Development and characterization of cell line from caudal fin of goldfish, *Carassius auratus* for the continuous propagation of Cyprinid herpes virus -2 (CyHV-2)

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Background: Herpesviral haematopoietic necrosis disease, caused by cyprinid herpesvirus-2 (CyHV-2), is responsible for massive mortalities in goldfish, *Carassius auratus.* Currently, few permissive cell lines were established from different tissues of goldfish for the isolation and propagation of CyHV-2 by sacrifying the fish. Therefore, in the present study, we developed a cell line, FtGF (Fantail Goldfish Fin) from caudal fin of goldfish using non lethal sampling. **Methods:** Caudal fin tissue sample was collected from goldfish without killing fish. We describe a simple protocol for successful establishment and characterization of a permissive cell line through explant method and continous propagation of CyHV-2 with high viral titre using this cell line. Cell culture of goldfish caudal fin cells was carried out using Leibovitz's L-15 (L-15) medium containing 20% FBS and 1X concentration of antibiotic antimycotic solution was added to the flask, which was incubated at 28 °C. Cells were characterized and origin of the cells was confirmed by sequencing of the fragment of 16S rRNA and COI gene. CyHV-2 was grown in the FtGF cells and passaged continuously for 20 times. The infectivity of the CyHV-2 isolated using FtGF cells was confirmed by experimental infection of naïve goldfish **Results:** The cell line has been passaged up to 55 times in L-15 with 10% FBS. Karyotyping of FtGF cells at $30^{th,}$ 40^{th} and 56^{th} passage indicated that modal chromosome number was 2n = 104. Species authentication of FtGF was performed by sequencing of the fragment of 16S rRNA and COI genes. The cell line was used for continuous propagation of CyHV-2 over 20 passages with high viral titer of $10^{7.8\pm0.26}$ $TCID_{50}$/mL. Following inoculation of positive for CyHV-2 tissue homogenate, FtGF cells showed cytopathic effect by 2 day post-inoculation (dpi) and complete destruction of cells was observed by 10 dpi. An experimental infection of naïve goldfish using supernatant from infected FtGF cells caused 100% mortality and CyHV-2

infection in the challenged fish was confirmed by the amplification of DNA polymerase gene, histopathology and transmission electron microscopy. These findings provide confirmation that the FtGF cell line is highly permissive to the propagation of CyHV-2.

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Running page head: CyHV-2 isolation in India

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Abstract

Background:

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Results:

The cell line has been passaged up to 55 times in L-15 with 10% FBS. Karyotyping of FtGF cells at $30^{th,}$ 40^{th} and 56^{th} passage indicated that modal chromosome number was 2n = 104. Species authentication of FtGF was performed by sequencing of the fragment of 16S rRNA and COI genes. The cell line was used for continuous propagation of CyHV-2 over 20 passages with high viral titer of $10^{7.8\pm0.26}$ $TCID_{50}$/mL. Following inoculation of positive for CyHV-2 tissue

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Introduction

Cyprinid herpesvirus-2 (CyHV-2), the etiological agent of herpesviral haematopoietic necrosis (HVHN) disease, is a pathogen of goldfish that has been associated with disease outbreaks in goldfish populations (Jeffery et al., 2007). The pathogen first reported as cause of outbreaks in juvenile goldfish in 1992 from Japan (Jung & Miyazaki, 1995), has subsequently been reported from more than 13 countries (Adamek et al., 2018). In India, CyHV-2 infection was associated with large-scale mortalities of goldfish in West Bengal (Sahoo et al., 2016). As the surviving fish become lifelong carriers of latent infection without exhibiting any clinical signs of the disease (Wang et al., 2012) and goldfish is one of the most traded ornamental fish globally, the international trade of such apparently healthy but latently infected goldfish is suspected to be responsible for global spread of CyHV-2 (Ito et al., 2013; Adamek et al., 2018).

