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Establishment and characterization of a continuous cell line from thymus of striped snakehead, *Channa striatus* (Bloch 1793)

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Abstract The establishment and characterization of a continuous cell line from the thymus of air-breathing fish *Channa striatus* are described. The cell line, designated *C. striatus* thymus (CST), has been subcultured over 71 times and shows optimal growth at 28°C in Leibovitz's-15 (L-15) medium supplemented with 20% fetal bovine serum. The CST cells exhibited low plating efficiency which improved with increase in seeding density. The karyotype analysis revealed that CST cells have a normal diploid karyotype with $2n=40$. Partial amplification and sequencing of two mitochondrial genes, viz. 16S ribosomal RNA (rRNA) and cytochrome oxidase I, confirmed that the cell line originated from *C. striatus*. CST cells were successfully transfected indicating their potential application for expression of recombinant proteins. In immunocytochemical staining, CST cells showed characteristics of epithelial cells. These cells were sensitive to extracellular products of *Vibrio cholerae* MTCC 3904 as well as to heavy metal mercuric chloride. The CST cell line would be a useful tool in functional genomic studies such as RNA interference and gene knockout as well as for cytotoxicity studies.

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Introduction

Striped snakehead, *Channa striatus* (Bloch 1793), belonging to the family Channidae, is a popular and the most economically important species of the genus *Channa* (Talwar and Jhingran 1992). This species has an extensive natural distribution particularly in Southeast Asian countries and has many advantageous characteristics, notably high market price, hardiness, and high tolerance to adverse environmental conditions. In addition, its flesh has high nutritive value and is said to have wound-healing effect and recuperative attributes (Courtenay and Williams 2004). *C. striatus* is highly suitable for aquaculture due to its air-breathing habit and is considered as a candidate species for aquaculture (NBFGR 2011). One of the major objectives of fish culture is to breed and cultivate fish to limit the impact on wild populations. However, various infectious diseases tend to occur during aquaculture. In this context, easily manageable tools such as in vitro test systems and optimal cell growth parameters are necessary (Grunow *et al.* 2011).

In vivo bioassays using fish are often applied, but assays using whole fish are inconvenient, time consuming, difficult to reproduce and require sacrificing the organisms (Wang *et al.* 2004). The inherent economic and ethical constraints associated with tests on live animals have encouraged the use of fish cell lines (Ní Shúilleabháin *et al.* 2004) which seem to be potential surrogates for the whole fish (Wang *et al.* 2004). The fish cell lines provide a readily available, stable, cost-effective, and reproducible system for analyzing identical cells that genetically resemble animal tissues and species (Bols *et al.* 1994; Smith 2006) and, therefore, are valuable for

studying tissue- and species-specific responses at the cellular level to different stimuli (Dewitte-Orr *et al.* 2006; Higaki *et al.* 2013). Moreover, the cell lines allow cellular phenomena to be studied in a controlled and completely defined environment, independent of the complexities and variability of systemic or larger physiological controls (Bols *et al.* 2005). The cultured cells also have the potential to be used as nucleus carriers for fish bearing valuable phenotype or genotype or when their gametes or embryos are not available (Han *et al.* 2011).

Fish cell lines have been widely used as in vitro models in physiology, nutrition, virology, immunology, and functional genomics (Fent 2001; Wise *et al.* 2002; Ní Shúilleabháin *et al.* 2004; Ariel *et al.* 2009; Cheng *et al.* 2010; Buonocore *et al.* 2011). The susceptibility of fish viruses can be species-specific and this makes a cell line derived from a particular fish species more appropriate for isolation of viruses (Dewitte-Orr *et al.* 2006; Swaminathan *et al.* 2010). In addition, virus isolation using cell lines can detect emerging or previously unknown viruses for which molecular assays are yet to be developed. The cell lines also allow to produce a sufficient stock of virus for infectivity trials and vaccine production (Frerichs *et al.* 1996; Ariel *et al.* 2009). Furthermore, cell lines are useful tools to study the cellular and molecular basis of physiological processes such as analysis of function of important immune genes (Thompson *et al.* 1999 Dewitte-Orr *et al.* 2006; Wang *et al.* 2010). Permanent cell lines have been employed for screening and relative toxicity ranking of chemicals and environmental samples (Davoren *et al.* 2005; Schirmer *et al.* 2008; Tan *et al.* 2008).

The present paper describes the establishment of a cell line from the thymus of *C. striatus*, using the explant method. The developed cell line was characterized in terms of growth at different temperatures and varying concentrations of fetal bovine serum. Confirmation of species of origin, histological origin, karyotyping, susceptibility to bacterial extracellular products and heavy metal as well as expression of foreign gene was also assessed.

Materials and Methods

Preparation of tissue for primary cell culture.

