher temperature; goldfish cells, for example, can be
incubated at 37°C but incubation at 15°C or lower markedly inhibits mitosis. Once the cells or tissues have been established in culture the use to which they are to be applied dictates incubation temperature (Wolf and Quimby, 1976).

A complete growth medium consists of a basal cell culture medium supplemented with ingredients such as sera, growth factors, trace elements, and hormones. Cell culture media are complex mixtures of salts, carbohydrates, vitamins, amino acids, metabolic precursors, growth factors, hormones, and trace elements. The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Carbohydrates are supplied primarily in the form of glucose. In some instances, glucose is replaced with galactose to decrease lactic acid build-up, as galactose is metabolized at a slower rate. Other carbon sources include amino acids (particularly L-glutamine) and pyruvate.

Wolf and Quimby (1962) discussed that the conventional physiological solutions and culture media intended for mammalian or avian work are wholly appropriate without modification for use with fresh water teleost cells and tissues. The culture of cells from fish has followed a similar process to that in warm-blooded animals and hence the types of media used are similar; L-15 or MEM (Earle’s, Hank’s, or Glasgow) being the most widely used. So in this study, Leibovitz 15 (L-15) with 15% FBS added was the best media for cell survival and growth. This is in agreement with other published studies where cells grown in EMEM and L-15 performed the best (Fernandez et al., 1993; Tung et al., 1991). The principle difference between media types are based on concentration of salts and variations in the amino acids present as well as the amount. For instance EMEM is high in bicarbonate whilst H-MEM is low. L-15 has higher levels of sodium pyruvate but lacks bicarbonate.
Levels of bicarbonate can have an important influence. Divalent cations e.g. Ca\(^{2+}\) are required by some adhesion molecules and can also influence whether cells proliferate or differentiate (Freshney, 2005). Bicarbonate also plays a role in pH especially with the low cell numbers found in primary cultures.

In addition to nutrients, the medium helps maintain the pH and osmolality in a culture system. The pH is maintained by one or more buffering systems; CO\(_2\)/sodium bicarbonate, phosphate, and HEPES are the most common. Sera will also buffer a complete medium. Phenol red, a pH indicator, is added to medium to colorimetrically monitor changes in pH. Most cultured cells have fairly wide tolerance for osmotic pressure. The osmolality of cell culture media for most vertebrate cells is kept within a narrow range from 260 to 320 mOsm/kg, even though most established cell lines will tolerate a rather large variation in osmotic pressure. In contrast, the osmolality requirements for some invertebrate cell lines fall outside of this range. For example, the snail embryo (ATCC® CRL-1494™) requires medium of about 155 mOsm/kg, while some insect cells prefer 360 to 375 mOsm/kg (Waymouth, 1970). The osmolality most commercially available tissue culture media will be kept within a narrow range from 260 to 320 mOsm/kg. So we have used Leibovitz 15 (L-15) (Invitrogen, USA) for the growth of cells from different tissues of E. merra.

In this study L-15 supplemented with 15% FBS was sufficient for survival and growth so there was no further need to refine the media. It was important to note that although in this case the serum used was sufficient for survival and growth, testing of different serum batches could play a role. Based on our experience in this study, HGF cell line grew well upon a particular serum batch whilst cell line will show poor or little growth on the same serum batch. Investigating different serum batches as done with
established cells lines may have improved growth in those cultures which exhibited lower growth rates.

The cells that attached and proliferated did not demonstrate any of the normal indicators of stress i.e. vacuoles, granulation or clumping so it was assumed that the media did not create unfavourable conditions for their survival. This however does not guarantee the optimum conditions for growth. Further development of specifically designed media for fish cell lines is rare as currently existing media are generally adequate. There is no need to refine media until a cell line has been established and growth needs to be optimized. There have been some cases were media has been specially developed during the primary culture stage. However, the basis of the media was still E-MEM and cultures were still isolated although attachment and growth was to a lesser extent (Kumar et al., 2001).

