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DEVELOPMENT OF CAUDAL FIN CELL LINE FROM HILL TROUT *Barilius bendelisis* (HAMILTON, 1807) FOR CYTOTOXICITY AND TRANSFECTION STUDIES

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ABSTRACT

A cell line named BBdF-1, established from the caudal fin of hill stream fish *Barilius bendelisis*, has been subcultured for more than 52 passages and is being maintained in L-15 media containing 20% FBS. Species origin of the cell line was confirmed using amplification of partial region of 16S and COI mitochondrial gene sequences. The optimum temperature for growth of BBdF-1 cell line was found to be 28°C. Karyotyping revealed diploid chromosome number as 50. Cells exhibited strong binding for cytokeratin marker and thus were found to be epithelial-like. Strong green fluorescence was observed in BBdF-1 cells transfected with phrGFP-II-N vector, indicating its suitability for utilization in gene expression and manipulation studies. Successful assessment of cytotoxicity of two heavy metals, *viz*. mercury and chromium, was performed. The cell line can serve as a useful resource material for early toxicity screening of pesticides/ pollutant and gene expression.

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INTRODUCTION

Barilius bendelisis, a hill stream minor carp belonging to the Cyprinidae family, characterized by a compressed body with blackish-blue bars and distant dorsal fin from the middle point of the body, is found widely distributed in Asian countries, viz. India, Nepal, Bhutan, Sri Lanka, Myanmar, Bangladesh, etc. (Talwar and Jhingran, 1991; Oo, 2002; Sukham et al., 2013; Singh et al., 2016). It is popularly called hill trout or Hamilton's barila (Hamilton, 1822) and constitutes an important food and ornamental fishery in several Himalayan regions of Uttarakhand, Assam, Meghalaya, Manipur and Arunachal Pradesh of India where efforts of raising exotic or Indian major carps have not been successful (Mir et al., 2015; Sahoo et al., 2009; Mishra et al., 2012; Sharma et al., 2015). They are also an important part of small-scale capture fisheries in hilly regions of Odisha, Chhattisgarh, Madhya Pradesh, Telangana and Maharashtra states of India. The species in Bangladesh has been categorized as endangered (IUCN Bangladesh, 2010) even though in India, *B. bendelisis* is categorized as a "least concern" species, the unknown reasons for natural reduction of this species are a matter of concern and therefore its preservation as a genetic resource is of much importance (Mir et al., 2015; Vishwanath, 2010).

Establishment of a cell line is one of the tools to preserve DNA as means of *ex situ* conservation. In India, National Repository of Fish Cell lines (NRFC), the world's largest fish cell line repository at ICAR-National Bureau of Fish Genetic Resources, Lucknow, has been maintaining more than 70 fish cell lines and making them available on-demand for researchers (http://mail.nbfgr.res.in/nrfc/cellline.php). *B. bendelisis* have been reported to be well adapted to most environmental conditions and are potential aquaculture candidate species. Further they are easy to maintain in labs or aquarias (Dewan et al., 2016). The present study was aimed to establish and characterize a new cell line from hill trout *B. bendelisis* and to evaluate it for cytotoxicity and transfection studies. Further, the cell line was also evaluated for optimum growth conditions, cryopreservation potential, chromosome analysis, etc. This is the first cell line to be reported from this species with potential applications for many *in vitro* studies.

MATERIALS AND METHODS

Preparation of tissue for primary culture

The procedure for the development of primary culture was followed according to the standard operating procedures of NRFC laboratory (http://mail.nbfgr.res.in/nrfc/sop.php). In brief, "live specimens of around 20 g body weight of *B. bendelisis* were collected from the Kalsa stream, near Chafi, Uttarakhand, India and acclimatized for a week at 25°C. A healthy specimen was allowed to swim in sterile water containing antibiotic-antimycotic solution (HiMedia cat# A002-20ML taking 10X solution from the 100X) for an hour and anaesthetized using clove oil before use for the study. Then, after surface sterilization of the specimen using 70% ethanol, a piece of caudal fin was aseptically excised and washed thrice with phosphate buffer saline (PBS) containing 2X concentration of antibiotic-antimycotic solution followed by a single wash with 1 ml fresh PBS containing 1X of antibiotic-antimycotic solution (HiMedia cat# A002-20ML). The fin tissue was minced into small pieces with the help of a sterile scalpel blade and transferred to a 25 cm^2 flask and incubated at 25 and 28°C. 100 µl fetal bovine serum (FBS) was added in the culture flask after 4-6 hours for facilitating good attachment of explants. Then 5 ml of Leibovitz's-15 (L-15) (HiMedia), supplemented with 20% FBS containing 1X antibiotic and antimycotic solution, was added to the flask and incubated again. The culture flask was observed daily for cell growth and any contamination. Half of the media was changed at every 4 days interval till the formation of a full monolayer."

