

# Establishment and Characterization of Eye Muscle Cell Line from Snow Trout, *Schizothorax richardsonii* (Gray, 1832), a Vulnerable Coldwater Fish, for *In Vitro* Studies

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## Abstract

*Schizothorax richardsonii*, commonly called snow trout is a vulnerable coldwater fish distributed in India and adjoining countries, is an important resource as food, ornamental and game fishery. A cell line, designated as SREM-1, has been established from the eye muscle of *S. richardsonii* using explant method and cultured in Leibovitz's L-15 medium. The growth studies at 20-32°C temperatures and 5-20% of fetal bovine serum (FBS) concentrations revealed that SREM-1 cells exhibited optimal growth at 28°C in L-15 medium containing 20% FBS. Cell type was confirmed primarily as epithelial in nature by immuno-phenotyping assay and was sub-cultured for >55 times since its development. The origin of the cell line was confirmed by sequencing of cytochrome oxidase c subunit I and 16S rRNA genes. SREM-1 cell line is free from mycoplasma contamination and has been cryopreserved at different passage levels with revival efficiency of 70-80% after 6 months. The SREM-1 cell line was successfully transfected and also employed for evaluating *in vitro* cytotoxicity against a heavy metal, mercuric chloride. The newly established cell line of this vulnerable species would be useful as a model for aquatic toxicological and transfection studies.

## Introduction

The common snow trout, *Schizothorax richardsonii* (Gray, 1832) belonging to Cyprinidae family, is an important coldwater fish species inhabiting high altitude hill streams of India and neighbouring countries like Afghanistan, Nepal and Pakistan. It is a much-sought after species for its delicacy in Indian upland areas. It has been studied as a potential model species for investigating thermal stress (Kapila *et al.*, 2002; Barat *et al.*, 2016). The fishery of this important species has declined, due to habitat destruction and over exploitation, and has been designated as a vulnerable species in the International Union for Conservation of Nature (IUCN) red list (Vishwanath, 2010). Availability of a cell line could stimulate further research which can lead to a better understanding of biological and molecular processes of this coldwater species. The

availability of a cell line could also be used in future, once the cloning and somatic cell nuclear transfer technologies develop for fishes.

Fish cell lines are presently considered as an ethical solution for sacrificing fish specimens for research purposes and also easy to work for supporting biomedical applications, useful for isolating virus and immunological studies, provide the best source of chromosome preparation for banding/ staining purposes etc. (Hightower and Renfro, 1988; Driever and Rangini, 1993; Villena, 2003; Nagpure *et al.*, 2016). The past few years have seen plenty of activities in applications of cell lines in fisheries specially for diseases, pharmacology and toxicology along with the opening of new and exciting research avenues.

Globally, few cell lines have been developed from important cold water fish species, like the *Salmo gairdneri*, *S. salar*, *Oncorhynchus keta*, *O. kisutch*, *O.*

*mykiss*, *O. nerka* etc. (Wolf and Quimby, 1962; Lannan *et al.*, 1984; Bols *et al.*, 1994; Rodriguez *et al.*, 2014). However, some fish cell lines were also developed in India, like *Puntius chelynoides* and *Tor putitora* (Lakra *et al.*, 2006; Goswami *et al.*, 2012), but still, there is a paucity of cell lines from the fishes of cold water ecosystems that needs to be developed for research and development (R&D) purposes. A fin cell line from *S. richardsonii* was developed by Goswami *et al.* (2013), which is no longer available in the National Repository of Fish Cell Lines (NRFC) due to technical problems. The present study describes the development and characterization of an eye muscle cell line from *S. richardsonii* as a material for germplasm conservation and its wide applications in toxicity testing as well as pathological and transfection studies.

## Materials and Methods

### Generation of Primary Culture and Routine Maintenance

Healthy live specimens of *S. richardsonii* (n=10 with 10-20 g weight) were collected from Kalsa stream, Chafi, Uttarakhand, India, and transported to the laboratory. Specimens were acclimatized for a week in water having 15°C temperature with proper aeration and feed, where one-third of the water was changed every alternate day. Fish specimens were handled following the strict guidelines issued by Control and Supervision of Experiments on Animals (CSEPA), Government of India and the experimental protocols were approved by Institutional Animal Ethics Committee (AEC) of ICAR-National Bureau of Fish Genetic Resources, Lucknow as per the guidelines of (CSEPA) Government of India.

Explant method was followed for the development of primary culture. Before setting explants, the specimens were kept overnight in aerated freshwater tank added with 1 ml of antibiotic-antimycotic solution (CELLclone, Genetix Brand) per 100 ml of water to minimise bacterial or fungal contamination. The specimens were then anaesthetized on ice and wiped with 70% ethanol before dissection. Eye muscle tissues were then aseptically removed using a sterile scalpel blade, minced into small pieces, washed repeatedly with 1 ml of phosphate buffer saline (PBS, HiMedia, India), containing 10X antibiotics-antimycotics mixture. The tissue fragments were then explanted into a 25 cm<sup>2</sup> cell culture flask (ThermoFisher Scientific, USA) added with 200 µl of fetal bovine serum (FBS, ThermoFisher Scientific, USA) and then the flask kept in a vertical position for 2 h to allow the culture to attach to the surface of the flask. Later, 5 ml of Leibovitz's L-15 medium (HiMedia, India) supplemented with 20% FBS and 100 µl of antibiotic and antimycotic solution were added to the flask and incubated at 28°C in an incubator, where half the medium was changed every 3 or 4 days. Upon reaching 80-90% confluency, the cells were sub-cultured at 1:2 ratio following standard trypsinization

method by trypsin-EDTA solution (trypsin 0.25%, EDTA 0.02% in PBS) (HiMedia, India). No antibiotics were used thereafter and the passage number was recorded every time after splitting the flask.

### Cryopreservation

The cells were cryopreserved at various passage levels for future use. Cells were trypsinized in the flask at 70–80% confluency level, harvested and collected in a 15 ml centrifuge tube by centrifugation at 1,500 rpm for 5 min. The cells were then washed once with 2 ml PBS and re-suspended in freezing medium (containing pre-cooled L-15 medium supplemented with 20% FBS and 10% dimethyl sulphoxide) at a density of  $1 \times 10^6$  cells/ml. The cells were then transferred to 1.8 ml cryovials and stored at -20°C. After 4 h, the cells were kept overnight at -80°C and then transferred to liquid nitrogen (LN2) vapour phase for cryopreservation. After 6 months, the cells were revived and checked for cell viability. The cryopreserved cells were thawed quickly at 37°C in a water bath and mixed drop-wise with complete L-15 medium in a 15 ml centrifuge tube. The cells were then centrifuged at 1500 rpm for 5 min and the pellet was re-suspended in 5 ml complete medium. Cell viability was checked with a haemocytometer following trypan blue staining. The revived cells were seeded into 25 cm<sup>2</sup> flask and incubated at 28°C for further culture.