As discussed earlier, cells rely on the external environment to an extent to stimulate growth. It is possible to encourage cells to proliferate by simulating these signals. However, any growth factors available commercially are primarily developed for mammalian cell culture. Although investigations into the use of bovine growth factors, basic fibroblast growth factor (bFGF) on HGF cells did not produce any significant increase in primary cell growth. bFGF is a potent mitogen for embryonic stem cells derived from sea perch (Chen et al., 2003a), lymphoid cells from grass shrimp Penaeus monodon (Hsu et al., 1995), embryonic cells from Japanese flounder Paralichthys olivaceus Temminck & Schlegel (Chen et al., 2004). Ye et al. (2006) found that the bFGF was found to stimulate proliferation of various cells from sea perch. It can be suggested that there fish-based growth factors may stimulate growth of fish cell lines when compared to the growth factors of bovine origin. So far no specific fish based growth factors have been developed.
Growth factors are primarily used as a replacement for factors in serum when serum-free media is required. Under normal circumstances, serum provides the necessary growth factors and so addition of further growth factors has no significant effect. Attempts to use fish serum as a growth stimulate were unsuccessful. The fish serum was toxic to the cells. Past studies also found fish serum to be inhibitory or toxic (Wolf and Quimby, 1969). While serum contains growth factors, it can also contain other substances which are cytostatic (Freshney, 2005). There has been use of specially modified media to increase growth of isolated cells (Kumar et al., 2001). Hashimoto et al. (1997) explained that fish serum in combination with FBS is extremely effective for investigations of fish cell growth and differentiation, especially in utilizing primary culture cells. Unfortunately growth factors will not change the fact that the majority of cell lines developed are finite. Media, growth factors and other supplements will simply optimize the conditions for growth. Conditioned media and feeder layers do provide benefits in keeping a cell monolayer viable which is useful for some types of testing e.g. metabolic studies (Zhao et al., 2003).

5.6. Transformation of cell line

None of the primary cultures that developed into confluent cultures gave rise to a continuous cell line. This is not surprising as most normal cells will not and they require transformation to become ‘immortal’ (Wolf and Quimby 1962). Although the cells appeared to proliferate between subculturing, there were a limited number of passages before proliferation ceased and senescence occurred. Freshney (2005) detailed that most finite cell lines have twenty to eighty possible population doublings before senescence. As most reported cell lines are not available commercially, it is impossible to determine whether these cell lines are transformed or have a limited number of passages too.
Immortal cell lines can either be derived from carcinomas or they can be generated through exposure to various agents (viral, chemical, or physical). Cells can transform spontaneously in vitro, however transformation can also be chemically or virally induced (Lannan et al., 1984). Hence the attempt to induce transformation by exposing cultures to a mutagen. There is little evidence of this being attempted in fish culture as most transformation appears to be spontaneous. Takarada et al. (1989) reported transforming a fin cell line with the mutagen MNNG (N-methyl-N-nitro-N-nitrosoguanidine). After two exposures to MNNG the cells exhibited loss of contact inhibition and increased growth rate and this method was not common as toxic nature of the chemical. Guo et al. (2003) reported spontaneous transformation of a flounder cell line occurred at around passage 171. The cells became neoplastic and formed spherical cell masses. There was no such success with this project either by using 4NQO as the chemical mutagen or through spontaneous transformation.

Several other methods exist for immortalizing mammalian cells in culture. Viral genes, including Epstein-Barr virus (EBV), Simian virus 40 (SV40) T antigen, adenovirus E1A and E1B, and human papillomavirus (HPV) E6 and E7 and through expression of the telomerase reverse transcriptase protein (TERT) by hTERT/oncogene plasmids can induce immortalization by a process known as viral transformation. Although the process is reliable and relatively simple, these cells may become genetically unstable (aneuploid) and lose the properties of primary cells. For the most part, these viral genes achieve immortalization by inactivating the tumor suppressor genes that put cells into a replicative senescent state (Takakura et al., 1999).