Subculture and maintenance

After complete monolayer formation, the cells were harvested and further subcultured, and passaged in the ratio of 1:2 using 30% 1X trypsin-EDTA solution diluted with PBS. The cells were passaged routinely.

Cryopreservation and revival

The cell line was cryopreserved after every five passages in liquid nitrogen (LN_2). When the tissue culture flask reached a confluency level of 70–80%, cells were harvested and collected by trypsinization method. After washing with 2 ml PBS, the cells were re-suspended in a freezing medium (containing pre-cooled L-15 medium supplemented with FBS and dimethyl sulphoxide (DMSO)) as cryopreservative at 9:1 ratio. The cells were then transferred to 1.8 ml cryovials at a density of 1×10^6 cells/ml and stored at - 20°C. After 4 h, the cells were kept overnight at -80°C and then transferred to liquid nitrogen (LN_2) vapour phase for cryopreservation. Cells were successfully revived after 6 months. The cryovials containing cells were taken out from LN_2, thawed quickly at 37°C in a water bath and re-suspended in 10 ml L-15 complete media. Cells were pelletized at 1,000 rpm for 5 min at room temperature (RT), re-suspended again in 6 ml L-15 complete media and seeded into 25 cm^2 cell culture flask. For measuring cell viability, trypan blue staining was used. Live cells were counted using a hemocytometer under an inverted microscope.

Growth studies

To optimize growth conditions, the caudal fin cells were grown at different FBS concentrations and incubation temperatures, keeping one of the factors constant. The cells were trypsinized at 28 passage number, counted and then seeded into 25 cm^2 flasks at 1×10^5 cells per flask and incubated at 28°C overnight for cell attachment. Next day,

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the old medium was replaced with fresh L-15 complete medium supplemented with 5, 10, 15 and 20% FBS concentrations and the flasks were incubated at 28°C in triplicate. Each day, three flasks of each FBS concentration were trypsinized and counted using a hemocytometer. A similar procedure was followed with cells at passage level 30 to study the effect of selected temperatures (*i.e.* 24, 28, 32 and 37°C) on cell growth.

Confirmation of origin of cell line by PCR

Though traditional taxonomic keys were used to identify the fish specimen, the cells were also used to confirm the source species, through amplification and sequencing of partial region of mitochondrial genes using a pair of primers specific to two universal barcoding regions, *i.e.* 16S rRNA and COI genes. Partial amplification of genes was done at 30 passage level. DNA from the cells was isolated using Purelink Genomic DNA mini kit (Invitrogen, USA). "The primers used for 16S were: 5'-CGCCT GTTTATCAAAAACAT-3' (L), 5'-CCGGTCTGAACTCAGATCACGT-3' (H) (Palumbi et al., 1991) and for COI were: 5'-TCAACCAACCACAAAGACATTGGCAC-3' (forward), 5'-TAGACT TCTGGGTGGCCAAAGAATCA-3' (reverse) (Ward et al., 2005)". The amplified products were analyzed at 1.5% agarose gels, stained with ethidium bromide and visualized with ultraviolet transilluminator, to confirm the size. The amplicons were sequenced on 3730xl DNA Analyzer (Applied Biosystems, USA) and were aligned against known sequences from the NCBI database.

Chromosome analysis

The caudal fin cells grown till 70-80% confluence were trypsinized and retained in the same 75 cm^2 size culture flask and were allowed to grow overnight for increasing the number of dividing cells. Next day, 25 µl colchicine (0.05% conc.) was added to the culture media of the flask and incubated at 28°C for 2 h. Afterwards, cells were trypsinized, collected in a tube and incubated with 5 ml hypotonic solution (0.56% KCl) for 20 min at 37°C. After hypotonic treatment, 1 ml chilled Carnoy's fixative (3:1 methanol and acetic acid) was added to the tube and cells were pelletized by centrifugation at 1,200 rpm for 10 min. The supernatant was then discarded leaving 1 ml solution with cell pellet, which was re-suspended in the remaining 1 ml solution followed by addition of 4 ml fresh fixative slowly for cell fixation. The same process was repeated 3-4 times for proper cell fixation. The chromosome preparation was made by conventional splash drop technique (Freshney, 2005). The number of chromosomes in each spread was counted under a light microscope (Leica DMLB-2). A total of 30 spreads were counted.