Cell lines are considered the gold standard for isolation and identification of viruses (Landry, 2009). Earlier, a number of cell lines from other fish species, namely Epithelioma papulosum cyprini cells (EPC), fathead minnow cells (FHM) and koi fin cell lines (KF-1) have been used for CyHV-2 cultivation, but these cell lines did not support its continuous propagation beyond 5th passage (Jeffery et al., 2007; Wang et al., 2012). It has been reported that cell lines derived from homologous species provide sensitive tools for virus propagation (Fryer & Lannan,

1994) and successful cultivation of CyHV-2 has been reported in the cell lines developed from goldfish, namely GFTF (Yan, Nie & Lu, 2011), GFF and SRTF (Ito et al., 2013) and GiCB (Ma et al., 2015). In our earlier study, although CyHV-2 produced cytopathic effects in CCKF cell line (Sahoo et al., 2016), the virus could not be propagated beyond 4th passage. However, no cell line from goldfish is available in India, therefore, the need of establishing a homologous cell line from goldfish was deemed necessary for the continuous propagation of CyHV-2.

In this paper, we report the development of a cell line from the caudal fin of fantail goldfish (FtGF). The cell line has been successfully employed for propagation of CyHV-2, and experimental reproduction of HVHN using supernatant from the CyHV-2 infected cell line (at 10th passage). The newly developed FtGF cell line will play significant role in future research on CyHV-2, including the development of strategies for the prevention and control of the disease caused by CyHV-2 in India.

Materials and methods

Generation of the primary cell culture

A healthy fantail goldfish weighing 20 g was purchased from a local commercial aquarium shop and kept in properly aerated and filtered fish tank. The fish was fed daily and water changes were performed on alternate days. The goldfish was anaesthetized using 3- aminobenzoic acid ethyl ester methanesulphonate (MS-222, Sigma-Aldrich, St. Louis, MO) at the dose rate of 150 mg/L of water for 15-20 min exposure and the caudal fin was excised following wiping with 70% ethanol. The excised tissue was washed three times in Phosphate Buffer Saline (PBS) containing the antibiotic-antimycotic solution (Life Technologies, Carlsbad, CA) (200 IU/mL penicillin, 200 μg/mL streptomycin and 0·5 μg/mL amphotericin B). The fin tissue was mechanically cut with a fine scalpel into smaller pieces and then seeded in a 25 cm^2 flask. The PBS was removed

and attachment of the tissues to the surface was facilitated by adding 200 µl of fetal bovine serum (FBS). After 2 h, about 7 mL of Leibovitz's L-15 (L-15) medium containing 20% FBS and 1X concentration of antibiotic antimycotic solution was added to the flask, which was incubated at 28 °C. The flask was observed daily under an inverted light microscope (Nikon Corporation, Tokyo, Japan).

Subculture and maintenance

After formation of monolayer, the cells were washed with PBS followed by trypsinization with 0.25% trypsin EDTA solution. The cells were observed under an inverted light microscope. The trypsin EDTA solution was discarded immediately on observing cell detachment. The flasks were gently tapped to release the cells from the surface and L-15 medium with 20% FBS was added to stop the action of trypsin. The cells were subcultured at a split ratio of 1:2. The flasks were observed regularly and the cells were subcultured after attaining 80-90% confluence. After 10 subcultures, the concentration of FBS in medium was reduced to 10%.

Cell growth characteristics

Cell growth studies were conducted at different temperatures and FBS concentrations in L-15 medium. The growth rate assays were conducted at temperatures ranging from 15 °C to 37 °C and FBS concentrations from 5% - 20%. A monolayer flask at 20^{th} passage was trypsinized and the cells were seeded into different 6 well plates. The plates were incubated overnight at 28 °C. Thereafter, the plates were incubated at different temperatures of 15 °C, 20 °C, 28 °C, 30 °C and 37 °C, and the growth rate was observed. Every day, the cells were harvested from the duplicate wells and counted with a Neubauer haemocytometer. A similar study was conducted using different 6 well plates incubated at 28 °C having different FBS concentrations of 5%, 7.5%, 10%, 15% and 20%.

Chromosome analysis

The chromosomal counts for FtGF were undertaken from the 30th, 40th and 56th passage flask. A flask with 80% confluence was treated with 1 μg/mL colchicine (Sigma, St Louis, MO, USA) for 4 h at 28 °C and then the supernatant was removed, the cells harvested and resuspended in a hypotonic 0.5% KCl solution for 10 min, and then fixed in a 3:1 methanol : acetic acid solution. The cells were pelleted at 367 g at 4 °C for 10 min and washed four times with methanol and acetic acid solution. Smears were prepared following a conventional drop-splash technique (Freshney, 1994), the slides were stained with 10 % Giemsa solution and air dried for 30 min. A total of 100 chromosome spreads were observed and counted under a light microscope (Leica, Germany). .