### Growth Studies

To determine the optimum temperature and serum concentration, the eye muscle cells were grown at different temperature and FBS concentrations. The effect of incubation temperature was determined by seeding cells at passage number 40 at a density of  $1 \times 10^5$  cells in different 25 cm<sup>2</sup> tissue culture flasks. After cell attachment, the tissue culture flasks were incubated at 20, 24, 28 and 32°C temperatures for growth studies. The experiment was conducted in triplicates and cell densities measured using a haemocytometer for five consecutive days. Effect of serum was determined by seeding cells at passage 46 at a density of  $1 \times 10^5$  cells in different 25 cm<sup>2</sup> tissue culture flasks with 5, 10, 15 and 20% FBS concentrations incubated for five successive days at the optimum temperature obtained from above experiment.

### Plating Efficiency and Doubling Time

Cell line at 42 passage was also used to determine the plating efficiency (in terms of cells' attachability and growth). 100, 500 and 1000 cells were seeded in 25 cm<sup>2</sup> tissue culture flasks in triplicates. The cells were incubated at 28°C in L-15 medium with 20% FBS, where 1/3<sup>rd</sup> of the culture medium was changed every 4<sup>th</sup> day. After 10 days of incubation, the culture medium was discarded and cells were washed with PBS and fixed

using methanol and stained with crystal violet. The individual cell colonies in a flask were then counted under the inverted microscope (Nikon Eclipse TS100, Japan) to know the plating efficiency (Freshney, 2005). The calculation of population-doubling time of the cell line was performed at two different passages, viz. 30 and 45, where cells were counted using a haemocytometer under an inverted microscope (Freshney, 1994).

## Cell Line Characterization

### i) Mitochondrial Genes Sequencing

To authenticate the origin of the cell line, COI and 16S rRNA genes were sequenced on ABI 3500 DNA analyzer (Applied Biosystems, USA) using DNA isolated from SREM-1 cells harvested at 35 passage level. Universal primers for COI [FishF1 5'-TCAACCAACCACAAAGACATTGGCAC-3'; FishR1 5'-TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward *et al.* 2005)] and 16S rRNA [F 5'-CGCTGTTTATCAAAAACAT-3'; R 5'-CCGGTCTGAAC TCAGATCACGT-3' (Palumbi *et al.* 1991)] genes were used. The resulting DNA sequences for both the genes were blasted and aligned with respective sequences of *S. richardsonii* available at National Centre for Biotechnology Information (NCBI) databases.

### ii) Chromosome Analysis

Cells at 47 and 59 passage levels were sub-cultured in a T-75 culture flask, where the cells were trypsinized and processed for chromosome analysis. Next day fresh 8 ml L-15 media along with 25 µl of 0.05% colchicine was added in the flask, which was then incubated at 28°C for 2 h. Cells were then harvested and collected in a 15 ml tube and incubated with 5 ml hypotonic solution at 28°C for 25 min and after this 1 ml chilled Carnoy's fixative was added and cells were pelleted after centrifugation. Later, the cells were re-suspended in fresh 5 ml fixative and kept for 2 h at 4°C for fixation of the cells. The same fixation step was repeated for 4-5 times. Slides were then prepared using the conventional drop-splash technique (Freshney, 2005). Diploid chromosome number in 100 metaphase spreads was counted under a light microscope (Leica DM LB2, Germany).

### iii) Immuno-Phenotyping

Morphological confirmation of the cells was done by immune-phenotyping. The cells at 35 passage level were grown at 28°C for 24 h on coverslips placed on 30 cm petri plate. The cells were then washed with PBS and fixed in methanol-acetone solution (1:1) at -20°C for 20 min. The solution was then discarded and blocking of unspecific sites was done with 3% Bovine Serum Albumin (BSA) dissolved in PBS for 1 hr at 37°C. The cells were then stained with primary antibodies, viz. mouse anti-cytokeratin (pan) and mouse anti-fibronectin

antibodies (Sigma-Aldrich, USA) at 1:100 dilutions and incubated overnight at 4°C. For control group, 1% BSA in PBS was used in place of primary antibodies. Next day, the cells were washed with PBS and incubated with secondary antibody, viz. fluorescein thiocyanate (FITC)-labeled rabbit anti-mouse IgG (Sigma-Aldrich, USA) diluted 1:100 times in PBS, for 1 hr at 37°C. After a final wash with PBS, the coverslip was mounted in buffered glycerol and monitored under a fluorescent microscope (Nikon Eclipse TS100, Japan).

### iv) Transfection

The cells at 37 passage levels were cultured in a 6 well plate in duplicates. After 24 h, sub-confluent monolayers were transfected with 1.5 µg of pAcGFP1-N1 eukaryotic expression vector (Takara Clontech, USA) using lipofectamine 2000 reagent (Invitrogen Corporation, Grand Island, NY, USA). After 48 h, the transfected cells were checked under a fluorescent microscope for the green fluorescent signals.

### v) Mycoplasma Detection

PCR based method was used for mycoplasma detection in the cell line. Cells at 37 passage level were allowed to grow in complete medium (L-15+20% FBS) without antibiotics for 5 days. Then, 2 ml of the supernatant from the flask was transferred to microcentrifuge tube and the tube centrifuged at 13000 rpm for 30 min. The pellet formed was dissolved in 50 µl of 1× TE buffer, vortexed and heated at 95°C for 10 min. The EZdetect™ PCR Kit (HiMedia, India), which is based on the amplification of the spacer region between 16S and 23S ribosomal RNA (rRNA) genic sequence, was used for this. PCR was carried out using the reagents and cycling conditions as per the manufacturers' instructions. The amplified products were separated in 2% agarose gel and visualized under UV transilluminator (UVP, USA).

### vi) Cytotoxicity Testing

Cytotoxicity studies were carried out using alamarBlue and Neutral Red assays for an important heavy metal, viz. mercury chloride as the standard protocol provided by the manufacturer.

#### a) alamarBlue Assay

Briefly, about 50,000 cells were seeded per well, in triplicates, in 96 well plate along with the control and the plate was incubated at 28°C. After 24 h, the culture medium was removed and 100 µl each of 11 concentrations of analytical grade HgCl<sub>2</sub> (Sigma-Aldrich, USA), viz. 5000, 4500, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 500 and 100 µM, prepared in complete culture medium, was poured in each well and kept the plate for 24 h exposure at 28°C. After 24 h of exposure,

10  $\mu$ l alamarBlue reagent was added to each well and incubated for 2 h. Then absorption readings at 570 and 600 nm wavelengths were recorded using a spectrophotometer. Inhibition constant ( $IC_{50}$ ) value was then calculated using GraphPad Prism 6 software.