Apparently healthy *C. striatus*, weighing 90 g, was procured from a local fish market. The fish was euthanized with an overdose of MS222 (Sigma-Aldrich, St. Louis, MO) and wiped with 70% alcohol. The thymus which is located on the dorsal body wall of the gill cavity (Fig. 1) was aseptically removed. The thymus tissue was washed thrice with phosphate buffer saline (PBS) containing 2× concentration of antibiotic-antimycotic solution (Gibco, Grand Island, NY) and transferred to a petri dish containing PBS with 1× concentration of antibiotic-antimycotic solution. The tissue was

Figure 1. Anatomical location of the thymus in *Channa striatus* (*arrow*). The operculum has been removed to reveal the thymus.

minced into small pieces (approximately 1 mm^3) with sterile scissors and transferred to a 25-cm^2 flask (Nunc, Roskilde, Denmark). The excess PBS was removed and 100 µl of heat-inactivated fetal bovine serum (FBS, Gibco) was added to facilitate the attachment of tissue explants to the surface of the flask which was incubated at 28°C. After 2 h, excess FBS was drained from the flask and 5 ml of growth medium, Leibovitz's-15 (L-15) medium supplemented with 10% FBS and 1× concentration of antibiotic-antimycotic solution, was added. About one third of the medium was replaced every 4th day till the radiating cells attained 90% confluency. After formation of a monolayer, the cells were trypsinized with 0.05% trypsin-EDTA solution (Gibco) and subcultured at a split ratio of 1:2 in growth medium.

Cryopreservation.

The subcultures were stored in liquid nitrogen after every 10th passage in the freezing medium, which consisted of FBS containing 10% dimethyl sulfoxide. After trypsinization, the cells were centrifuged at 1500*g* for 5 min. The pellet was resuspended in 1 ml of the freezing medium and the cell suspension was transferred to a cryovial (Nunc). The cryovial was kept at −80°C overnight in a Mr. Frosty Freezing container and finally stored in liquid nitrogen (−196°C). For revival, the cryovial was thawed quickly in a water bath at 28°C and transferred to a 25-cm^2 tissue culture flask containing about 10 ml of growth medium. The viability of the revived cells was estimated by trypan blue staining using a Neubauer hemocytometer. After overnight incubation, the medium in the flasks was replaced with fresh medium.

Effect of temperature and FBS concentration on cell growth.

Growth studies were carried out to determine the optimum temperature and FBS concentration for the *C. striatus* thymus (CST) cell line. A total of 1×10^5 cells ml^{-1} at passage 45 were inoculated into 25-cm^2 cell culture flasks and incubated at 28°C for 2 h to allow attachment of cells. Afterwards, the

batches of culture flasks were incubated at selected temperatures of 24, 28, 32, and 37°C for growth studies. This study was performed using growth medium. Every day, three flasks at each temperature were trypsinized to count the number of cells and the study was carried out for 4 d. A similar study was carried out to determine the effect of different concentrations of FBS (5, 10, 15, and 20%) on cell growth with passage 46 cells at 28°C.

Plating efficiency (PE).

The plating efficiency of the CST cell line was determined at passage 67 in 8.8-cm^2 tissue culture dishes (Nunc). The wells were seeded with CST cells at densities of 100 and 250 cells $dish^{-1}$ and cultured in L-15 medium at 28°C. Half of the medium was replaced every 4th day. After 10 d, the medium was discarded and cells were washed with PBS. Thereafter, the cells were fixed with methanol and stained with crystal violet. The individual colonies were counted under a microscope, and plating efficiency was calculated using the formula: PE (%)=number of cell colonies/number of cells seeded×100 (Freshney 2005).

Chromosome analysis.

The karyotype was prepared using the standard procedure with passage 64 cells (Freshney 2005). Briefly, the cells were grown in a 25-cm^2 tissue culture flask till 70–80% confluency was attained. Colchicine solution (Life Technologies, Carlsbad, CA) was added to the cells at a final concentration of 0.2 μg ml^{-1}. The flask was incubated for 2 h at 28°C. After trypsinization, the cells were collected by centrifugation at 200*g* for 5 min and treated with a hypotonic solution of 0.56% potassium chloride for 20 min. Thereafter, the cells were fixed in acetic acid/methanol solution (1:3) for 5 min at room temperature. Slides were prepared using a conventional drop technique and stained with 5% Giemsa solution. Chromosomes were observed and counted under a light microscope.

Confirmation of the origin of the cell line.

The origin of the developed CST cell line was authenticated by partial amplification and sequencing of two mitochondrial genes, viz. 16S ribosomal RNA (rRNA) and cytochrome oxidase I (COI), from CST cells following Swaminathan *et al.* (2010). Briefly, DNA was isolated from CST cells (5×10^6) at passage 42 as well as from the muscle of *C. striatus*, and fragments of the two genes were amplified by PCR using the published primers (Table 1). The thermal cycling conditions included an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 45 s, and a final extension of 5 min at 72°C. The amplified PCR products were sequenced in Applied Biosystems ABI 3730xl capillary sequencer through a commercial sequencing facility. The DNA sequences from the CST cell line were aligned with sequences from *C. striatus* muscle. The sequences for both the mitochondrial genes amplified from the CST cell line were also checked for similarity with existing sequences submitted in NCBI GenBank.

Transfection.

The CST cells at the 60th passage were seeded in a six-well plate at a density of 1×10^5 cells $well^{-1}$. After 24 h, the subconfluent monolayers were transfected with 2 μg of phrGFP II-N mammalian expression vector (Stratagene, La Jolla, CA) which contains a CMV promoter, bovine growth hormone polyadenylation signal, and a gene for neomycin/kanamycin resistance. The CST cells were transfected using SatisFection reagent (Stratagene), and the green fluorescence signals were observed under a fluorescent microscope 48 h after transfection (Qin *et al.* 2006).