Confirmation of cell morphology

The cells were grown on a sterile coverslip and incubated overnight at 28°C for attachment. Next day, the cells were washed twice with PBS followed by cell fixation and permeabilization with 1:1 methanol-acetone solution for 20 min at -20°C and then incubated with 3% BSA in PBS (3% P-BSA) solution for 1 h at 37°C. After 1 h, the cells were washed twice with PBS and incubated overnight at 4°C with primary antibodies [mouse anti-cytokeratin pan (Sigma), anti-fibronectin (Sigma) and mouse anti-vimentin antibodies] diluted in 1% PBSA. For control, 1% PBSA without primary antibodies was used. Cells were then washed with PBS and incubated at 37°C for 1 h with rabbit anti-mouse IgG FITC conjugate (diluted 1:100 in PBSA). Finally, the coverslip was washed again with PBS, mounted in 1:1 of PBS & glycerol and checked for fluorescence.

Plating efficiency and doubling time

Doubling time and plating efficiency were calculated as described by Freshney (2005). Plating efficiency, also known as clonogenic assay or colony-forming assay, was determined at 33 passage level. Briefly, cells were trypsinized, counted and seeded into 25 cm^2 culture flasks at a density of 100, 500 and 1,000 cells per flask in triplicates. L-15 medium supplemented with 20% FBS was used to culture the seeded cells at 28°C for the study. On every 4^{th} day, half of the medium was changed. After 12 days, flasks were decanted, cells washed with PBS, fixed using methanol and stained with crystal violet. The individual cell colonies were then counted under an inverted microscope. The population doubling time of the cell line was calculated at two passages, 32 and 40.

Mycoplasma detection

The cell line was checked for mycoplasma contamination using a PCR-based test. Cells were subcultured at passage level 35 in L-15 complete medium, containing 20% FBS without antibiotics, for 5 days. Afterwards, 2 ml of the supernatant from the flask was collected and centrifuged at 13,000 rpm for 30 min. Obtained pellet was dissolved in 50 µl of 1× TE buffer, then vortexed and heated for 10 min at 95°C. The EZdetectTM PCR kit (HiMedia, India) for mycoplasma detection was used as per the manufacturer's protocol using positive control (as provided in the kit), negative control (where no DNA template used) and cell supernatant. For this, amplification of the region between 16S and 23S rRNA was carried out, and the amplified products were electrophoresed at 2% agarose gel and visualized under UV transilluminator.

Cytotoxicity assays

The cytotoxicity of two salts of heavy metals, mercuric chloride ($HgCl_2$) and potassium dichromate ($K_2Cr_2O_7$), was evaluated using the cell line through alamarBlue assay. Briefly, 3×10^4 cells per ml in 100 µL of L-15 medium supplemented with 20% FBS were seeded in 96 well plates and incubated overnight at 28°C. The culture medium was then discarded and 100 µL of L-15 containing different concentrations (3000, 1500, 750, 375, 187.5, 93.75, 46.87, 23.43, 11.71 µM) of analytical grade $HgCl_2$ (Sigma-Aldrich, USA) was added to the wells in triplicates and incubated at 28°C. Toxicant concentration was prepared

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in L-15 supplemented with 4% FBS. In control, the cells present in the wells were incubated only with 100 µL of L-15 medium. After 24 h, 10 ul alamarBlue reagent was added to the wells and incubated for 2 h. Then, light absorption was measured at 2 wavelengths, 570 and 600 nm, using a spectrophotometer (Synergy, BioTek Instruments, Winooski, VT, USA) and their respective readings were recorded. Similar methodology was also used for analytical grade $K_2Cr_2O_7$ (Sigma-Aldrich, USA), where concentrations ranged from 1000 to 3.9 µM. The recorded absorption readings were then subjected to dose-response curve fitting using GraphPad Prism 6 Software and IC_{50} value was calculated.

Transfection

The caudal fin cells at 51 passage level were seeded into 6 well plates and incubated overnight at 28°C for attachment. Sub-confluent monolayers of cells were transfected with 1.5 µg of phrGFP-II-N vector (Clontech, Takara Bio USA, Inc.), using lipofectamine 2000 (Invitrogen, Carlsbad, USA). After 48 h, the 6 well plates were observed under a Nikon fluorescence microscope.

RESULTS

Primary cell culture and subculture

While the radiation of cells from the fin tissues of *B. bendelisis* was observed on the 4th day of setting of explants, good radiation from most of the explants was noticed only after 7 days.

Formation of a complete monolayer of cells took around 20-25 days, after which they were harvested and subcultured every 5-6 days at a ratio of 1:2. The morphology of the subcultured cells didn't change after many successive passages and seemed stable. As the flasks started getting confluent very fast, the percentage of FBS concentration was reduced to 15% after the 35th passage. The cell line was subcultured until 52 passages over a period of one year and designated as BBdF-1 (Fig. 1A, B).