### b) Neutral Red (NR) Assay

For NR assay, the SREM-1 cells were seeded, in triplicates, in 96 well plates at a density of  $1 \times 10^5$  cells/well along with control and the plate was incubated at 28°C for cell attachment. After cell attachment, 100  $\mu$ l each in 6 concentrations of analytical grade  $HgCl_2$  (Sigma-Aldrich, USA), viz. 375, 187.5, 93.75, 46.875, 23.43 and 11.71  $\mu$ M, prepared in culture medium, were added in each well and incubated at 28°C for 24 h for exposure. After 24 h, the medium was discarded and wells were washed with PBS followed by addition of 100  $\mu$ l NR working solution and incubation at 28°C. After 3 h of incubation, the NR medium was decanted, the wells washed with PBS and 150  $\mu$ l of NR de-staining solution was added. Then, absorption readings at 540 wavelengths were recorded using spectrophotometer and the  $IC_{50}$  value was calculated using GraphPad Prism 6 software.

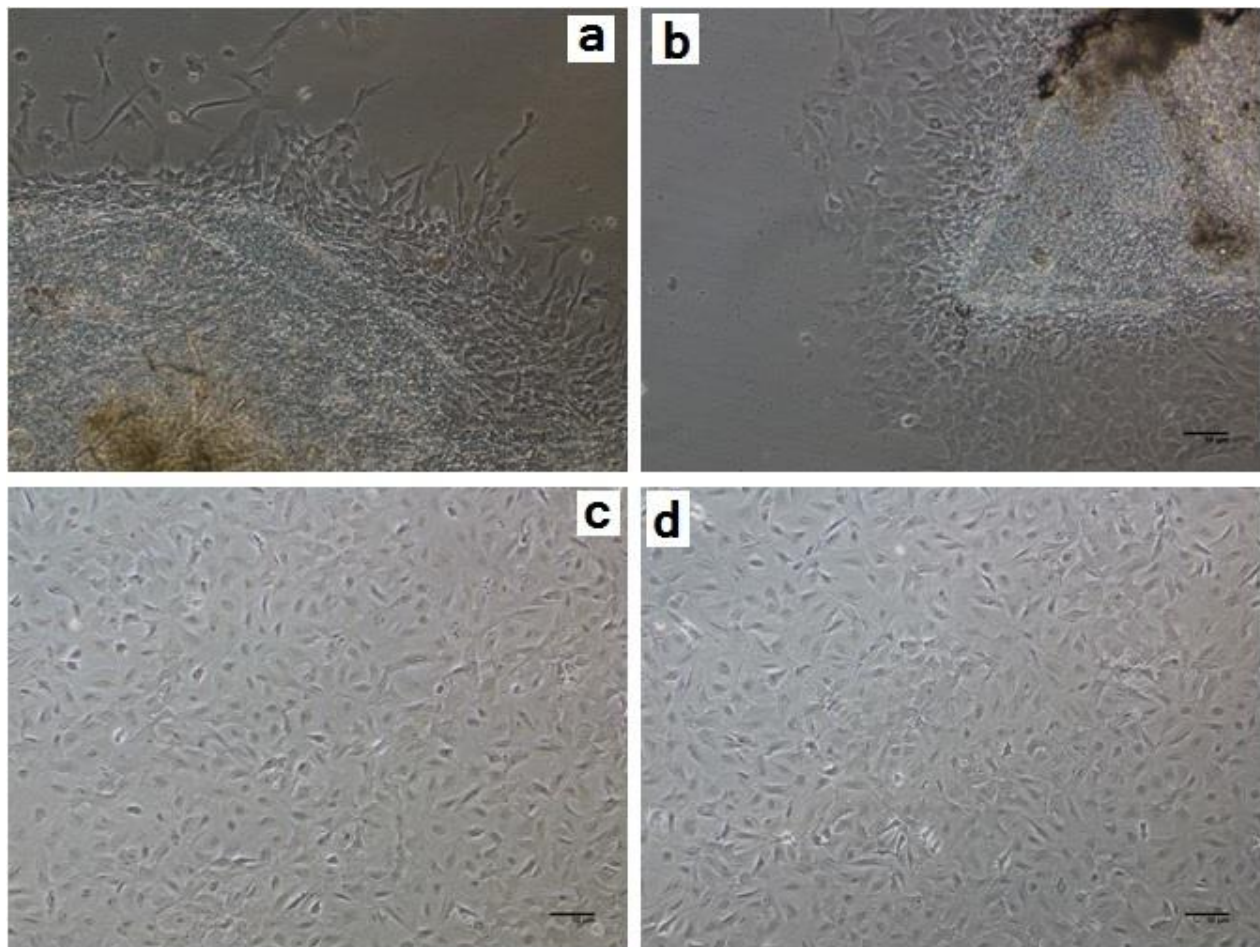
## Results

### Primary Culture

Primary cell cultures were initiated from eye muscle tissue of *S. richardsonii* using explant culture method. The cells migrated from the tissue fragments on 3<sup>rd</sup> day and formed monolayer within 18 days that could be sub-cultured at an interval of 4-5 days. Initial cultures consisted of heterogeneous cell population but later passages exhibited epithelial-like cell types. The cells were split at 1:2 ratio and this cell line were sub-cultured for >55 times since its initiation and has been named as *S. richardsonii* Eye Muscle (SREM-1) cell line (Figure 1).

### Cryopreservation

SREM-1 cells were cryopreserved at various passage levels and were successfully revived after 6 months with  $78 \pm 3\%$  viability. The revived cells formed a monolayer within 5 days at 28°C. The cells retained their normal growth, doubling time and attachment ability without showing any morphological changes.



**Figure 1.** Photographs depicting stages of SREM-1 cell line: (a) eye muscle explant showing radiation on day 4, (b) eye muscle explant on day 19, (c) monolayer of cells at 35 passage level, and (d) monolayer of cells at 50 passage level

### Effect of Temperature and FBS on Growth and Plating Efficiency

SREM-1 cells could be grown at varying temperatures ranging from 20 to 32°C, but maximum growth was observed at 28°C (Figure 2a). Similarly, SREM-1 cells could grow at different FBS concentration ranging from 5 to 20%. There was an increase in growth rate with increase in FBS concentration, in which maximum growth was seen at 20% and least growth was seen at 5% (Figure 2b). SREM-1 cells seeded at the densities of 100, 500 and 1000 cells per flask exhibited 6.3, 10.6 and 15.23% plating efficiency, respectively. The doubling time was estimated to be 34 h.