Morphological confirmation with immunocytochemistry.

Immunophenotyping of the CST cell line was carried out following Mauger *et al.* (2009) at passage 47. Briefly, CST cells were grown on sterile coverslips for 24 h. The cells were subsequently fixed and permeabilized with methanol at −20°C for 30 min. For immunostaining, the coverslips were preincubated with PBS containing 1% bovine serum albumin (BSA) for 1 h at 37°C and then incubated overnight at 4°C with mouse anticytokeratin (pan), clone AE1/AE3 antibodies (Invitrogen), or mouse antivimentin antibodies (Invitrogen). In control coverslips, only PBS with 1% BSA was used in place of the primary antibodies. After PBS washing, cells were incubated for 1 h with rabbit antimouse IgG FITC conjugate (diluted 1:50 in PBS containing 1% BSA). The coverslips were washed again in PBS, mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA), and observed under a fluorescent microscope.

Table 1. List of mtDNA primers

Sl. no.	Primers		Sequence (5′–3′)	No. of bases	Reference
1	COI	F	TCAACCAACCACAAAGACATTGGCAC	26	Ward *et al.* (2005)
		R	TAGACTTCTGGGTGGCCAAAGAATCA	26	
2	16S rRNA	L	CGCCTGTTTATCAAAAACAT	20	Palumbi *et al.* (1991)
		H	CCGGTCTGAACTCAGATCACGT	22	

Cytotoxicity test.

Bacterial extracellular products.

The cytotoxicity of bacterial extracellular products (ECPs) from *Vibrio cholerae* MTCC 3904 was tested with CST cells. The ECPs from *V. cholerae* were prepared following Balebona *et al.* (1998). Briefly, 0.5 ml of a 24-h-old broth culture of *V. cholerae* was spread on sterile cellophane sheet, overlaying brain heart infusion agar plate, and incubated at 37°C for 48 h. Bacterial cells were harvested from the cellophane sheet with PBS. The cell suspension was centrifuged at l3,000*g* for 20 min. The supernatant was filtered through a 0.22-μm membrane filter and used as crude preparation of ECPs. The monolayers of CST cells grown in a 24-well plate using growth medium were inoculated with 0.1 ml of a twofold serial dilution of crude ECPs in triplicates. For negative controls, sterile saline was used in place of ECPs. Plates were incubated at 28°C and the effects of ECPs on the cells were observed up to 48 h.

Heavy metal.

Neutral red uptake assay was carried out to measure plasma membrane integrity in CST cells following exposure to different concentrations of mercuric chloride, as per Repetto *et al.* (2008). Briefly, wells of a 96-well plate were seeded with 100 μl of 1×10^5 CST cells ml^{-1} and incubated overnight at 28°C. Thereafter, the culture supernatant was removed and 100 μl of L-15 containing mercuric chloride (SRL, Mumbai, India) in increasing concentration (1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 μg ml^{-1}) was added to the wells in triplicates and incubated at 28°C for 24 h. In control wells, 100 μl of growth medium was added. Following washing with PBS, 100 μl of neutral red working solution (40 μg ml^{-1} of growth medium) was added to the wells. The plate was again incubated for 2 h at 28°C and then the neutral red solution was decanted. The wells were washed with 150 μl of PBS followed by fixation of cells by adding 5% glutaraldehyde for 2 min. Then, 150 μl of neutral red destaining solution was added to each well and the plate was kept on a plate shaker for 10 min. The optical density (OD) of the neutral red extract was taken at 540 nm in a microplate reader.

Results

Primary cell culture and subculture. The cells migrated out from the edges of thymus explants of *C. striatus* and formed a complete monolayer by 3 wk. Subsequently, the cells were split at a ratio of 1:2 and monolayer formation took about 10–11 d during earlier passages. After about 50 passages, the monolayer was formed in 6–7 d. The cells have been subcultured for 71 passages and the cell line has been designated CST cell line. Observation of radiating cells from the explants revealed cells with epithelial as well as fibroblastic morphology (Fig. 2). During the initial passages, there was a mixed population of cells: cells with long cytoplasmic processes which were quite prominent, and in between these cells, there were cells with epithelial morphology which appeared very light. After 40 passages, the epithelial cells became predominant with a few cells having long cytoplasmic processes.

Growth studies. The CST cells grew well at 28 and 32°C; however, the maximum growth was observed at 28°C. The cells did not grow at 37°C (Fig. 3*a*). Growth of the cell line in media supplemented with varying concentrations of FBS is shown in Fig. 3*b*. Faster growth of cells was observed with increasing concentration of FBS, with maximum growth in L-15 medium supplemented with 20% FBS. After 4 d of culture, the growth of CST cells was very slow in the medium containing 5% FBS than in the media containing a higher concentration of FBS.

Cryopreservation and plating efficiency. Revival of CST cells after 8 mo of storage in liquid nitrogen showed over 90% viability after thawing. The cells grew to form a monolayer in 5 d and did not show any change in morphology. Plating efficiency of CST cells was determined to be 2.5 and 6.4% at seeding concentrations of 100 and 250 cells, respectively, in 8.8-cm^2 tissue culture dishes. The efficiency was low but improved with increase in seeding density.