5.7. HGF cell line

In the initial passages, HGF cells were composed of a heterogeneous mixture of fibroblastic-like and epithelial-like cells. After 15 subcultures, they
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were predominantly fibroblastic-like cells. Too many passages and excess trypsin digestion could adversely affect the biological characteristics of cells, especially the hereditary characteristics (Wolf and Quimby 1976); hence we restricted the passages of HGF cells up to 35 and then cryopreserved at 196°C in liquid nitrogen. The morphological results indicated that there were both epithelial-like cells and fibroblastic-like cells present during the primary and early passages of the HGF cells. Different tolerances to trypsin were evident as the fibroblasts fell off first when treated with trypsin and were adherent again quickly after passage, while most epithelial cells were not adherent or stably adherent and fell off only when treated with mechanical agitation (Ren et al., 2002). This could be a possible reason for the homogenous population of fibroblastic-like in the RTF cells after 15 passages. A similar morphological change has also been observed in orange spotted grouper, Epinephelus coicoides fin (GF-1) cells (Chi et al., 1999), and yellow grouper, Epinephelus awoara fin (GF), and heart (GH) cells (Lai et al., 2003), Asian seabass fry, Lates calcarifer (Bloch), (SF) cells (Chang et al., 2001), L. calcarifer (Parameswaran et al., 2006a), and Catla catla and Labeo rohita (Ahmed et al., 2009b), Puntius denisonii (RTF) cells (Swaminathan et al., 2011) The change could be due to a common serum-derived factor present in all sera, which has strong mitogenic effect on fibroblast cells thereby inhibiting the epithelial cell proliferation as reported in other animal cells (Freshney, 2005).

Optimal growth temperature for HGF cells ranged 26–30°C which was identical with other fish cell lines reported previously (Swaminathan et al., 2010). However, maximum growth was obtained at 28°C. HGF cells were also able to spread and grow well at 20°C, although it took 48 hours for the cells to become well attached When incubated at 30°C, the cells proliferated fast during the first 48 hours, but the growth and proliferation slowed
dramatically, and the cells appeared to age rapidly. Although cells remained viable during the test period when incubated at 37°C, cell growth was minimal and individual cells looked abnormal. Culture morphology changed at different maintenance temperatures.

In a previous study, after a week at the temperature extremes, 5 and 36°C, monolayers of PBLE remained intact, although monolayer appearance changed subtly. With time at 5°C, the cells became shorter and rounded, whereas at 30 and 36°C the cells became more epithelial-like (Dewitte-orr et al., 2006). Cell lines from coldwater fish proliferate at temperatures as low as 5°C and as high as up to 24- 26°C (Fryer and Lannan, 1994). Cell lines of warm-water fish generally grow optimally around 30°C (Chen et al., 2003a). Also, as pointed out by several authors (Kang et al., 2003), cell lines that grow over a wide range of temperatures are potentially valuable for isolating and studying both coldwater and warm-water fish viruses. Thus, HGF may prove to be a valuable tool for understanding many specific characteristics of temperature tolerance, cell death pathways and antiviral mechanisms.

The growth rate of HGF cells increased as the FBS proportion increased from 2 to 20% at 28°C. Cells exhibited poor growth at 5% concentrations of FBS, relatively good growth at 10% but maximum growth occurred with the concentrations of 15 and 20% FBS. In this study, it was noted that the growth of cultures required that the L-15 medium be supplemented with fetal bovine serum (FBS). Both cell attachment and proliferation were influenced by FBS. At lower cell densities, cells attached similarly, with or without FBS; however, at higher cell densities FBS aided in cell attachment. With time, cell number increased slightly or not at all in cultures with L-15 alone. Dewitte-orr et al. (2006) explained that the PBLE cells proliferated better with more FBS when tested with a range of 10 to 20% FBS. However, in this study a 15% concentration of FBS also provided
relatively good growth, and this was an advantage in maintaining the cell line at low cost.

