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Development, characterization and *in vitro* applications of a thymus cell line from *Pangasianodon hypophthalmus* (Sauvage 1878)

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ABSTRACT

Pangasianodon hypophthalmus is an economically important catfish species cultured in India. In the present study, a continuous cell line from the thymus of *P. hypophthalmus* (PHT) was established using the explant method and subcultured 52 times since development. PHT cells showed optimal growth in L-15 medium containing 20% fetal bovine serum at 28°C. The species of the cell line from striped catfish was confirmed through PCR amplification and sequencing of 16S, and COI genes. The cell line was found to be Mycoplasma free. The modal chromosome number of PHT cells was 60 (2n). Immunophenotyping using different antibodies showed the epithelial nature of the cells. Cytotoxicity of arsenic and mercury was assessed using Neutral red and MTT assay, which revealed reduced cell survival with an increase in toxicant concentration. Cells transfected successfully with the GFP reporter gene using lipofectamine reagent indicated the suitability of the cell line for expression studies. The cell line has been submitted to NRFC, Lucknow with accession no. NRFC-078 at ICAR-NBFGR, Lucknow. The developed cell line will have applications in suspected viral disease investigation, transgenic, and immunological studies.

Keywords: Chromosome, *Pangasianodon hypophthalmus,* Thymus

Pangasianodon hypopthalmus is one of the important cultured catfish species in Southeast Asian countries including India, Bangladesh, Thailand, Vietnam, Indonesia, Malaysia, and Philippines (Globefish 2012). In India, the culture of *P. hypopthalmus* is spreading fast (Singh *et al.* 2012, Mugaonkar *et al.* 2019) due to fast growth, easy culture, good acceptability to artificial feed, and survival ability in adverse conditions like high salinity and low oxygen level (Ali *et al.* 2013). However, intensification of culture poses the risk of several diseases including infection with the Channel catfish virus (Siti-Zahrah *et al.* 2014), epitheliocystis (Sood *et al.* 2018), etc.

The thymus is the primary lymphoid organ located in the dorsal side of the branchial cavity in teleosts (Barraza *et al.* 2020). While the thymus plays an important role in the immune system of vertebrates, its role in fishes is not completely understood. However, some reports indicate that the thymus plays a pivotal role in the fish's immune response to pathogenic infections (Chilmonczyk 1992, Vasiliev and Polevshchikov 2014). A few fish thymus cell lines are available (Chaudhary *et al.* 2013, Rebello *et al.* 2014, Sood *et al.* 2015) for such studies but to date, none is reported from *P. hypophthalmus*. Due to the growing interest in *P. hypophthalmus* culture, a new epithelial cell line has been established from the thymus of this species and the cells have been employed successfully for cytotoxicological studies of mercury and arsenic.

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MATERIALS AND METHODS

Primary culture and routine maintenance: The explant method was used to perform primary culture from *P. hypophthalmus* thymus. A healthy fish weighing approximately 1 kg was purchased from a local market. Thereafter, the fish was anesthetized using MS-222 and the surface was disinfected using 70% ethyl alcohol solution. Thymus was excised from the opercular cavity, washed with PBS containing 2× Antibiotic-Antimycotic Solution (AAS), (Gibco, GI, USA), and explants were then transferred to a 25 cm^2 cell culture flask and allowed to attach at 28°C for 4 h. Afterwards, 5 ml L-15 medium containing 20% FBS with 2× AAS was added, and 2.5 ml medium was replaced after every five days. After monolayer formation, trypsin solution (Sigma, St. Louis, USA) was used to subculture the cells.

Cryopreservation and revival: Cryopreservation is an ideal method for the storage of developed cell lines that otherwise may not survive due to various reasons, including contamination, and senescence. PHT cells were cryopreserved at different passage levels (5, 10, 15, 20, 30, 40). Briefly, cells were grown up to 90% confluence in a 75 cm^2 cell culture flask, trypsinized, centrifuged, and resuspended into 1 ml freezing medium (FBS and Dimethyl sulphoxide in a ratio of 9:1) and transferred to a cryovial. The vial was finally transferred to liquid nitrogen after

keeping it at -80°C for 24 h. To revive cells at a passage 15, 25, and 35, the cryovial was kept in the water bath at 37°C for quick thawing. Cells suspension was then added in a dropwise fashion to a 10 ml L-15 medium containing 20% FBS and centrifuged at 1500 rpm for 10 min. Medium was discarded and cells were transferred to a 25 cm^2 flask after resuspending them into 5 ml fresh medium. Live cells were counted in the hemocytometer using a trypan blue solution.