Cryopreservation

The monolayers with 80 – 90% confluency were used for cryopreservation at various intervals of subculturing (7th, 10th, 20th, 30th and 40th). The media was changed one day prior to cryopreservation. Next day, the cells were trypsinised in PBS and centrifuged at 500 *g* at 4 °C. The supernatant was discarded and the cells were suspended in recovery cell-culture freezing medium (Life Technologies) at a density of 5 x 10^6 cells/mL. The cell-culture freezing medium containing cells was aliquoted into 2 mL sterile cryovials (Nunc), followed by holding at 4 °C for 2 h, -20 °C for 1 h, -80 °C for overnight and then transferred into liquid nitrogen (LN_2) containers. The cells stored at 20th and 40th passage were revived after 2 months of storage. Briefly, the frozen cells were thawed quickly at 37 °C and added drop-wise to 15 mL complete medium in a centrifuge tube. The cells were centrifuged at 825 *g* at 4 °C and the pellet was resuspended in 7 mL of complete medium. Cell viability was checked with a haemocytometer

following trypan blue staining. The revived cells were seeded into 25 cm^2 flask and incubated at 28 °C.

Molecular characterization of the cell line

DNA isolation was carried out by salting out method following Miller, Dykes & Polesky (1988). The DNA was extracted from FtGF cells at 40th passage and muscle tissue of goldfish. PCR was carried out as per Swaminathan et al., (2016b). PCR products of 562 bp and 642 bp were amplified for mitochondrial 16S rRNA and cytochrome c oxidase subunit I (COI) genes from FtGF cells and goldfish muscle, using universal primers ((F 5′CGC CTG TTT ATC AAA AAC AT 3′and H 5′CCG GTC TGA ACT CAG ATC ACG T 3′ (Ward, Zemlak, Innes, Last & Hebert, 2005) and F 5′TCA ACC AAC CAC AAA GAC ATT GGC AC 3′and R 5′TAG ACT TCT GGG TGG CCA AAG AAT CA3′ (Palumbi et al., 1991) respectively. The PCR products of both the fragments were sequenced in an ABI 3730 DNA analyser (Applied Biosystems). The sequences of both the mt DNA gene PCR fragments were compared with the published and known sequences in the National Centre for Biotechnology Information database by the basic local alignment search tool (BLAST) (Altschul et al., 1990).

Detection of *Mycoplasma* contamination in FtGF cell line

Mycoplasma contamination was tested by PCR with 15th and 40th passage FtGF cells grown for 4 days in L-15 medium without antibiotics. Briefly, the harvested cells were centrifuged at 200 *g* for 10 min and the supernatant was transferred into micro-centrifuge tubes and centrifuged at 250 *g* to remove debris. The *Mycoplasma* contamination was checked using EZdetect PCR Kit (HiMedia) based on amplification of spacer region between 16S rRNA and 23S rRNA. The amplification products were analysed in 1.5% agarose gel.

CyHV-2 isolation on FtGF cells and viral titer determination

Diseased goldfish obtained from a farm in Kerala, India were confirmed to be infected with CyHV-2 by PCR as per Jeffery et al. (2007). External examination of moribund goldfish revealed clinical signs including simple loss of scale, pale gills and gills covered with mucus. Internally, all visceral organs of affected fish were congested and enlarged and also white nodules were found on the spleen and the kidney of fish. Gill, kidney and spleen tissues from diseased fish were collected aseptically, and then homogenized in Dulbecco's Phosphate Buffered Saline (DPBS), freeze-thawed for 3 cycles and the tissue homogenate was centrifuged at 3000 *g* for 30 min at 4 °C. The supernatant was then filtered through a 0.22 µm filter (Millipore) and checked for bacterial contamination before use. Thereafter, 500 µl of the filtered tissue homogenate was inoculated onto 25 cm^2 flasks after removing the medium and incubated in a shaking incubator (45 rpm) at 28 °C. In control flasks, maintenance medium was used in place of tissue homogenate. After 1 h adsorption, 6.5 mL of the maintenance medium (L-15 medium with 2% FBS) was added to the FtGF flasks, which were incubated at 28 °C. The inoculated flasks were checked daily for cytopathic effects (CPE). The cells from flasks exhibiting 80 - 90% CPE were harvested along with supernatant. This cell suspension was frozen at -80 ^oC for further use. For titration, FtGF cells were grown in a 96-well plate with a confluence of 70% to 80%. After removing the medium, filtered tissue homogenate was diluted ten-fold (10^{-1} to 10^{-9}), and 0.1 mL of diluted filtrate was inoculated in wells of a 96-well plate in triplicate and allowed to adsorb for 1 h. In control wells, DPBS was added in place of filtered tissue homogenate. Thereafter, 0.2 mL of maintenance medium was added to each well and the plate was incubated at 28 °C. The wells were examined daily for the appearance of CPE up to 2 week. The virus titer was determined by 50% tissue culture infective dose ($TCID_{50}$) assay using Reed & Muench (1938) calculations.