Growth studies

Fin cells of *B. bendelisis* showed different growth at different culture temperatures, viz. 24, 28, 32 and 37°C. The maximum growth rate was obtained at 28°C, while the cell growth was modest at 24 as well as 32°C but lowest at 37°C. The growth rate of cells at 28°C temperature increased as the FBS concentration increased from 5% to 20%. At 5% FBS concentration, the cell growth was lowest but the cells were healthy. Although the cells grew well at 10% and 15% FBS concentration, the maximum growth was observed with 20% FBS concentration (Fig. 2A, B).

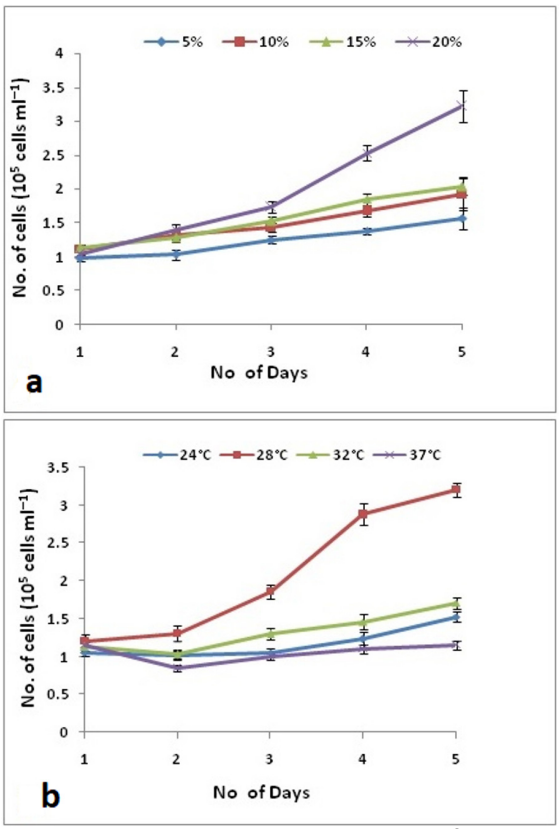

Fig 2. Graphs showing growth responses of BBdF-1 for various: (a) fetal bovine serum concentrations, and (b) culture temperatures

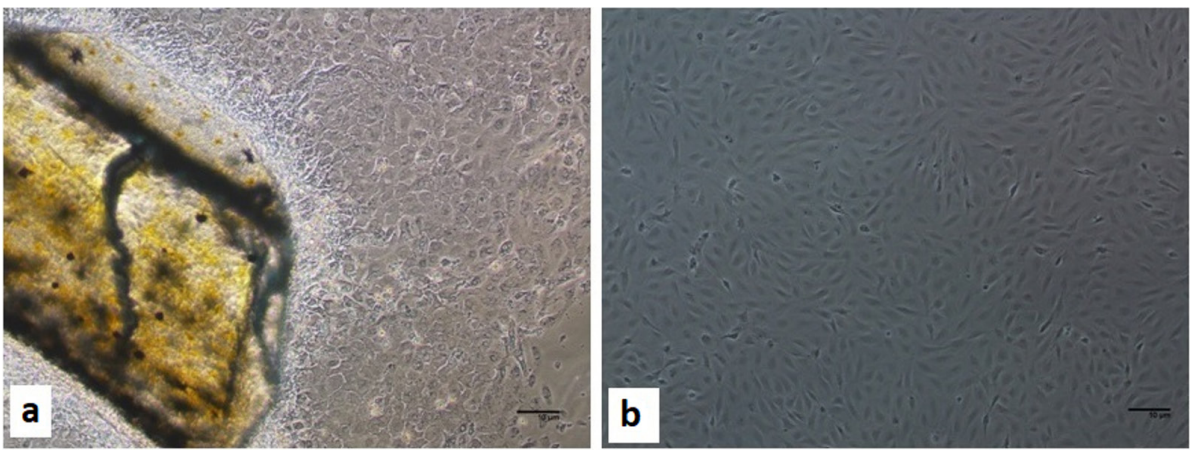

Fig 1. Image of *B. bendelisis* cells derived from fin tissue: (a) primary culture on the 5th day after tissue explant, (b) monolayer of cells at 25 passage

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Cryopreservation

The BBdF-1 cells were cryopreserved successfully and revived with 75±4.98% viability after 4 months of storage in LN_2. The cells grew well and reached confluence within 6 days in L-15 media containing 20% FBS after revival.