Effect of FBS concentration and temperature on cell growth: The optimum temperature and FBS concentration for PHT cells were also determined. At 28th passage, a total of 1×10^5 cells were seeded in T-25 cell culture flasks (NuncTM, Thermofisher, Denmark) and incubated at selected temperatures i.e. 24, 28, 32, and 37°C. The growth of cells was monitored for five consecutive days. At the same passage with the same cell number per flask effect of FBS concentrations was also determined; cells were grown in L-15 medium supplemented with different FBS concentrations, i.e. 5, 10, 15, and 20% at 28°C. A total of three flasks were used for each selected temperature and FBS concentration, where cells were harvested every day by trypsinization and counted using a hemocytometer.

DNA Barcoding: To authenticate the species of PHT cells at the 35th passage, two universal primers 16S, and COI, were used (Palumbi 1991, Ward *et al.* 2005). For this, DNA was isolated from PHT cells using a DNA extraction kit (Qiagen, Germany) as per the protocol recommended by the manufacturer. DNA purity was analyzed with a spectrophotometer. Sequencing of PCR products was done using ABI 3730xl sequencer (Applied Biosystems, USA) and obtained sequences similarity was checked using BLAST against the available DNA sequences at NCBI Genbank.

Detection of Mycoplasma contamination: At the 38th passage, PHT cells were checked for *Mycoplasma* contamination using EZdetectTM PCR kit (HiMedia Laboratories, India). The test was done as per the manufacturer's protocol. Final PCR products were run on 2% agarose gel and visualized using a UV transilluminator.

Chromosome analysis: PHT cells at the 34th passage were used to prepare chromosomal spreads (Freshney 2015). After preparation and proper drying, slides were stained with Giemsa and observed at 100× magnification using a light microscope, and chromosomes in 100 spreads were counted.

Immunocytochemistry: To confirm the morphology of PHT cells at passage 32, antibodies against different cell surface markers were used (Mauger *et al.* 2009). Immunostained cells on coverslips were finally mounted with Vectashield mounting medium and checked for fluorescence under a microscope.

Cytotoxicity: MTT assay and Neutral red assay were carried out to assess the toxicity of selected heavy metals, i.e. Arsenic (As) and Mercury (Hg) on PHT cells. The salts used were mercuric chloride ($HgCl_2$) and sodium arsenate dibasic heptahydrate ($Na_2HAsO_4.7H_2O$). The serial dilution method was used to prepare different dilutions of two salts from the highest to the lowest dose. The range of dilution for $HgCl_2$ was 1000-3.9 µM and for $Na_2HAsO_4.7H_2O$ range was 1500-5.85 µM. IC50 value, where cell survival is inhibited to 50% after 24 h of exposure to metal salts, was calculated using prism7 software. Briefly, cells were trypsinized, resuspended into the L-15 medium containing 20% FBS, and counted using a hemocytometer. A total of 2.5×10^4 cells were seeded in each well of a 96-well plate and incubated at 28°C for cell attachment. All dilutions of both metals salts along with controls were taken in triplicates.

Neutral red uptake assay: PHT cells at passage level 36th were used to perform the neutral red assay (Repetto *et al.* 2008) where absorbance was measured using a spectrophotometer at 540 nm to calculate IC50.

MTT assay: MTT assay for PHT cells was carried out at passage 40 (Borenfreund *et al.* 1988). Absorbance was checked using a spectrophotometer at 570 nm and obtained values were used to calculate IC50.

Plating efficiency: PHT cells at the 30th passage were used to determine plating efficiency (Freshney 2015).

Transfection: At 32nd passage, PHT cells were detached using trypsin and plated into a six-well plate at a concentration of 1×10^5 cells per well. Thereafter, the L-15 medium was replaced with fresh opti-MEM (Gibco) medium and incubated at 28°C for one hour. After 30 minutes of media replacement, transfection reagent was prepared by mixing 1.5 µg phrGFP-II-N vector (Clontech, Takara) in 5 µl lipofectamine 2000 (Invitrogen, Carlsbad USA) in a 1.5 ml tube and incubated for 30 minutes at RT. This transfection reagent was then added to the semiconfluent monolayer and the plate was kept at 28°C. Cells were checked for transfection after 48 h under the fluorescent microscope.

RESULTS AND DISCUSSION

Fish cell culture is being used by researchers worldwide. It has become a valuable biological tool to conduct various studies under *in vitro* conditions. It is also preferred over *in vivo* studies because of ethical issues involved in the usage of live animals (Kumar *et al*. 2019). Keeping the same in consideration, a new thymus cell line was established and characterized from *P. hypophthalmus*. The cell line was passaged 52 times and designated as PHT.