Experimental challenge studies on goldfish using CyHV-2 propagated in FtGF cells

Clinically healthy goldfish (12-15 cm; 16-23 gm), procured from a local ornamental fish farm, were divided into two groups of 30 fish each and acclimatized in the laboratory aquarium tanks for a week. The tissues from randomly collected goldfish (n=5) were screened for CyHV-2 using PCR following Jeffery et al. (2007). The fish were anaesthetized with MS-222 (Sigma-Aldrich). Each fish in one group was challenged with an intraperitoneal (IP) injection of 0.5 mL of 10th passage CyHV-2 FtGF cell culture supernatant and this constituted infected group, whereas fish in the control group were injected with 0.5 mL maintenance medium. The water temperature was maintained at 28 °C during the experiment and fish were observed daily for clinical signs and mortality. Three goldfish each in the infected and control group were selected randomly after 7 days post-injection (dpi) and screened for CyHV-2 by PCR assay as described earlier for confirmation of CyHV-2 infection. The experimental challenge study in fish was carried out following ARRIVE guidelines and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. The experimental challenge trials were evaluated and approved by Institute Animal ethics Committee (IAEC) of ICAR National Bureau of Fish Genetic Resources (NBFGR) vide approval Number G/IAEC/2019/1 dated 04th October 2019.

Confirmation of the CyHV-2 virus

Polymerase chain reaction

The naturally infected tissues from goldfish, supernatant along with cells collected from FtGF flask and infected tissues from experimentally infected goldfish were processed for confirmation by PCR. The harvested FtGF cells were centrifuged and DNA was isolated from cell pellet using DNeasy blood and tissue kit (Qiagen). Concentration and purity of the extracted DNA was

determined by measuring OD at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer (Nano Drop Technologies Inc). The samples were stored at -20 °C for further analysis. PCR was performed using published oligonucleotide primers CyHVpol-FOR and CyHVpol-REV primers (Jeffery et al., 2007) for confirmation. Briefly, amplification was performed in 25 µl reaction mixture containing 2.5 µl of 10X Taq buffer, 1.5 µl (10 pmol) of each primer, 0.5 µl of dNTPs (2 mM), 0.25 µl of Taq DNA polymerase (5 U μl^{-1}), 1 µl of total DNA and ddH_2O to make final volume to 25 µl. The reaction mixture was pre-heated at 95° C for 3 min followed by 40 cycles of 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were visualized following electrophoresis in 1.5% agarose gel. Representative PCR amplicons from each primer set were purified and sequenced by Sanger sequencing facility (Scigenom Pvt. Ltd). These sequences of amplified PCR products from all the cases (natural infected goldfish, experimentally infected goldfish and infected FtGF cells) were confirmed by BLAST analysis.

Histopathology

For histopathological examination, tissues including gills, kidney and spleen were collected from the moribund fish at 7 dpi and fixed in 10% neutral buffered formalin. Tissues were dehydrated in ascending grades of ethanol, cleared in chloroform and embedded in paraffin wax. Thin tissue sections, 4-5 µm thick were cut and stained with haematoxylin-eosin for examination under a compound microscope.

Transmission electron microscopy

The experimentally infected tissues were fixed with 2.5% glutaraldehyde, post fixed with osmium tetroxide for 1 h at 4 °C, then dehydrated and embedded. A microtome (Leica ultracut UCT) was used to cut thin sections of about 60–70 nm. Sections were stained with uranyl acetate

and alkaline lead citrate after mounting on copper grids. Sections were observed and photographed under a Tecnai T12 Spirit transmission electron microscope at 60 kV. Supernatant from CyHV-2 infected FtGF cells displaying CPE were studied by electron microscopy. Subcultured samples were negatively stained with phosphotungstic acid and examined using a High Resolution Transmission Electron Microscope Facility, (FEI-TECNAI-G2 20 TWIN) at 7800× magnification and an accelerating voltage of 120 kV.