Newborn calf serum alone or in combination with FBS had growth-promoting effects on fin cells in early passage and was effective as same as FBS on cell growth. The complementary effects of the combined use of NBCS and FBS might be due to some factors that are present or abundant in NBCS but not in FBS and vice versa. In this study, we observed morphologic changes in fin cells in early passage especially in the presence of NBCS. The findings are in corroboration with the earlier findings in goldfish fin cells when carp serum was used in combination with FBS (Hashimoto et al., 1997). Furthermore, primary cultures of cells from fish gills and kidneys were developed using serum from *Clarias gariepinus* (Rathore et al., 2001). Fujiwara et al. (2007) found that the red seabream (*Pagrus major*) fish serum promoted cell adhesion to and cell growth Chinese hamster ovary (CHO) cells on collagen-coated dishes. In another study, Nanda et al. (2009) suggested that the goat serum at 10% concentration can be used as effectively as newborn calf serum for routine culture of *Cirrhinus mrigala* cells. Newborn calf serum may contain the factor(s) that are responsible for the alteration of the normal cell shape of HGF cells. Our research implies that NBCS in combination with FBS was effective for investigations of fish cell growth especially in utilizing primary culture cells.

Ganassin et al. (1994) suggested that the standardization is the requirement and source of autologous, allogenic and/or xenogenic serum supplements or replacements in culture media for fish cells. A valid approach to solve this problem is to develop serum-free media. Such standardized media are available for some fish tissue cultures and cell lines used in immunology and toxicology (Segner and Cravedi, 2001). Some attempts to
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use a defined culture medium have been done in fish (Wang et al., 2011), but with little success for the long-term growth of the cells.

The HGF cells were cryopreserved at 10th, 20th and 35th passages. The cells were recovered after six months from storage and grew to confluency within 5 d. The average viability of the cells after cryopreservation was estimated to be 83% with the same morphology. Cryopreservation of cell lines is necessary for long-term storage. The viability of different cell lines was demonstrated that all the cells have the ability to survive following storage at -196°C by various workers (WSF, WSBM and WSHST cells – Wang et al., 2003; SISK- Hameed et al., 2006; LCF and LCE – Lakra et al., 2006a; RE and CB cells – Ahmed et al., 2009a; PSF cells – Swaminathan et al., 2010; RTG cells – Swaminathan et al., 2011).

Although these HGF cells remained viable following liquid nitrogen storage, their plating efficiency was rather low (<16%). Since general characteristics for non-transformation status of this cell line was further evidenced by their chromosomal typing, showing a diploid chromosomal count of 48 and which has been documented in the literature for this aquatic species (Ohno et al., 1974; Zheng et al., 2005).

Partial sequence information of 16S rRNA (590 bp) and COI (655 bp) were used to further confirm the origin of the newly established HGF by PCR. The amplified PCR fragment was sequenced and this DNA fragment matched perfectly with the genomic sequence of 16SrRNA and COI previously reported for E. merra (e values 0.0 and 0.0 respectively; NCBI Genbank accession numbers AY947629.1 and DQ107899.1). The mitochondrial 16SrRNA, 12SrRNA and COI gene sequence alignment has been used as reliable molecular methods to accurately identify the origin of cell lines of many fish species such as rohu (Ahmed et al., 2009a), grouper (Ku et al., 2009), pearl spot (Swaminathan et al., 2010) and redlined torpedo
(Swaminathan et al., 2011). The sequence data confirmed that the newly established HGF cell lines were derived from *E. merra*.