After the removal of the thymus from the gill chamber, a small fragment of it was placed on a slide. This fragment was pressed using a coverslip, and checked under a microscope and a large number of thymic lymphocytes were observed (Fig. 1A and 1B). Subsequently, the explant method was used to initiate culture from the thymus tissue of *P. hypophthalmus*. Cell migration from the explants was observed after one week of primary (Fig. 1C) culture and the monolayer was obtained on day 35 (Fig. 1D). The cells were trypsinized, followed by splitting at a ratio of 1:2, and formation of the monolayer was observed after 5-6 days of passaging.

PHT cells were cryopreserved in liquid Nitrogen and

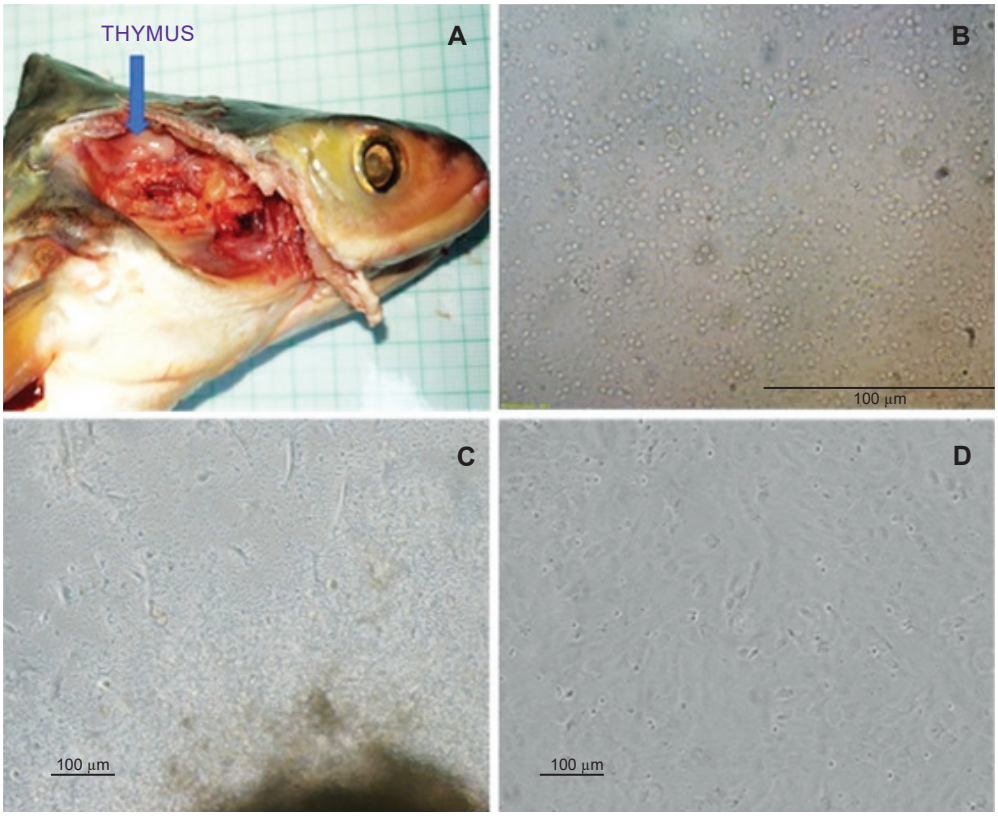

Fig. 1. (A) Location of thymus in *P. hypophthalmus,* (B) Wet mount of thymus showing thymic lymphocytes, (C) Primary culture thymus cells after 7 days, (D) Monolayer of PHT cells at passage 25.

revived successfully. The cell viability of PHT cells was 71.42±5.58%. Moreover, after attachment and passaging, no alteration in morphology and growth pattern was recorded. The revival rate is similar to that of other fish cell lines (Soni *et al.* 2018, Yashwanth *et al.* 2020).

The optimum growth of PHT cells was found with 20% FBS which gradually reduced to 15% and 10% FBS while 5% FBS was not supportive of its growth. Growth of PHT cells was good at 24, 28, and 32°C but very slow at 37°C. Cells were grown at 28°C from starting and the same temperature was found optimum for the cell line's growth (Supplementary Fig. 1A and 1B). L-15 is a very suitable medium for the development of fish cell lines in the temperature range (24-37°C) with optimum growth at 28°C (Chaudhary *et al.* 2012, Chen *et al.* 2019, Sathiyanarayanan *et al.* 2022).