Chromosome analysis. The results of chromosome counts of 100 metaphase spreads from CST cells at passage 64 revealed that the number of chromosomes varied from 31 to 49 (Fig. 4*a*). However, the majority of CST cells (62%) had a modal chromosome number of 40. The metaphase spreads with normal diploid number of 40 displayed normal karyotype morphology (Fig. 4*b*).

Identification of the cell line. Amplification of cytochrome oxidase I and 16S rRNA genes from CST cells and *C. striatus* muscle yielded PCR products of approximately 654 and 569 bp, respectively (Fig. 5). Comparative sequence analysis revealed 100% identity among the COI genes from CST cells and *C. striatus* muscle, as well as with the known *C. striatus* mitochondrial DNA sequences available in NCBI GenBank. Similarly, the sequence of the 16S rRNA gene of CST cells showed 100% similarity with 16S sequence from *C. striatus* muscle and known mitochondrial DNA sequence available in GenBank. The sequences of COI and 16S rRNA gene of CST cells have been submitted to GenBank (accession numbers KP226701 and

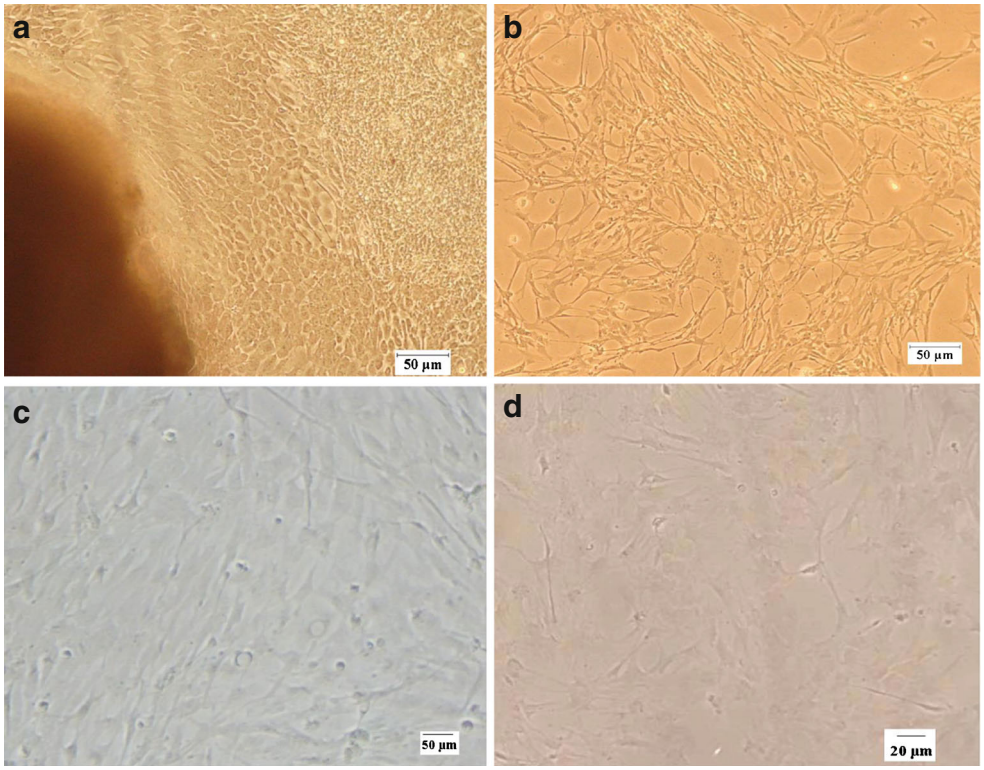

Figure 2. Photomicrographs of CST cells derived from *Channa striatus*. (*a*) Thymus explant showing migration of cells, (*b*) multiplying cells with large cytoplasmic processes during 5th passage, (*c*) monolayer of CST cells at 25th passage, and (*d*) monolayer of CST cells at passage 71.

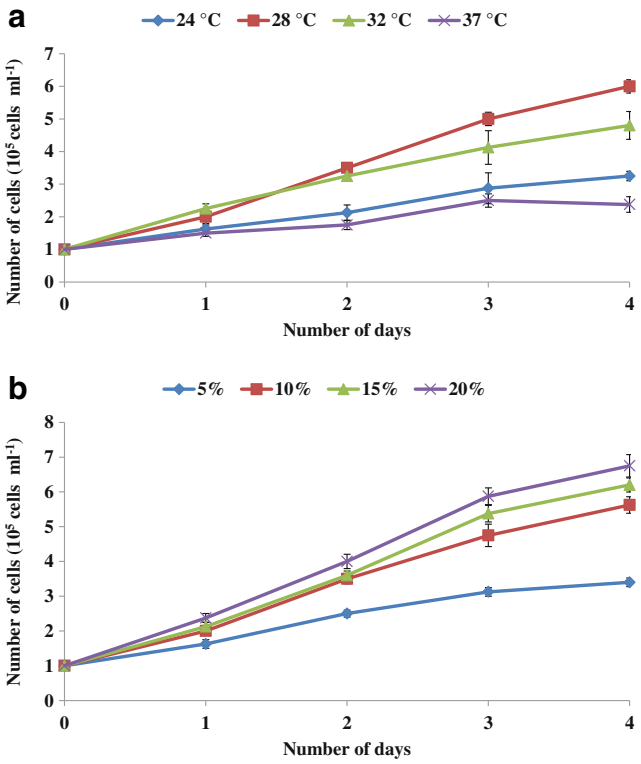

Figure 3. Growth response of CST cells at different temperatures in L-15 medium supplemented with 10% FBS at 45th passage (*a*) and in L-15 medium supplemented with different concentrations of FBS with passage 46 cells at 28°C (*b*).