### Mitochondrial Gene Sequencing and Chromosome Analysis

The sequencing of amplified products (Figure 3), generated 637 bp and 586 bp fragment length of COI and 16S rRNA genes respectively. This sequence information for two fragments showed 95.45 and 99.65% match, respectively, with the known *S. richardsonii* mtDNA sequences, submitted in NCBI database, confirming the origin of the cells from the undertaken species. The counting of 25 metaphase spreads revealed the presence of varied diploid chromosome numbers ranging from 83-98 with some spreads showing polyploidy having very tiny chromosomes in SREM-1 cells (Figure 4).

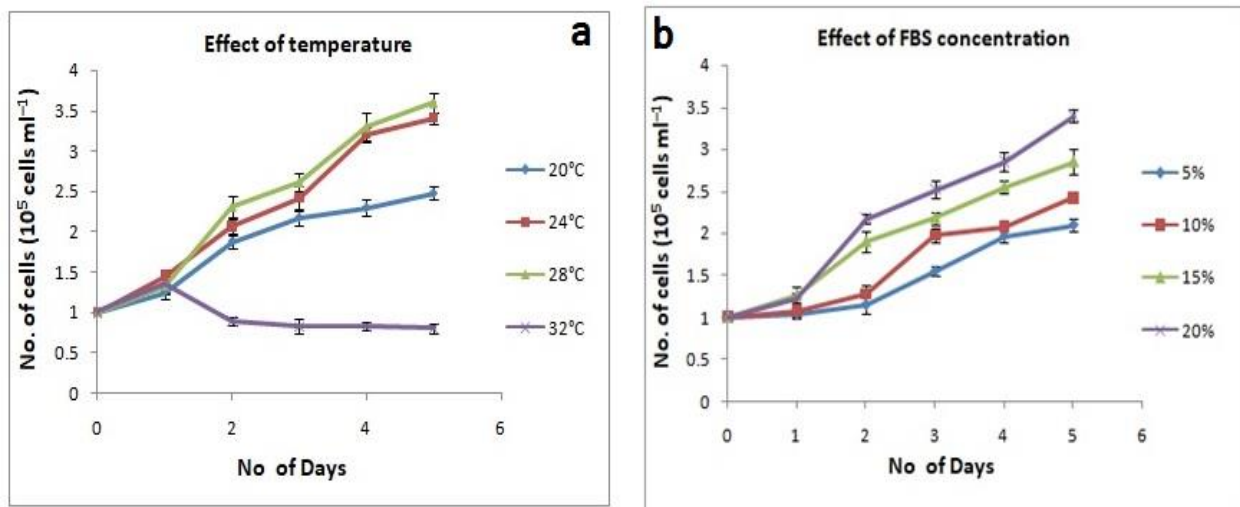


Figure 2. Graphs showing the effect of: (a) temperature, and (b) serum on SREM-1 cells.

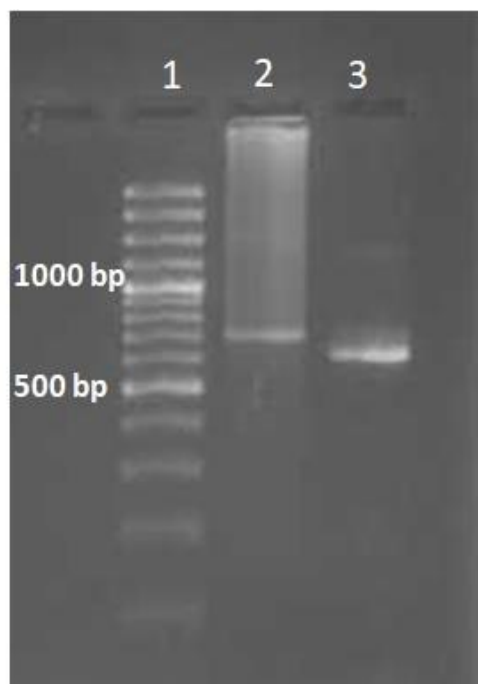


Figure 3. Gel image of PCR product: Lane 1: 100 bp DNA marker, Lane 2: COI gene amplicon, Lane 3: 16S rRNA gene amplicon

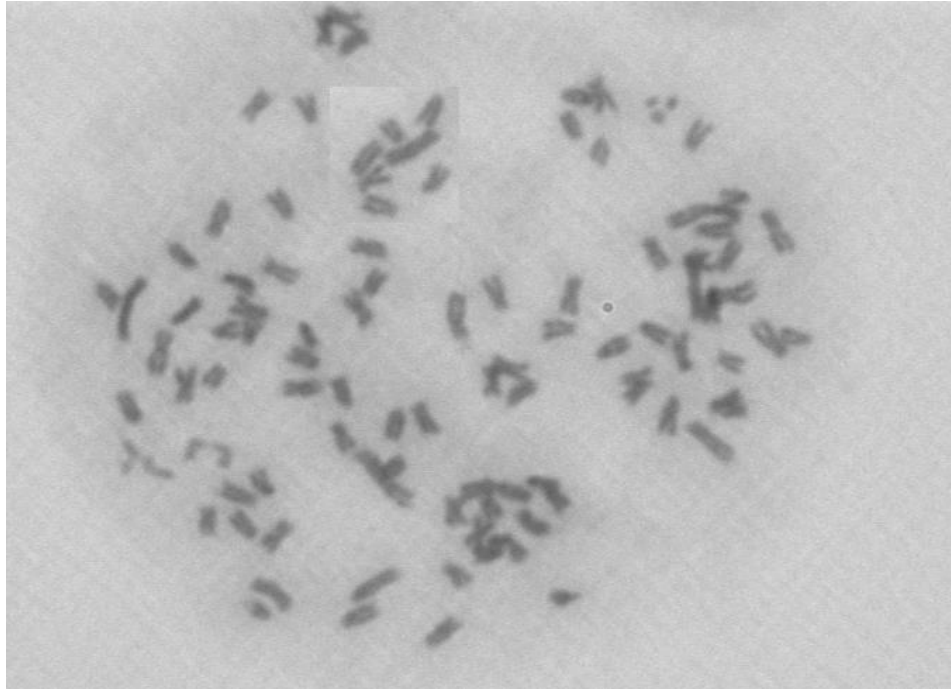
### Immuno-Phenotyping and Mycoplasma Detection

Immuno-staining results showed a strong positive signal in the cells incubated with cytokeratin, but not in fibronectin, which confirmed that SREM-1 cells are epithelial-like (Figure 5). It was confirmed from the amplification that the cell line is free from any mycoplasma contamination, as there was no