Species validation is a must to confirm the origin of a cell line. Among modern advanced technologies, DNA barcoding using universal primers for the amplification of mitochondrial genes including COI, 16S, 12S, and 18S is a recommended method for species identification (O'Donoghue *et al.* 2011, Jing *et al.* 2017). In the present study, partial regions of 16S rRNA and COI genes were amplified and sequenced for the PHT cell line's species authentication (Fig. 2A). The sequences of COI and 16S rRNA amplicons showed 99.49% and 99.24% sequence similarity, respectively with available sequences of both genes of *Pangasianodon hypophthalmus* in NCBI Genbank (Accession numbers for 16S rRNA- MT872507; COI - MT863239).

Microbial contamination is a major issue during the development and maintenance of a cell line. Bacterial and fungal contamination can be easily observed due to *p*H change and/or turbidity. Though, to check presence of mycoplasma in culture is a tedious task as it is the smallest living microorganism. *Mycoplasma* can persist in culture for

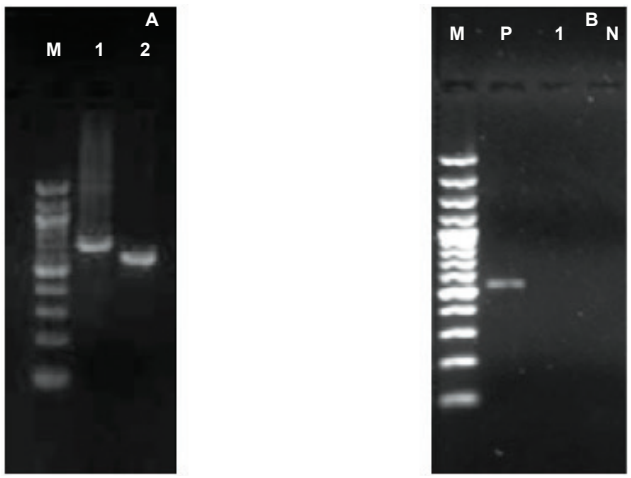

Fig. 2. Agarose gel images. (A) PCR amplification of COI and 16S rRNA genes (Lane M-100 bp marker, Lane 1-COI gene , Lane 2-16S rRNA gene), (B) Mycoplasma detection; (Lane M-100 bp Marker, Lane P-Positive control, Lane 1-PHT cell's supernatant used as template, Lane N-negative control).