KP226702). The data confirm that the CST cell line is derived from *C. striatus*.

Cell transfection. CST cells were successfully transfected with phrGFP II-N vector. The expression of GFP in the form of clear and strong green fluorescence signals was observed 48 h after transfection (Fig. 6).

Morphological confirmation by immunocytochemistry. The CST cells exhibited a strong reactivity for cytokeratin, an epithelial cell marker (Fig. 7). No reactivity was observed in CST cells incubated with antivimentin antibodies or in control coverslips in which PBS with 1% BSA was used in place of primary antibodies.

Cytotoxicity studies. The ECPs from *V. cholerae* MTCC 3904 proved to be cytotoxic to the CST cell line. The cytotoxic effects could be observed even with a dilution of 1:16, within 12 h of inoculation of ECPs. The morphological alterations in CST cells included rounding, detachment, and destruction of the monolayer by 48 h (Fig. 8). In addition, neutral red uptake assay was used to measure the integrity of the plasma membrane of CST cells in response to a toxicant, viz. mercuric chloride. The cells were incubated with growth medium containing increasing concentration of mercuric chloride and subsequently examined for its effect on neutral red uptake. A decrease in the uptake of neutral red was

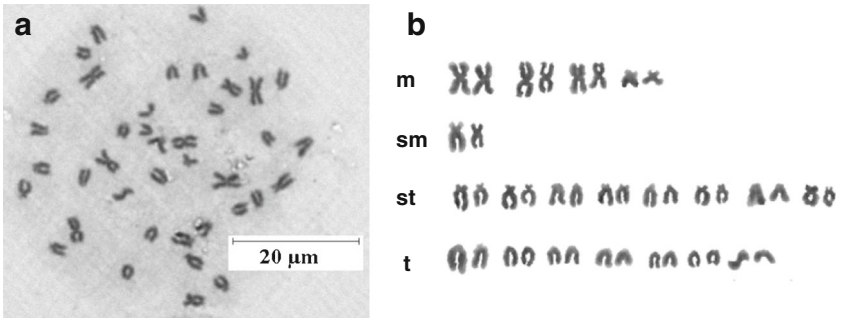

Figure 4. Karyotype analysis of CST cells at passage 64. (*a*) Chromosome number distribution, metaphase spread; and (*b*) diploid karyotype of CST cell. The main chromosome number was 40 which consisted of 4 pairs of metacentrics, 1 pair of submetacentrics, 8 pairs of subtelocentrics, and 7 pairs of telocentrics (2*n*=8 m+2 sm+16 st+14 t).

evident with increasing concentration of mercuric chloride (Fig. 9).

Discussion

In the present study, a cell line has been established from the thymus of an air-breathing fish, *C. striatus*, using the explant method. The cell line, designated CST cell line, has been subcultured over 71 passages and the cells exhibit epithelial morphology. Till date, there are only a few reports on the establishment of cell lines from the thymus of fishes (Katakura *et al.* 2009; Chaudhary *et al.* 2013, 2014; Rebello *et al.* 2014). In teleosts, the thymus is a paired lymphoid organ and primary site for the development and maturation of T lymphocytes. Thymic parenchyma consists of leukocytic cells called thymocytes, and stromal cells (Le Douari and Jotereau 1975). The stromal cells are composed of thymic epithelial cells, macrophages, interdigitating/dendritic cells, myoid cells, and also other cell types in fewer numbers (Zapata *et al.* 1996) and provide signals to support the diverse processes of thymocyte development that are essential for the supply of circulating T cells (Williams *et al.* 1986). The thymic cell lines of epithelial origin can be critical to evaluate the role of these cells in the induction and/or regulation of thymocyte differentiation and maturation (Chaudhary *et al.* 2013).