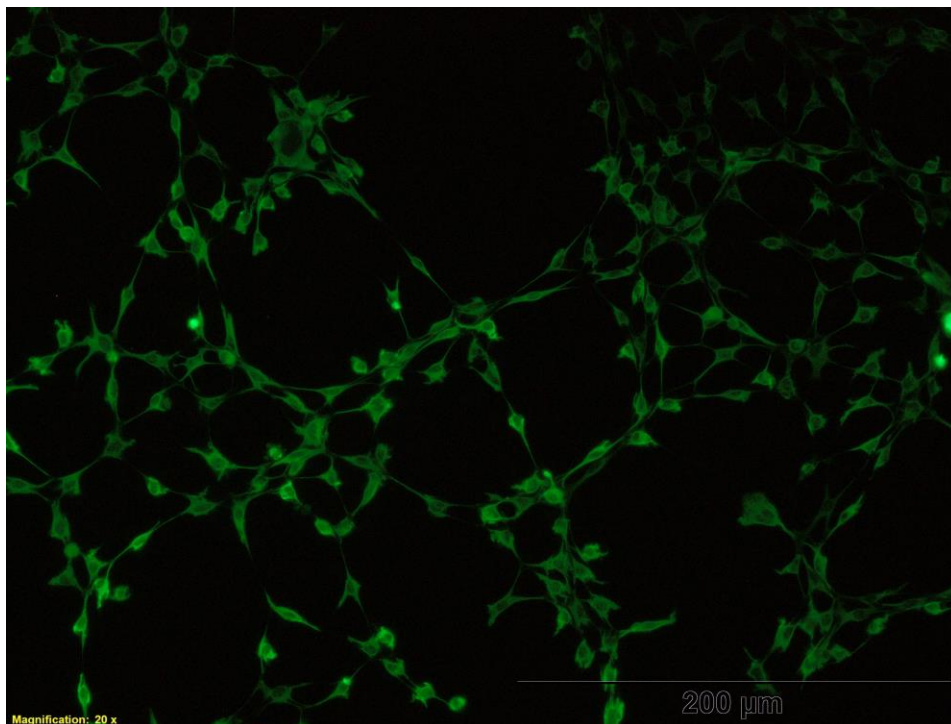
amplification of mycoplasma specific DNA content in the cell growth medium of SREM-1 cells at 40 passage level (Figure 6).

### Cell Transfection

The transfection of SREM-1 cells with green fluorescent protein (GFP) plasmid pAcGFP1-N1



**Figure 4.** Metaphase chromosome spread in SREM-1 cell line.



**Figure 5.** SREM-1 cells showing positive to cytokeratin marker

exhibited fluorescent signals at 48 h post-transfection, with  $5\pm 1.2\%$  transfection efficiency thus, indicating the suitability of these cells for transfection and gene expression studies (Figure 7).

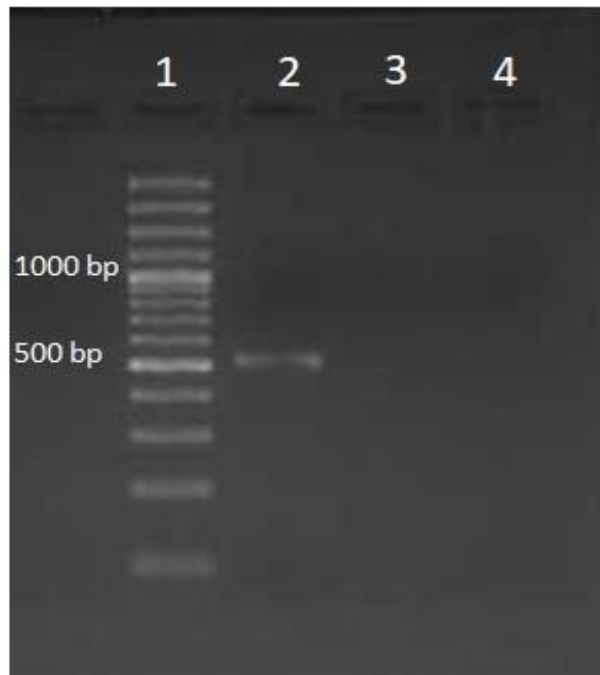
### Cytotoxicity Tests

Cytotoxicity induced due to heavy metal, mercury chloride, was noticed in SREM-1 cells at 24 h after exposure. Cells in control wells had a regular shape, whereas treated cells lost their regular shape and size. Change in cell morphology, such as cell shrinking, rounding and floating of dead cells, was observed at

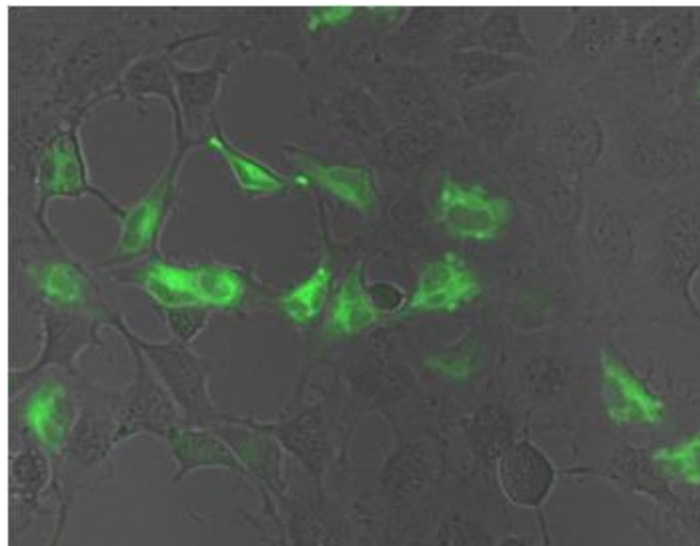
higher doses. The cytotoxic effects on morphology were found to be concentration dependent. A decrease in absorption values was noticed with increasing concentration of  $\text{HgCl}_2$ . At the highest dose, all cells appeared to be dead.  $\text{IC}_{50}$  values for  $\text{HgCl}_2$  were determined to be  $411.0\pm 2.5$  and  $321.9\pm 1.77$   $\mu\text{M}$  using alamarBlue and NR assays, respectively (Figure 8a, b).

### Discussion

Schirmer (2006) proposed to improve and utilize cell lines as an alternative for regulatory testing of chemicals and effluents using fish. Fish cell lines have



**Figure 6.** Gel image of PCR products used for mycoplasma testing: Lane 1: 100 bp DNA ladder, Lane 2: positive control, Lane 3: negative control, and Lane 4: SREM-1 cell supernatant.