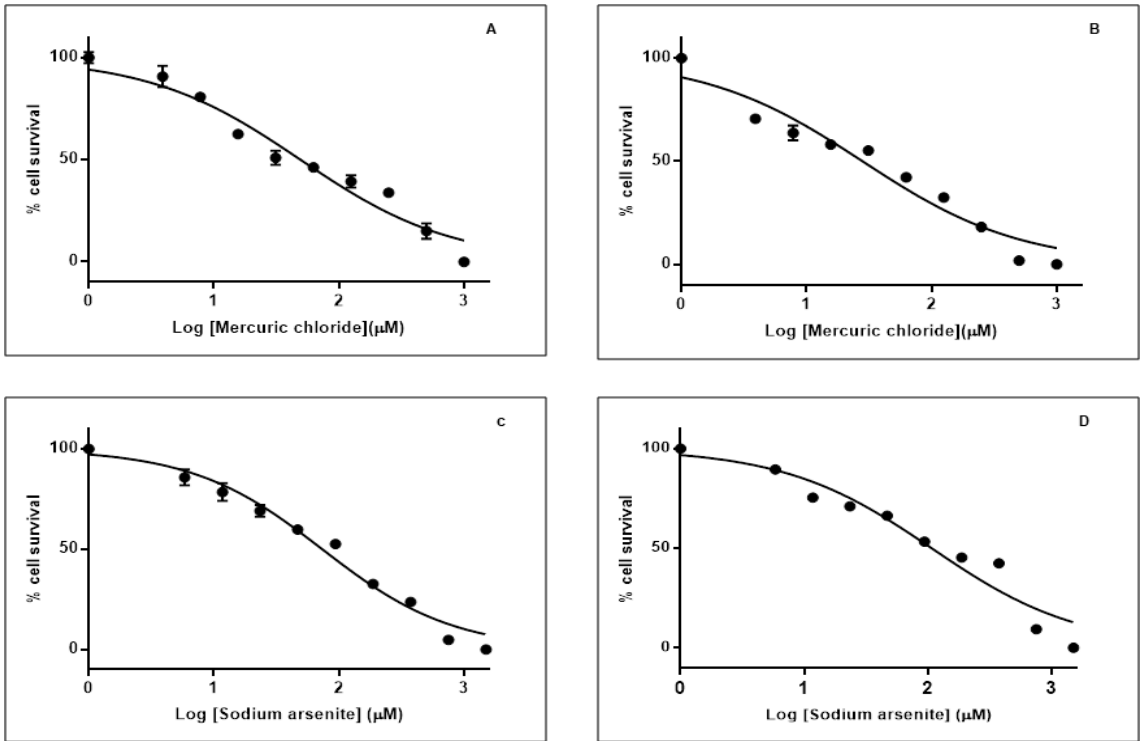

Fig. 3. Result of statistical analysis for IC50 calculation. (A) Mercuric chloride using MTT assay, (B) Mercuric chloride using NR assay, (C) Sodium hydrogen arsenate heptahydrate using MTT assay, (D) Sodium hydrogen arsenate heptahydrate using NR assay (where base of log values -Log_{10}).

a long period. It can further alter the cells and consequently the final result of experiments being performed on infected cells (Frerichs 1996). PHT cells were found to be negative for mycoplasma contamination using PCR based kit along with positive control (Fig. 2B).

Chromosome preparation demonstrates whether a cell line is genetically stable or not. They carry the genetic information from one generation to the next. The modal chromosome number of PHT cells was found to be 60 (2n). This is similar to that reported for *P. hypophthalmus* (Magtoon and Donskul 1987, Khuda-Buksh 2007) (Supplementary Fig. 2A and 2B). Though the chromosome count of a cell line does not always coincide with its original species (Hsu 1973) yet unchanged number of chromosomes shows that the cell line is stable and additionally serves as a cytogenetic marker for species identification (Thangaraj *et al.* 2021).

PHT cells comprised epithelial and fibroblastic cell characteristics during their development, comparable to other fish cell lines (Swaminathan *et al.* 2010, Fu *et al.* 2015). Cells became homogenous with successive passages due to epithelial cells that dominated over fibroblastic cells. In immunocytochemistry, strong fluorescent signals were observed with the anti-pancytokeratin antibody whereas no reactivity was observed with the anti-fibronectin antibody (Supplementary Fig. 3). Thymus cell lines from *Channa striatus* and *Catla catla* were also found to be epithelial in origin (Chaudhary *et al.* 2013, Sood *et al.* 2015).

PHT cell line proved to be a valuable tool for the toxicity assessment of two heavy metals. Here mercury and arsenic were selected since both are considered potential ecotoxicants (Pandey and Bhatt 2015). Decreased cell survival was noticed with the gradual increase in the concentration of metal salts. The IC50 value was calculated for both salts and it was found that the cytotoxic effect was directly proportional to the concentration of salt. Obtained values for mercuric chloride IC50 were 49.35±2.38 μM using MTT assay (Fig. 3A) and 28.10± 0.44 μM using NR assay (Fig. 3B); while IC50 values for sodium hydrogen arsenate heptahydrate were 73.77±9.34 μM using MTT assay (Fig. 3C) and 107.2±2.41 μM using NR assay (Fig. 3D). MTT and neutral red uptake assays are considered sensitive and provide accurate cytotoxicity assessment and have been successfully used for assessment of cytotoxicity of different chemical compounds on other cell lines developed from fish (Goswami *et al.* 2014, Swaminathan *et al.* 2016).

Plating efficiency is a sensitive test that defines colony formation from every single cell in the population. Cell lines generally show high plating efficiency with high seeding density. Similar results were obtained in PHT cells where plating efficiency increased with increasing cell number, i.e. 4.66±.088% for 100 cells, 4.73±2.90% for 500 cells, and 7.76±4.48% for 1000 cells. The results conform to other thymus cell lines (Rebello *et al.* 2014, Sood *et al*. 2015).

The fish cell line can serve as a genetic resource for fish. They can be utilized to complement studies related to fish biology and the function of genes (Thangaraj *et al.* 2021). Successful transfection of PHT cells with the GFP gene

(Supplementary Fig.4) proves its potential utility for gene manipulation studies. Transfection also serves the purpose of studying the function of gene and their products as well as the production of recombinant proteins inside the cells (Wurm 2004).

In conclusion, *P. hypophthalmus*, is an economically important cultured fish species. A thymus cell line from *P. hypophthalmus* has been established and characterized using molecular and cytogenetic markers and transfection. Plating efficiency of the cell line was also tested. The cell line was successfully maintained in the L-15 medium. It has been used for various studies including cytotoxicity, cytogenetics, and transgenics, and has the potential to be used for many other studies related to fish biology.

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