Confirmation of cell origin

To confirm the origin of the cell line, partial regions of 16S rRNA and COI mitochondrial genes were amplified using template DNA of BBdF-1 cells. Partial COI gene has been submitted to NCBI GenBank database (Accession no: MN539107). The sequenced regions were blasted against available sequences in NCBI database, and the similarity for 16S rRNA and COI regions of these were found to be 96.49 and 100%, respectively, against known *B. bendelisis*.

Chromosome analysis

Chromosome counts of 30 metaphase spreads from BBdF-1 cells at 28 passage level revealed the variation in diploid chromosome numbers from 40 to 58. However, the majority of the BBdF-1 cells showed 50 as a model diploid chromosome number in metaphase spreads (Fig. 3).

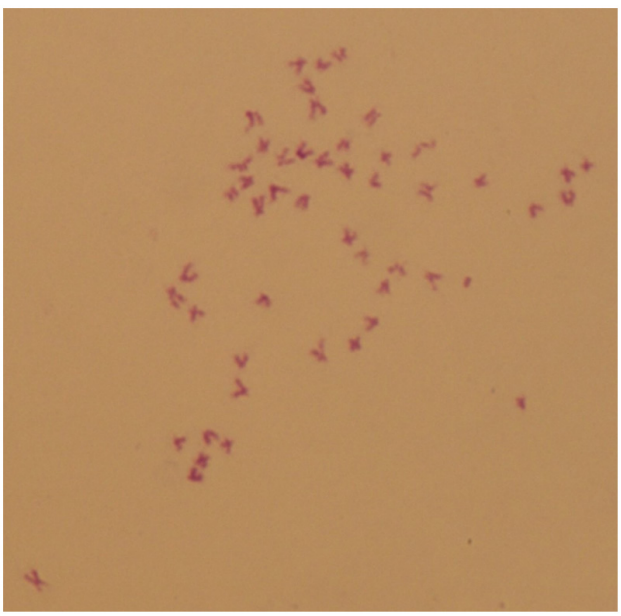

Fig 3. Metaphase chromosome complement of BBdF-1 cells

Plating efficiency and doubling time

The BBdF-1 cells seeded at a density of 100, 500 and 1000 cells per flask showed 5.3, 6.5 and 8.06% plating efficiency, respectively. The plating efficiency improved with an increase in the seeding density. Doubling time of the BBdF-1 cell line was found to be 68 h in L-15 medium supplemented with 20% FBS.

Mycoplasma detection

BBdF-1 cells were found to be free from mycoplasma contamination (Fig. 4).

Fig 4. Gel image showing PCR product for mycoplasma detection (Lane M: 100 bp DNA ladder, Lane 1: positive control, Lane 2: negative control, and Lane 3: BBdf-1 cell supernatant)

Transfection

The BBdF-1 cell line was successfully transfected with lipofectamine 2000 transfection using phrGFP-II-N plasmid. Green fluorescence was observed after 48 h of transfection, which indicated the expression of green fluorescent protein (GFP) reporter in BBdF-1 cell line. The result suggests the suitability of the BBdF-1 cell line for gene expression studies (Fig. 5).

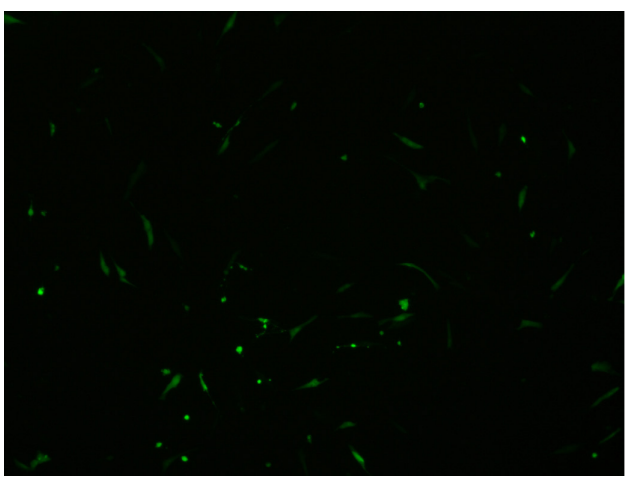

Fig 5. Expression of GFP gene in BBdF-1 cell line at 51 passage level

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Cell type

In cell type determination of BBdF-1 cells by immuno-phenotyping using antibody markers, strong positive green fluorescence signals for anti-cytokeratin pan antibody was recorded while no signals were observed in control anti-fibronectin and anti-vimentin antibodies, which indicated epithelial-like cell type (Fig. 6).