The ECPs from *Vibrio cholerae* MTCC 3904 proved to be cytotoxic for HGF cell line. Cytotoxic effects could be observed within 10 h after inoculation. The morphological changes detected in these cell lines were rounding, detaching and finally monolayer destruction. The ECP from *Vibrio cholerae* MTCC 3904 was cytotoxic to the HGF cell line. Many fish cells have proven suitable for demonstrating the cytotoxic effects of fish pathogenic bacteria, such as members of the various genuses (Ahmed et al., 2009b; Ku et al., 2009; Swaminathan et al., 2011). The susceptibility of cell lines to viral infection is the basis for isolating and characterizing fish viruses. Nodavirus, a marine fish virus (the only fish virus reported from India) was tested on the HGF cell line but was not susceptible to the virus. No CPE was detected in HGF cells inoculated with the filtrate of the tissue homogenate from the field samples up to 2 weeks of observation and also even after 10 blind passages. Similarly no CPE could be detected in both BF-2 and FHM cell lines infected with the test samples filtrate even after 15 d of incubation and after 10 subsequent blind passages. The PCR testing of the samples revealed that all the samples were negative for VNN, iridovirus, VHS, IHN, IPN, KHV, BKD, *Aeromonas salmonicida*, *A. Hydrophila*, *Yersinia ruckeri* and *Vibrio cholerae*.

5.8. Virus detection and isolation

In the present study, all 93 grouper samples collected from Mandapam and Keelakarai showed negative results in screening of nodavirus and iridovirus by RT-PCR and PCR respectively. PCR technique could detect the viral nucleic acid even if there was very minimal copy of numbers of viral particle in the tissue (Bercovier et al., 2005). So these results suggested that there was no nodavirus and iridovirus infection in all the samples tested in this study during that period.
Viruses are the major cause of serious diseases associated with high losses for which no effective treatment is available. Two different viruses, iridovirus (Danayadol et al., 1995) and betanodavirus (Danayadol et al., 1996), have been identified as pathogens capable of causing high mortality in Thailand. These two kinds of viruses have also been reported in other marine cultured fishes from many countries in this region and other parts of the world. A recent review of betanodavirus infections in finfish indicated that the virus caused problems to finfish aquaculture in most parts of the world except the African continent (Munday et al., 2002). However, very little information is available about the occurrence of viral diseases in the groupers cultured in Indian waters, which can be due to the lack of appropriate cell lines. Cell culture methodologies are essential tools for the diagnosis of viral diseases in farmed fish (Wolf, 1988). Such techniques are mainly based on the use of fish cell lines sensitive to different viruses (Ahne, 1985), in which viral infection is primarily demonstrated by the cytopathic (due to cell lysis) or syncytial (produced by cell-cell fusion) effects. As the occurrence of cytopathic/syncytial effects may take several days, and in some cases no such effects occur under some culture conditions.

Scientists found that fish cell lines such as striped snakehead (SSN-1) (Frerichs et al., 1996) and grouper fin cell lines (GF-1) (Chi et al., 1999) gave good support to betanodavirus isolation and the Epithelioma papulosum cyprini (EPC) fish cell line was generally suitable for iridovirus isolation. At least two different marine fish viruses viz., betanodavirus and iridovirus have been isolated from diseased groupers using freshwater fish cell lines, SSN-1 and EPC inn Thailand (Kanchanakhan et al., 2005). Most iridoviruses cause CPE in cell cultures consisting of cytoplasmic inclusion bodies in focally infected cells followed by rounding and cell lysis (Ahne et al., 1989). Both PCR based detection and isolation of virus by in vitro assays did not reveal any viral infection in this study, indicating the absence of virus particles in
the tested samples collected from the South-east coast of India. It has been suggested that more surveillance for viral detection using both the molecular methods and isolation of virus by cell line should be carried out with more samples from a larger geographic area in all seasons.