**Figure 7.** Fluorescence image showing the expression of GFP gene in SREM-1 cell line transfected with pAcGFP1-N1 plasmid.

become an important resource in recent years with numerous applications in virology, aquatic toxicology, immunology, endocrinology, developmental biology, drug discovery etc. (Castano *et al.*, 2003; Huang and Han, 2014; Nagpure *et al.*, 2016; Soni *et al.*, 2018). This is evident from the availability of fish cell lines in various cell line repositories viz. 70 fish cell lines accessions in National Repository of Fish Cell Lines (NRFCL) (<http://mail.nbfgr.res.in/nrfc/cellline-available.php>), ICAR- National Bureau of Fish Genetic Resources (NBFGR), Lucknow, India, 25 in European Collection of Authenticated Animal Cell Cultures (<https://www.euro-culturecollections.org.uk/products/celllines/generalcell/search.jsp?searchtext=fish&dosearch=true>), 8 in American Type Culture Collection ([https://www.atcc.org/en/Products/Cells\\_and\\_Microorganisms/Cell\\_Lines/Animal/Fish.aspx](https://www.atcc.org/en/Products/Cells_and_Microorganisms/Cell_Lines/Animal/Fish.aspx)) etc. Cellosaurus-a knowledge resource on cell line lists 714 fish cell lines (<https://web.expasy.org/cgi-bin/cellosaurus/search>).

The snow trout, *S. richardsonii*, has an enormous aquaculture potential, but at the same time, it is susceptible to various pathogens (Mallik *et al.*, 2010; Tandel *et al.*, 2020), aquatic pollution etc (Khan, 2004). In the present study, a new cell line, named SREM-1, was established from eye muscle tissue of *S. richardsonii* and characterized using various markers to support its potential applications in toxicology, virology and gene expression studies in coldwater fishes. The cell line also serves as an *in vitro* model of this important vulnerable coldwater fish species. The SREM-1 cell line has been deposited in NRFCL at ICAR-NBFGR, Lucknow, India, with accession code NRFCL060 for further storage and distribution to researchers for R&D works.

Eye cell lines have been developed from a few fish species, like rohu and zebrafish (Ahmed *et al.*, 2009; Babu *et al.*, 2011; Nambi *et al.*, 2015). The SREM-1 cell line was maintained in L-15 medium with 20% FBS without need for any growth factors. The L-15 culture medium has been used in a number of other fish cell lines as being the preferred media (Lakra *et al.*, 2011; Chen *et al.*, 2019). Similar serum concentrations were

used in other fish cell lines (Rodriguez *et al.*, 2014; Singh *et al.*, 2019; Chen *et al.*, 2019). It was demonstrated in the present study that even though the SREM-1 cells could be cultured at wide temperature range of 20-32°C in L-15 medium supplemented with 5-20% FBS, the optimum growth was observed at 28°C with 20% FBS concentration. There is a little bit of elasticity with respect to growth when compared to human or other animal cell lines. Similar results were obtained in other fish cell lines viz. Retinal cell line from *Danio rerio* (Nambi *et al.*, 2015), caudal fin cell line from *Pangasianodon hypophthalmus* (Soni *et al.*, 2018), kidney cell line from *Anguilla anguilla* (Chen *et al.*, 2019) etc. The SREM-1 cell line was sub-cultured for more than 55 times over a period of 10 months. Continuously maintaining cell lines is not feasible either from handling point nor is it a good practice from genetics point. Further, there can be chances of loss of culture due to contamination or other reasons. Hence, cryopreservation is essential and required for long term storage and to prevent genetic changes. SREM-1 cell line exhibited 70-80% viability on revival after cryopreservation period of 6 months in vapour phase of LN2. There were no noticeable changes in cell morphology during revival of cryopreserved cells. Similar observations have been reported by others. Rodriguez *et al.* (2014) reported 80% cell viability after storage in liquid storage in pronephros cell line of *Salmo salar*. Nambi *et al.* (2015) reported recovery of 75%-80% of the *D. rerio* retinal cells after thawing after cryopreservation with no changes in growth or morphology.

Mitochondrial DNA genes, viz. COI and 16S rRNA, were amplified and sequenced from the SREM-1 cell line that confirmed the origin of the cell line to be *S. richardsonii*. In fish cell lines, the dominant cell types have been reported to be either fibroblast-like or epithelial-like cells (Lakra *et al.*, 2011). In this study, SREM-1 cells were confirmed as epithelial-like by immuno-staining with epithelial marker, cytokeratin. It has been reported that the modal chromosome number

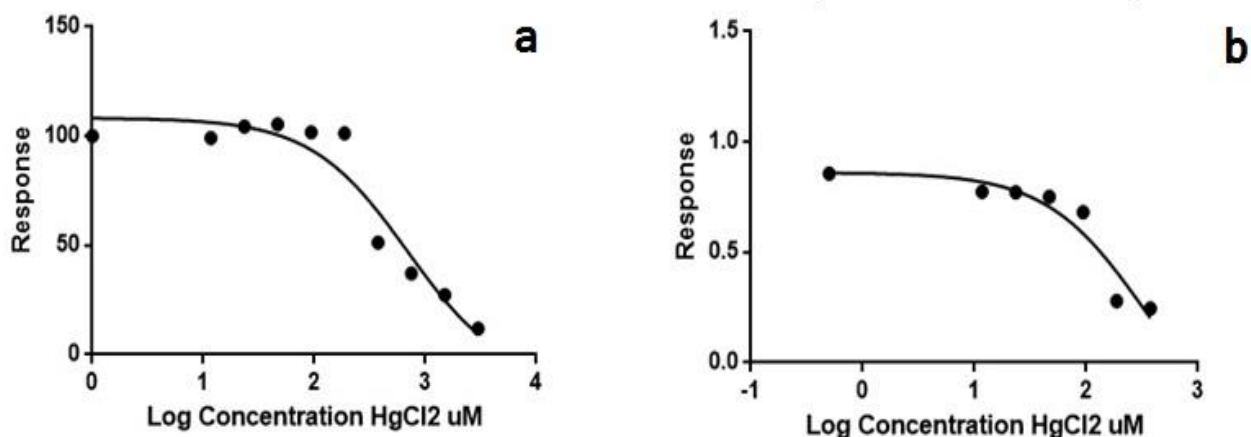


Figure 8. Dose response curve of HgCl<sub>2</sub>, assessed by: (a) alamarBlue, and (b) NR assays.



for the species is 98 (Sharma *et al.*, 1992; Lakara *et al.*, 1997) and 96 (Vasave *et al.*, 2016). In the present study, a majority of the SREM-1 cells revealed diploid chromosome number (2N) as 98. There was asymmetry of cells observed in some metaphase spreads. Similar observation was made by Goswami *et al.* (2013). High plating efficiency was observed at very cell densities only. The cell doubling time was estimated to be 34 h. Marques *et al.* (2007) had reported it to be 36 to 48 h in *Sparus aurata* vertebral cell line. Dhararatnam *et al.* (2020) reported doubling time of 33.9 h in caudal fin cell line of *Carassius auratus*. The cell growth and doubling time is generally better in fish cell lines than human or other animal cell lines. Mycoplasma contamination has become a major issue in cell line research from the last decade as they can unnoticed and unrecognized for a long time but are deleterious to cells (Drexler *et al.*, 2017; Corral-Vazquez *et al.*, 2017). Screening for mycoplasma has become essential, especially if the intention is to distribute the cell line to other labs for research purposes. The SREM-1 cells were found free from mycoplasma.