Cytotoxicity studies

The BBdF-1 cells were tested for their applicability for cytotoxicity evaluation. A decrease in absorbance was recorded with increasing concentration of both metal salts. The observed IC_{50} values were 190.2 µM (139.2-259.9) for $HgCl_2$ and 56.48 µM (43.7-73.01) for $K_2Cr_2O_7$ using alamarBlue assay (Fig. 7).

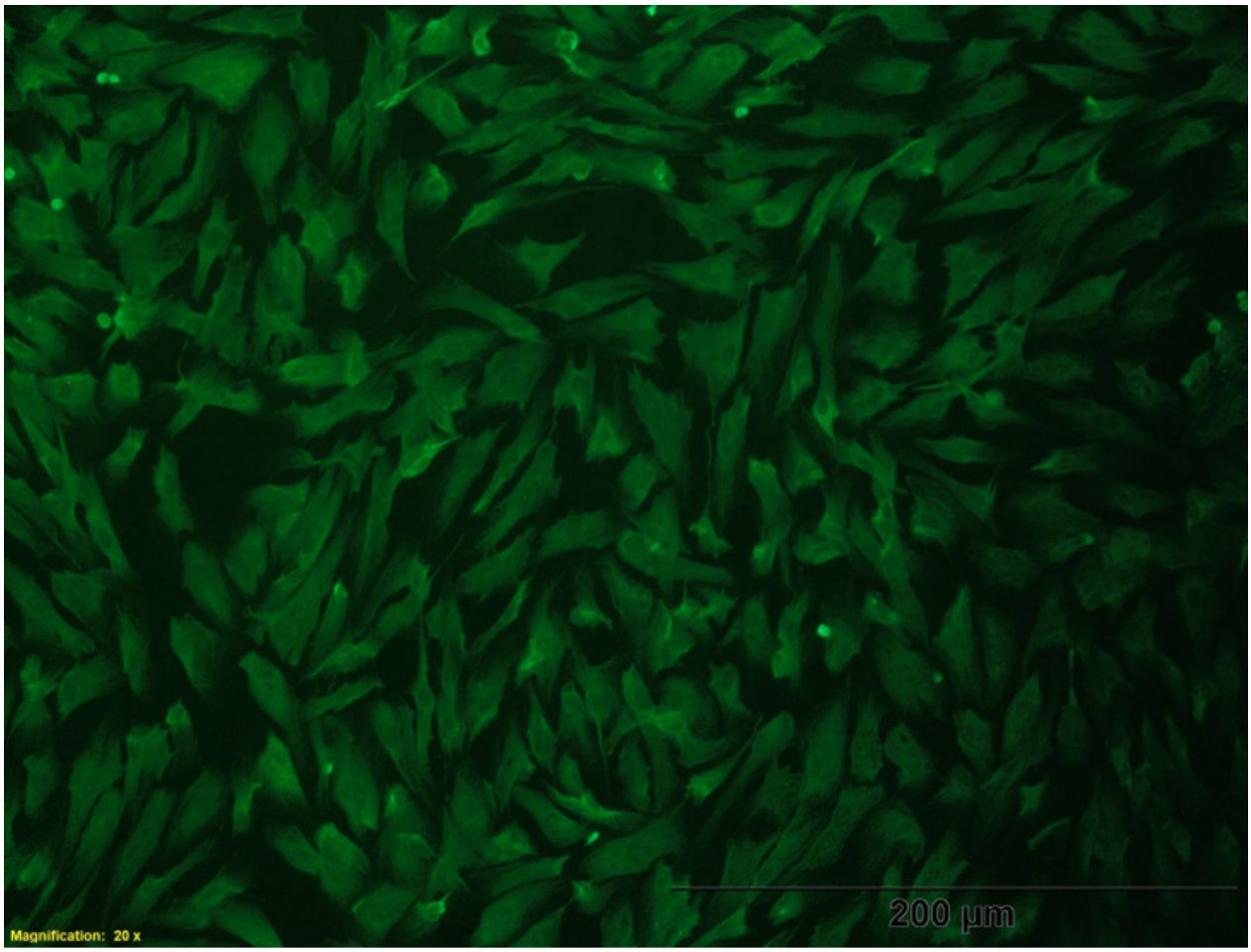

Fig 6. Fluorescent photomicrographs showing the presence of cytokeratin marker in BBdF-1 cells at) 20× Magnification