Some pathogenic viruses are known to be organ- and tissue-specific, which makes the establishment of additional cell lines from different organs and tissues of a host species essential for proper monitoring of viral diseases (Luc Rougéé et al., 2007). In the absence of susceptible cell culture systems, other methods and techniques, such as electron microscopy and bioassays, may be relied upon for the diagnosis of viruses which are expensive and not as easily reproducible as in vitro cell cultures. So, the development of HGF cell line would be valuable for isolation of the virus in any disease outbreaks in *E. merra* and also for studying species-specific responses of the viruses at the cellular level.

In this present study, the general characteristics of HGF cell line was not matching with the properties of continuous cell lines such as reduced serum requirement, reduced density limitation of growth, high plating efficiency, aneuploidy, etc. Our findings suggest that the HGF cell line was not transformed in the passages for which they were tested. The cells in a primary culture such as HGF cell line in the present study may vary less from the original fish cells unlike a transformed cell, therefore the former is of greater use in providing details of the mode of infection and replication of viruses within the host fish (Freshney, 2005).

Further, short-lived or finite cell cultures also have their place for other types of research other than virological requirements. Cell culture has also been used for the detection of some intracellular bacterial fish pathogens, as in *Rickettsiae* spp. (Fryer and Lannan, 1996) and *Renibacterium salmoninarum* (McIntosh et al., 1997). Several cell lines, including,
hepatocyte and astrocyte primary cell cultures from different fish species, were used to study in vitro the elongation and desaturation of different polyunsaturated fatty acids (PUFA) (Villena, 2003). Short-term primary cultures and cell lines from fish tissues have provided successful cell culture systems for environmental toxicology (Segner and Cravedi, 2001). Cell culture methods can be helpful to select the appropriate genes and methods to obtain stable transgenic animals (Iyengar et al., 1996) and to search for vectors that may be used to obtain transgenic farmed fish (Du et al., 1992).

The potential of cell culture techniques and associated methodologies to provide tools and strategies for disease control in aquaculture has not been yet achieved, mainly due to the poor standardization of fish cell culture methods, and to the lack of suitable techniques to develop cell lines from shellfish. The HGF cell line developed in the present study could be used for many more application like isolation and diagnosis of intracellular pathogens, nutritional experiments, toxicological experiments, transgenic research in addition to the virological studies.

Cell cultures would be crucial for many more purposes. It is expected that the increasing economic importance of disease in aquaculture will impel the progress of cell culture methods for rational health management in aquaculture (Villena, 2003). So the HGF cell line could be used for diagnosis of infectious agents in the event of any disease outbreaks in grouper. Ideally, cell and tissue cultures are substitutes of whole-animal models, in which in vitro assays can be applied to address specific biological issues.

A major problem in fish cell culture is that there is a large number of fish species that are being used as research models. Many of such species live in different aquatic environments and show disparate physiological requirements, and there is a lack of knowledge of the nutritional and physiological requirements of fish and shellfish cells grown in vitro. Many
investigators in fish cell culture have borrowed mammalian cell culture techniques (Bols and Lee, 1991), and little research has been done on the specific requisites of fish/shellfish cells in culture such as the response of several cell lines from marine and freshwater fish to different culture media, variations of saline strength and osmotic pressure, and to the temperature of incubation (Fernandez et al., 1993).

Further research on the newly established HGF cell line regarding their biological properties and functions will facilitate the establishment of these cells as a permissive biological tool in the study of grouper viruses. After such establishment and full characterization (like cell morphology, Growth curve analysis, karyotyping, DNA content analysis, RNA and protein expression, Enzyme activity, antigenic markers, Species verification by Isoenzymology and DNA fingerprinting, Mycoplasma and other microbial detection, identification of cross- contamination, etc) this new cell line will be available to scientists all over the world for the advancement of research in this field. The combination of cell/tissue cultures and in vitro assays will reduce the variability of the in vivo responses (in animals), which are due to the unavoidable effects of stress, environmental influences, and genetic backgrounds of farmed fish and shellfish species.