In the present study, the SREM-1 cell line was also tested for its use in cytotoxicity studies. To determine its suitability for cytotoxicity testing as a model, its response to a heavy metal, mercury chloride was studied. The alamarBlue and NR uptake assays are one of the most commonly used *in vitro* cytotoxicity tests. Mercury chloride is a toxic heavy metal element used in several industrial components, like batteries, pesticides, fireworks, laboratory chemicals, cosmetics, medical instruments etc., which can then find its way to the aquatic environment. It has also an inorganic form of ionic mercury that has widely been used in skin creams and other cosmetics. In the present study, SREM-1 cell line responded to the presence of HgCl<sub>2</sub>. After exposure of cells to higher HgCl<sub>2</sub> concentrations, the cells started dying. With increasing concentration of the toxicant, there was a gradual decrease in absorption values in both alamarBlue and NR assays. The results are similar to those observed by researchers in other cell lines viz. primary haemocyte culture of *Penaeus monodon* (Jose *et al.*, 2011) and neural cells of *Chelonia mydas* (Tong *et al.*, 2016), indicating its potential to be used for cytotoxicity or eco-toxicological studies. The transfection efficiency of SREM-1 cells was also found good, indicating its potential for conducting transfection or genetic manipulation and gene expression studies. Barat *et al.* (2016) generated large scale next generation sequencing (NGS) data for thermal tolerance in this species. The SREM-1 cell line could be used for validating such results.

## Conclusion

A new cell line, named SREM-1, was successfully developed from the eye muscle tissue of an important coldwater fish species of Himalayan waters, viz. *S. richardsonii*, that was found suitable for toxicological

and gene expression studies. The cell line has been deposited in NRFC at ICAR-NBFR, Lucknow, India, and any researcher who wish use the cell line for R&D work could easily access from the NRFC on request.

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## References

- Ahmed, V.P., Chandra, V., Sudhakaran, R., Kumar, S.R., Sarathi, M., Babu, V.S., Bhonde R., & Hameed, A.S.S. (2009). Development and characterization of cell lines derived from rohu, *Labeo rohita* (Hamilton) and catla, *Catla catla* (Hamilton). *Journal of Fish Diseases*, 32, 211-218. DOI: 10.1111/j.1365-2761.2008.00966.x
- Babu, V.S., Chandra, V., Nambi, K.S.N., Majeed, A.S., Taju, G., Patole, M.S., & Hameed, A.S.S. (2011). Development and characterization of novel cell lines from *Etroplus suratensis* and their applications in virology, toxicology and gene expression. *Journal of Fish Biology*, 80: 312-334. <https://doi.org/10.1111/j.1095-8649.2011.03167.x>
- Barat, A., Sahoo, P.K., Kumar, R., Goel, C., & Singh, A.K. (2016). mRNA-Transcriptional response to heat shock in liver of snow trout (*Schizothorax richardsonii*)-a vulnerable Himalayan Cyprinid fish. *Functional and Integrative Genomics*, 16:1-11. <https://doi.org/10.1007/s10142-016-0477-0>
- Bols, N.C., Barlian, A., Chirintorejo, M., Caldwell, S.J., Goegan, P., & Lee, L.E. (1994). Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *Journal of Fish Diseases*, 17:601- 611. <https://doi.org/10.1111/j.1365-2761.1994.tb00258.x>
- Castano, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L.E.J., Mothersill, C., Part, P., Repetto, G., Sintes, J.R., Ruffli, H., Smith, R., Wood, C., Segner, H. (2003). The use of fish cells in ecotoxicology-the report and recommendations of ECVAM workshop 47. *Alternatives to Lab Animals*, 31: 317-351.
- Chen, B., Zheng, Z., Yang, J., Chi, H., Huang, H., & Gong, H. (2019). Development and characterization of a new cell line derived from European eel *Anguilla anguilla* kidney. *Biology Open*, 8. <https://doi.org/10.1242/bio.037507>
- Corral-Vazquez, C., Aguilar-Quesada, R., Catalina, P., Aguilar, G.L., Ligerio, G., Miranda, B., Carrillo-Avila, J.A. (2017). Cell lines authentication and mycoplasma detection as minimum quality control of cell lines in biobanking. *Cell and Tissue Banking*, 18, 271-280. <https://doi.org/10.1007/s10561-017-9617-6>
- Dharmaratnam, A., Kumar, R., Valaparambil, B.S., Sood, N., Pradhan, P.K., Das, S., & Swaminatha, T.R. (2020). Establishment and characterization of fantail goldfish fin (FtGF) cell line from goldfish, *Carassius auratus* for *in*