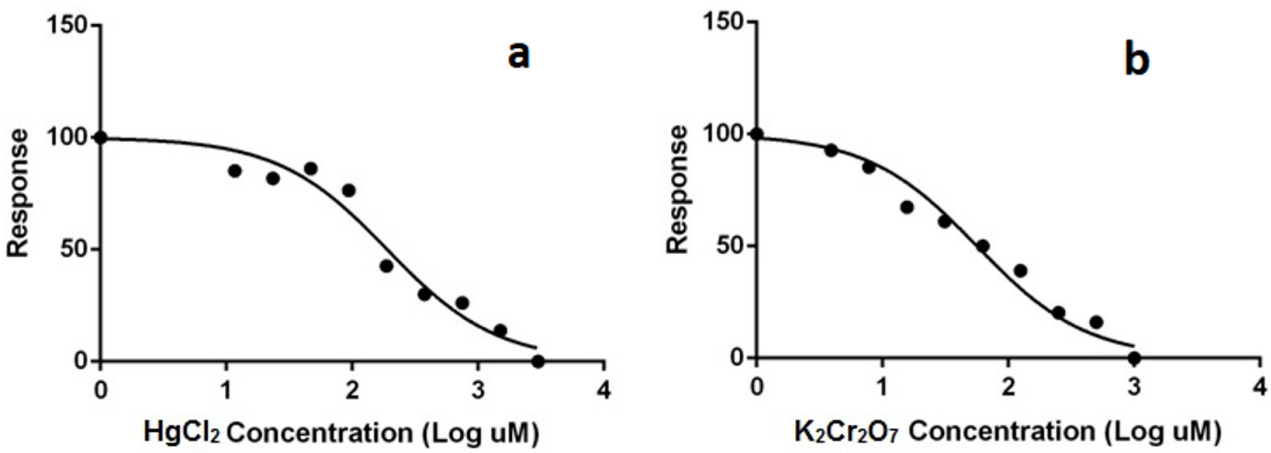

Fig 7. Viability of BBdF-1 cells after 24 h exposure to different test concentrations of $HgCl_2$ and $K_2Cr_2O_7$ using alamarBlue assay

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DISCUSSION

Cell lines from different organs, *viz*. gill, kidney, spleen, eye, muscle, swim bladder, skin, liver, etc., of different fish species have been established. Cellosaurus, a bioinformatics portal on cell line lists 714 fish cell lines (https://web.expasy.org/cgi-bin/cellosaurus/search). This phenomenal number of fish cell line development is because of growing applications of fish cell lines in toxicology, gene expression studies, developmental biology, biomedical research, etc. (Hightower and Renfro, 1988; Ryan et al., 2008; Lakra et al., 2011).

In the present study, a new fish cell line has been established from the caudal fin of *B. bendelisis* using explant method and designated as 'BBdF-1'. Explant method for primary culture is preferred over enzymatic method due to its ease, rapidity to avoid cell damage and has already been proven to be more suitable for many species as reported by various other researchers (Avella et al., 1994; Lakra et al., 2010; Chaudhary et al., 2014; Soni et al., 2018). The suitable temperature range for BBdF-1 cells was 24-32°C with optimum growth at 28°C, which has also been reported by many researchers (Tong et al., 1997; Ku et al., 2009; Lakra et al., 2011) for several cell lines. The cells were barely surviving at 37°C and showed almost negligible growth, probably because the habitat of this species is hilly and low-lying regions where temperatures do not reach this limit. Likewise, the optimum serum concentration (*i.e.* FBS) was found to be 20% in L-15 medium. For long term culture and experiments, 5% FBS containing L-15 medium is not recommended for BBdF-1 cells due to comparatively lower growth rate. Cells also grew at 10% FBS in L-15 medium indicating that BBdF-1 cells could also be maintained in L-15 medium (10% FBS) to reduce the operating cost and the probability of contamination due to high serum content. Further, the culture of BBdF-1 cells is cost-effective as they are grown in L-15 and there is no requirement of expensive CO_2 incubators. They can easily be grown in normal BOD incubators, unlike human and popular animal cell lines.

The average doubling time of 68 h, reported for BBdF-1 cells at different passages, is higher than *Schizothorax richardsonii* (48 h, Goswami et al., 2013), *Scophthalmus maximus* (45.6 h, Fan et al., 2010), *Channa striata* (29 h, Majeed et al., 2015) but the cell line exhibits slow and stable growth rate. Plating efficiency, determined as around 8% for 1000 cells per flask for BBdF-1, though not very high, exhibits an increase in efficiency with increasing seeding density resulting in better cell growth. There are several other reports on various fish cell lines with low plating efficiencies (Swaminathan et al., 2012; Ma et al., 2013; Rodriguez et al., 2014).