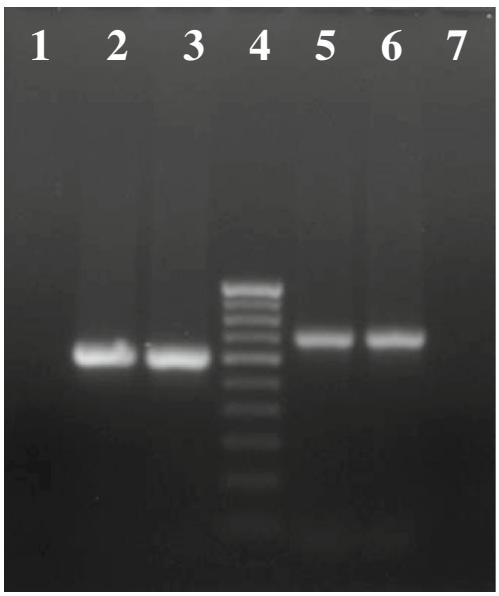

Figure 5. PCR amplification of ~600 and 700 bp sequences of the *Channa striatus* genome using universal oligonucleotide primers of the 16S rRNA and cytochrome oxidase I (COI) genes, respectively. Mitochondrial DNA amplification with 16S rRNA primers: *lane 1*—negative control, *lane 2*—*C. striatus* muscle, *lane 3*—CST cells, *lane 4*—100 bp DNA ladder (Thermo Scientific, Waltham, MA USA); mitochondrial DNA amplification with COI primers: *lane 5*—CST cells, *lane 6*—*C. striatus* muscle, *lane 7*—negative control.

Previously, a number of cell lines have been established from *C. striatus*, viz. SSN-1 from whole fry tissue (Frerichs *et al.* 1991): SHMS, SHHT, and SHSB from muscle, heart, and swim bladder, respectively (Zhao *et al.* 2004); *C. striatus* kidney (CSK) from kidney (Abdul Majeed *et al.* 2013); and *C. striatus* gill (CSG) from gill tissue (Abdul Majeed *et al.* 2014). The cells from all these cell lines have fibroblast morphology except CSG which has epithelioid morphology.

The optimum temperature for growth of CST cells was determined by incubating the flasks at different temperatures. The cells show maximum growth at 28°C. Most of the cell lines derived from freshwater fishes from tropical countries have been reported to grow optimally at 25–30°C (Cheng *et al.* 2010; Ku *et al.* 2010; Chaudhary *et al.* 2014, 2013; Ma *et al.* 2013; Swaminathan *et al.* 2014). Similarly, maximum growth of CSK cells was observed in L-15 medium supplemented with 20% FBS. However, the cells also showed good growth in the medium supplemented with 10% FBS which can be used for routine growth and maintenance of this cell line. These results are in conformity with earlier reports on different fish cell lines (Ku *et al.* 2009; Cheng *et al.* 2010; Ma *et al.* 2013 Swaminathan *et al.* 2014). In the present study, the CST cells showed very good viability (>90%) even after 8 mo of storage in liquid nitrogen and formed a monolayer within 5 d of revival. Furthermore, the cells did not show any alterations in morphology following freezing and thawing. These

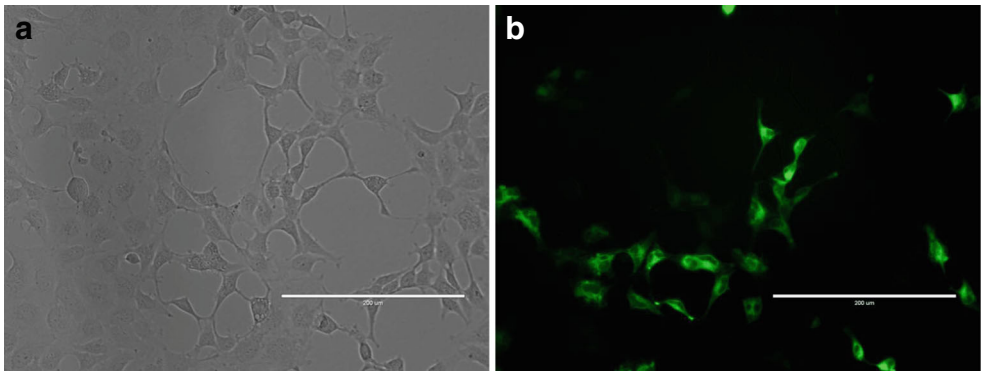

Figure 6. The CST cells at passage 60 were transfected with phrGFP II-N mammalian expression vector using SatisFection reagent and observed under a fluorescent microscope after 48 h. Phase contrast microphotograph of CST cells (*a*) vs. fluorescent microphotograph (*b*) (*scale bar*= 200 μm).

results are in accordance with most of earlier studies (Ku *et al.* 2010; Abdul Majeed *et al.* 2013, 2014; Swaminathan *et al.* 2014).

Plating efficiency or clonogenic assay is a measure of colonies originating from single cells and is a phenotypic marker of transformed cells. This assay essentially tests every cell in the population for its ability to undergo unlimited division. It is a very sensitive test, often used for determining the nutritional requirements of cells, testing serum lots, measuring the effects of growth factors, and testing toxicity. This is also the method of choice to determine cell reproductive death after treatment with ionizing radiation (Franken *et al.* 2006). Plating efficiency was low but improved with increase in seeding density. In conformity with our results, low plating efficiency has been reported previously for the cell line from red sea bream (Ku *et al.* 2010) and rockfish grouper (Ku *et al.* 2009). On the contrary, very high plating efficiency has been observed for cell lines derived from the kidney and gills of *C. striatus* (Abdul Majeed *et al.* 2013, 2014).