- in vitro* propagation of Cyprinid herpes virus-2 (CyHV-2). PeerJ 8: e9373. <https://doi.org/10.7717/peerj.9373>
- Drexler, H.G., Dirks, W.G., Macleod, R.A.F., & Uphoff, C.C. (2017). False and mycoplasma-contaminated leukemia-lymphoma cell lines: time for a reappraisal. *International Journal of Cancer*, 140(5):1209-1214. <https://doi.org/10.1002/ijc.30530>
- Driever, W., & Rangini, Z. (1993). Characterization of a cell line derived from zebrafish embryos. *In vitro Cellular and Developmental Biology-Animal*, 29A (9): 749-754. <https://doi.org/10.1007/BF02631432>
- Freshney, R.I. (1994). *Culture of Animal Cells: A Manual of Basic Technique*. Wiley-Liss, New York.
- Freshney, R.I. (2005). *Culture of Animal Cells - A Manual of Basic Techniques*. Wiley-Liss, New York, NY, USA, pp. 641.
- Goswami, M., Sharma, B.S., Bahuguna, S.N., Nagpure, N.S., & Lakra, W.S. (2013). A SRCF Cell line from *Schizothorax richardsonii*: Development and Characterization, *Tissue Cell*: 219-256. <https://doi.org/10.1016/j.tice.2013.02.002>
- Goswami, M., Sharma, B.S., Tripathi, A.K., Yadav, K., Bahuguna, S.N., Nagpure, N.S., Lakra, W.S., & Jena, J.K. (2012). Development and characterization of cell culture systems from *Puntius (Tor) chelynooides* (McClelland). *Gene*, 500(1):140-147. <https://doi.org/10.1016/j.gene.2012.03.016>
- Hightower, L.E., & Renfro, J.L. (1988). Recent applications in fish cell culture to biomedical research. *Journal of Experimental Zoology*, 248: 290-302. <https://doi.org/10.1002/jez.1402480307>
- Huang, Y.C., & Han, Y.S. (2014). Determining anti-betadodavirus compounds through a GF-1 cell-based screening platform. *Antiviral Research*, 105: 47-53. <https://doi.org/10.1016/j.antiviral.2014.02.018>
- Jose, S., Jayesh, P., Mohandas, A., Philip, R., & Singh, I.S.B. (2011). Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. *Marine Environmental Research*, 71: 169-177. <https://doi.org/10.1016/j.marenvres.2010.12.008>
- Kapila, R., Kapila, S., & Basade, Y. (2002). Impact of temperature variation on haematology and serum enzymes of *Schizothorax richardsonii* (Gray). *Indian Journal of Fisheries*, 49: 187-192.
- Khan, M.A. (2004). Environmental Appraisal of depleting Fish diversity of Kashmir Valley. Chapter 23. *Fishery Management*. Ed. Arvind Kumar, A.P.H Publishing Corporation. New Delhi. pp. 175-179.
- Lakra, W.S., Bhonde, R.R., Sivakumar, N., & Ayyappan, S. (2006). A new fibroblast like cell line from the fry of golden mahseer *Tor putitora* (Ham). *Aquaculture*, 253:238-243. <https://doi.org/10.1016/j.aquaculture.2005.09.021>
- Lakra, W.S., John, G., & Barat, A. (1997). Cytogenetic studies on endangered and threatened fishes.2. Karyotypes of two species of snow-trout, *Schizothorax richardsonii* (Gray) and *S. kumaonensis* (Menon). *Proceedings of the National Academy of Sciences, India, Section B: Biological Sciences*, 67(1): 79-81.
- Lakra, W.S., Swaminathan, T.R., & Joy, K.P. (2011). Development, characterization, conservation and storage of fish cell lines: A review. *Fish Physiology and Biochemistry*, 37(1): 1-20. <https://doi.org/10.1007/s10695-010-9411-x>
- Lannan, C.N., Winton, J.R., & Fryer, J.L. (1984). Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro*, 20: 671-676. <https://doi.org/10.1007/BF02618871>
- Mallik, S.K., Shahi, N., Das, P., Pandey, N.N., Haldar, R.S., Kumar, A., & Chandra, S. (2015). Occurrence of *Ichthyophthirius multifiliis* (White spot) infection in snow trout, *Schizothorax richardsonii* (Gray) and its treatment trial in control condition. *Indian Journal of Animal Research* 49 (2):227-30. <http://dx.doi.org/10.5958/0976-0555.2015.00113.2>
- Marques, C.L., Rafael, M.S., Cancela, M.L., & Laize, V. (2007). Establishment of primary cell cultures from fish calcified tissues, *Cytotechnology*, 55: 9-13. <https://dx.doi.org/10.1007%2Fs10616-007-9098-8>
- Nagpure, N.S., Mishra, A.K., Ninawe, A.S., Rasal, A., Dubey, A., Kumar, A., Goswami, M., Kumar, R., & Jena, J.K. (2016). Molecular and cytogenetic characterization of fish cell lines and its application in aquatic research. *National Academy Science Letters*, 39(1): 11-16. <https://doi.org/10.1007/s40009-015-0365-5>
- Nambi, K.S.N., Majeed, A.S., Taju, G., Sivasubbu, S., Raj, N.S., Madan, N., & Hameed, A.S.S. (2015). Development and Use of Retinal Pigmented Epithelial Cell Line from Zebrafish (*Danio rerio*) for Evaluating the Toxicity of Ultraviolet-B. *Zebrafish*, 12(1): 21-32. <https://doi.org/10.1089/zeb.2014.1012>
- Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., & Grabowski, G. (1991). *The simple fool's guide to PCR, Version 2.0*. Privately published. Honolulu: Department of Zoology, University of Hawaii.
- Rodriguez, S.J., Gonzalez, C., Monras, M., Romero, A., Ballesteros, N., Enriquez, R., & Perez- Prieto, S. (2014). Establishment and characterization of a new cell line (SSP-9) derived from Atlantic salmon *Salmo salar* that expresses type IIFN. *Journal of Fish Biology*, 85: 1526-1545. <https://doi.org/10.1111/jfb.12503>
- Schirmer, K. (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology*, 224:163-183. <https://doi.org/10.1016/j.tox.2006.04.042>
- Sharma, O.P., Gupta, S.C., Tripathi, N.K., & Kumar, R. (1992.) On the chromosomes of two species of fishes from Jammu. *Perspectives in cytology and genetics* (eds. Manna GK, Roy SC). 7: 1211- 1215.
- Singh, N., S, Murali., Kushwaha, B., Srivastava, J.K., Srivastava, S., Mishra, A.K., & Kumar, R. (2019). *In vitro* culture and characterization of testis-derived cells from *Clarias magur* (Hamilton, 1822). *Proceedings of the National Academy of Sciences, India, Section B: Biological Sciences*, 89: 1017-1025. <https://doi.org/10.1007/s40011-018-1020-y>
- Soni, P., Pradhan, P.K., Swaminathan, T.R., & Sood, N. (2018). Development, characterization and application of a new epithelial cell line from caudal fin of *Pangasianodon hypophthalmus* (Sauvage 1878). *Acta Tropica*, 182: 215-222. <https://doi.org/10.1016/j.actatropica.2018.03.015>
- Tandel, R.S., Dash, P., Bhat, R.A.H., Sharma, P., Kalingapuram, K., Dubey, M., & Sarma, D. (2020). Molecular Characterization of *Saprolegnia* Spp. From Himalayan Snow Trout, *Schizothorax richardsonii*: A Case Study Report. *Aquaculture*, 531,735824. <https://doi.org/10.1016/j.aquaculture.2020.735824>

- Tong, J., Wang, Y., & Lu, Y. (2016). *In vitro* evaluation of inorganic and methyl mercury mediated cytotoxic effect on neural cells derived from different animal species. *Journal of Environmental Science*, 41:138-145. <https://doi.org/10.1016/j.jes.2015.04.036>
- Vasave, S., Saxena, A., & Srivastava, S. (2016). Karyotypic diversity between rainbow trout (*Oncorhynchus mykiss*, Walbaum) and snow trout (*Schizothorax richardsonii*, Gray). *Journal of Plant and Agriculture Research*, 2(1):1-5.
- Villena, A.J. (2003). Applications and needs of fish and shellfish cell culture for disease control in aquaculture. *Reviews in Fish Biology and Fisheries*, 13: 111-140.
- Vishwanath, W. (2010) *Schizothorax richardsonii*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2
- Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., & Hebert, P.D. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360:1847–1857. <https://doi.org/10.1098/rstb.2005.1716>
- Wolf, K., & Quimby, M.C. (1962). Established eurythermic line of fish cells *in vitro*. *Science*, 135: 1065–1066. <https://doi.org/10.1126/science.135.3508.1065>