It was possible to successfully subculture BBdF-1 cells for more than 50 passages in a period of 7 months. With increasing passages, cultured cells may attain some genotypic or phenotypic changes. Moreover, risk of culture loss is always there due to contamination and/or natural death of cells. To save the culture, cryopreservation is of much importance. BBdF-1 cells were cryopreserved at different passages and revived successfully with no morphological alternations. Maximum cell viability revived cells after cryopreservation was found to be 75±4.98% for BBdF-1 cells, whereas other researchers have reported 80-85% (Goswami et al., 2013), 90-92% (Majeed et al., 2015), etc.

Different molecular markers are used to confirm the origin of cells. Partial 16S rRNA and COI genes have largely been employed for species characterization and phylogenetic analysis of many different species including fish and have proved to be an effective barcoding marker (Masuda et al., 1990; Marshall, 2005; Singh et al., 2016; Lakra et al., 2016). These two genes helped authenticate the origin of BBdF-1 cells from *B. bendelisis*. Diploid model chromosome number of 50 further authenticated its origin from the same species, as also documented in other studies (Sukham et al., 2013). Different cell markers are used to confirm the types of cells. Strong fluorescence observed for cytokeratin antibodies revealed the epithelial-like type of the BBdF-1 cell line. Such fluorescence-based antibody marker tests were also used by other researchers with similar results (Revest et al., 2001; Gjessing et al., 2018; Soni et al., 2018).

BBdF-1 cells transfected with the GFP gene showed green fluorescent signals after 48 h. Although the transfection efficiency was not calculated using flow cytometry, it was estimated to be 8-9% based on spot counting, which indicates that the BBdF-1 cells can also play a pivotal role in gene manipulation studies in this omics era.

Cytotoxicity testing using *in vitro* approach is one of the most important aspects at the current time. Fish cell lines have been used and are also continuously being employed for toxicity studies because fish are in direct contact with many eco-toxicants (Babich and Borenfreund, 1987). Moreover, reproducibility of results, ease and no requirement of animal killing make *in vitro* studies more suitable. In the present study, the IC_{50} values for BBdF-1 cells exposed against $HgCl_2$ were higher (190.2 µM) than $K_2Cr_2O_7$ (56.48 µM), which shows that chromium is more toxic than mercury (Tchounwou et al., 2012; Bakshi and Panigrahi, 2018). A gradual decrease in absorbance was recorded as per increase in toxicant concentration. Similar results were also obtained by different researchers for mercury (Sood et al., 2015; Tong et al., 2016) and chromium (Tan et al., 2010; Di et al., 2017). The *B. bendelisis* cell line has been deposited at NRFC at ICAR-NBFGR, Lucknow, Uttar Pradesh (India) (NRFC Accession no: NRFC061), thus making it available to researchers globally for R&D work.

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CONCLUSION

Hill streams harbor wide biological diversity and are also natural habitat of a large number of fish species. They also play an important role in maintaining ecological balance. As very few cell lines from cold water and hill stream fishes are available, *B. bendelisis* cell line BBdF-1 has been developed and characterized using several standard parameters. This cell line has proved to be useful for various studies, *viz*. toxicological and gene transfection studies. This cell line can also be a beneficial source for virus isolation not only in this species but also for other hill stream fishes if any viral disease is reported. The developed cell line thus can lead researchers to expand further their experimental insights for investigating disease outbreaks, environmental pollutant monitoring and other studies.

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RAZVOJ STANIČNE LINIJE REPNE PERAJE IZ BRDSKE ŠARANKE, *Barilius bendelisis* (HAMILTON, 1807), ZA STUDIJE CITOTOKSIČNOSTI I TRANSFEKCIJE

SAŽETAK

Stanična linija, nazvana BBdF-1, uspostavljena iz repne peraje *Barilius bendelisis*, presađena je na više od 52 „oblika"(prolaza) a održavala se u medijima L-15 koji sadrže 20% FBS. Porijeklo stanične linije potvrđeno je pomoću pojačanja djelomičnog područja 16S i COI mitohondrijskih genskih sekvenci. Utvrđeno je da je optimalna temperatura za rast stanične linije BBdF-1 28°C. Kariotipizacijom se utvrdio diploidni broj kromosoma kao 50. Stanice su pokazale snažno vezanje za citokeratinski marker i, prema tome, utvrđeno je da sliče epitelu. Jaka zelena fluorescencija primijećena je u stanicama BBdF-1 transficiranim vektorom phrGFP-II-N, što ukazuje da je prikladna za upotrebu u ispitivanjima ekspresije i manipulacije gena. Izvršena je i uspješna procjena citotoksičnosti dvaju teških metala, žive i kroma. Stanična linija može poslužiti kao koristan izvorni materijal za ranu provjeru toksičnosti pesticida/zagađivača i ekspresije gena.

Ključne riječi: *Barilius bendelisis*, stanična linija, citotoksičnost metala, NRFC

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