The main purposes of transfection are to study the function of genes or gene products, by enhancing or inhibiting specific gene expression in cells, and to produce recombinant proteins in the cells (Wurm 2004). The high transfection efficiency of CST cells indicated that conventional transfection procedures and heterologous CMV promoter adapted to mammalian cells can be applied to CMT cells. Therefore, it can be inferred that this cell line can be used as an in vitro model for exogenous gene manipulation. Similar results have been reported earlier for many fish cell lines (Qin *et al.* 2006; Ku *et al.* 2009, 2010; Cheng *et al.* 2010; Wang *et al.* 2010).

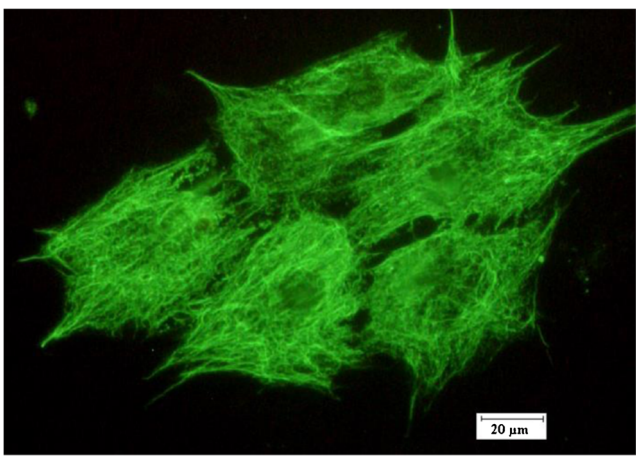

Figure 7. Photomicrograph of CST cells showing the presence of cytokeratin at passage 47.

The modal chromosome number in the CST cell line was reported to be 40 which is identical to the chromosome number reported for *C. striatus* (Kumar *et al.* 2013) as well as cell lines derived from *C. striatus* (Abdul Majeed *et al.* 2013, 2014). On the contrary, Zhao *et al.* (2004) reported that all the three cell lines derived from different tissues of *C. striatus* had a chromosome count of 44. Furthermore, Courtenay and Williams (2004) observed that chromosome counts of *C. striatus* had been reported to be 40 as well as 44, indicating that *C. striatus* represents a complex species. Mitochondrial DNA typing has been recommended for identifying the species of origin of established cell lines at molecular level (Cooper *et al.* 2007; O'Donoghue *et al.* 2011). A number of mitochondrial genes, viz. 12S rRNA, 16S rRNA, 18S rRNA, and cytochrome oxidase I gene, have been used for authenticating the origin of fish cell lines (Ding *et al.* 2006; Rougée *et al.* 2007; Ishaq Ahmed *et al.* 2009; Cheng *et al.* 2010; Chaudhary *et al.* 2014). In this study, we analyzed and compared partial mitochondrial 16S and COI gene sequences from CST cells with genes of *C. striatus*. A BLAST search indicated 100% sequence identity among the 16S and COI genes from CST cells and *C. striatus* indicating that this cell line was indeed derived from *C. striatus*.

The dominant cell types in fish cell lines have been reported to be fibroblast-like cells or epithelial-like cells (Lakra *et al.* 2011). The presumed origin of cells is mostly based on cell morphology (Bols *et al.* 2005); however, the relevance of cell morphology criterion to characterize the cultured cell population is questionable as cultured cell morphology changes quickly (Mauger *et al.* 2009). Therefore, immunochemical markers of a cytoskeleton have been developed in mammals to distinguish the two cell types and these antibodies of

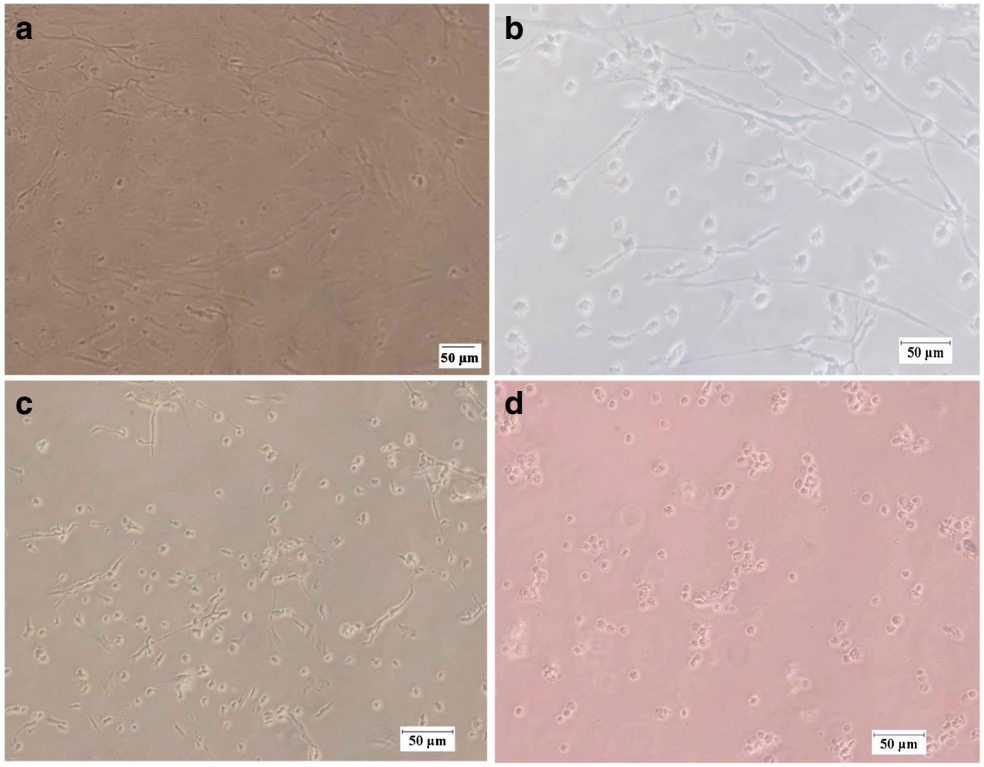

Figure 8. Cytotoxic effects of extracellular products (ECPs) of *Vibrio cholerae* MTCC 3904. (*a*) A monolayer of CST cells, (*b*) CST cells at 12 h following inoculation of ECPs, (*c*) CST cells at 24 h postinoculation of ECPs, and (*d*) complete detachment of the CTM cells was observed after 48 h.

fibroblastic or epithelial markers have also been applied to confirm the lineage of fish cell lines (Himizu *et al.* 2003; Butler and Nowak 2004; Ishaq Ahmed *et al.* 2009; Chaudhary *et al.* 2014). In the present study, CST cells showed strong reactivity to anticytokeratin antibodies indicating that the cell line is epithelial in origin.

Many cell lines have proven suitable for demonstration of cytotoxic effects exerted by extracellular products of fish pathogenic bacteria (Ku *et al.* 2009, 2010). The ECPs from *V. cholerae* were cytotoxic to CST cells and the morphological changes in subconfluent monolayers were similar to those described previously (Bejar *et al.* 1997; Ishaq Ahmed *et al.* 2009; Swaminathan *et al.* 2010; Chaudhary *et al.* 2014).

Neutral red (NR) uptake assay is a viability assay, based on the ability of viable cells to incorporate and bind supravital dye NR which concentrates in the lysosomes. The dye is then extracted from viable cells using an acidified ethanol solution and absorbance of solubilized dye is quantified using a spectrophotometer (Repetto *et al.* 2008). Any alterations of the cell surface or lysosomal membrane produced by toxic substances cause decreased uptake and binding of NR, making it possible to distinguish between viable, damaged, or dead cells via

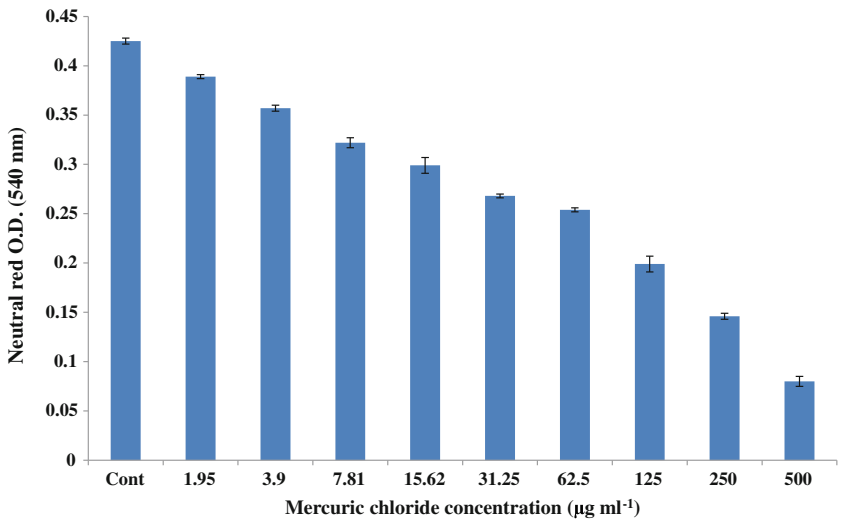

Figure 9. Neutral red uptake assay; CST cells were exposed to varying concentrations of mercuric chloride (1.95 to 500 µg ml^{-1}) for 24 h followed by incubation with neutral red medium for 2 h. A decrease in uptake of neutral red was evident with increasing concentration of mercuric oxide.

spectrophotometric measurements. It is one of the most used cytotoxicity tests (Brandão *et al.* 1992; Repetto and Sanz 1993) and believed to be a highly sensitive test in detecting cytotoxic events (Fotakis and Timbrell 2006). In the present experiment, lysosomal integrity of CST cells appeared to have been affected following incubation with L-15 medium containing varying concentrations of mercuric chloride, and it was evidenced by the concentration-dependent decrease in uptake of NR by CST cells. Hence, the CST cell line can be used for in vitro screening of chemicals for cytotoxicity and successfully employed as a biological alternative to the use of whole fish in toxicity screening, in accordance with earlier studies (Ní Shúilleabháin *et al.* 2004; Davoren *et al.* 2005; Fotakis and Timbrell 2006). There are a number of reports on evaluating cytotoxic effects of mercury salts using cell lines (Devlin and Clary 1998; Issa *et al.* 2003).

The results suggest that the CST cell line appears as a suitable system for expression of recombinant vertebrate proteins as well as an in vitro monitoring system for short-term cytotoxicity assays. The cell line can also be a useful tool to study and identify new biomarkers and can provide experimental insights into their basis.

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