Results

Primary cell culture and maintenance

The cells were seen emerging from the sides and edges of caudal fin tissues (Fig. 1A) and they readily attached to the bottom of the flask. A monolayer culture was obtained within 10 days at 28 °C . The monolayer was passaged at 1:2 ratio every 6-7 days. The growth pattern of lag phase (1-3 days), log phase (3-5 days) and stationary phase (5-7 days) was observed. During the initial passages, mixed fibroblast-like and epithelial-like cells were observed and after 10 passages, epithelial cells dominated over fibroblastic cells (Fig. 1B). The FtGF cells have been subcultured for over 55 passages and the cell line has been designated as Fantail Goldfish Fin (FtGF) cell line. The morphology of FtGF cells at 25^{th} passage and at 56^{th} passage are shown in the figure C & D

Characterization of FtGF cell line

The most favourable conditions for culturing of FtGF cells were tested at different incubation temperatures viz., 15, 20, 28, 30 and 37 °C as well as at different concentrations of FBS viz., 5, 7.5, 10, 15 and 20%. The cells incubated at 15 and 37 °C showed rounding and started detaching on 2^{nd} day. Cells grew in the flask at 20 °C, but the growth rate was slow. Maximum growth of the cells was observed at 28 °C. At 30 °C, FtGF cells proliferated very fast initially, but became

248 puffy and were dying by the 2nd day. Culture of FtGF cells grew better in L-15 with 20% FBS
249 and a slower growth was recorded with decreasing concentration of FBS. The growth kinetics of
250 FtGF cells did not show much differences in 10, 15 and 20% FBS, but cells in 7.5 and 5% FBS
251 had slower growth. The suitable temperature and FBS concentration for the culture of FtGF cells
252 were found to be 28 °C and 20% FBS, respectively (Fig. 2A & B). The FtGF cell growth was
253 arrested in metaphase using Colchicine at final concentration of 1 µg/mL. Karyotyping results
254 obtained by counting 100 spreads at metaphase showed 82–114 chromosomes, with a distinct
255 peak for the cell line at 104 diploid chromosomes at 30th, 40th and 56th passage. The majority of
256 the cells (60%) had a diploid chromosome number (2n=104) at all three passage levels. The
257 diploid karyotype of FtGF cells and its frequency distribution at passage 30 are shown (Fig. 3A
258 & 3B) respectively. The FtGF cells at 20th and 40th passage were revived and these exhibited 70-
259 75% viability. The cells were incubated at 28 °C and a monolayer was established within 15
260 days. The origin of FtGF cell line was confirmed by partial amplification and sequencing of 16S
261 rRNA and COI genes from FtGF cells and goldfish muscle and comparison with available COI
262 and 16S rRNA sequences in GenBank. Nucleotide sequence analysis revealed 100% similarity
263 with sequences from goldfish muscle and maximum similarity (99%) with COI (Accession
264 number KX145542 and KX145499) and 16S rRNA sequences (Accession number AJ247070,
265 KY231826) of goldfish in GenBank. These data confirm that the origin of the developed FtGF
266 cell line is from goldfish. No target band of spacer region between 16S and 23S rRNA of
267 *Mycoplasma* was observed indicating that the FtGF cells were free of *Mycoplasma*
268 contamination.

269 **CyHV-2 isolation on FtGF cells and virus titration assays**

The FtGF cell line (Fig. 4A) infected with CyHV-2 began to show morphological changes from the 2nd day (Fig. 4B) and the CPE such as cell elongation, rounding, and cell fusion with cytoplasmic vacuolation were observed from 4th day (Fig. 4C) at 28 °C. Cell death started from the 6th day (Fig. 4D) and complete detachment was observed by 10 days. The infected FtGF cells and the supernatant were confirmed for the presence of CyHV-2 by PCR and sequencing. No CPE was observed in the control cells inoculated with maintenance medium. The $TCID_{50}$ of the culture suspension harvested from infected FtGF was found to be $10^{7.8\pm0.26}$ $TCID_{50}$/mL. The CyHV-2 could be propagated in FtGF flasks for 20 passages.

Virulence of CyHV-2 propagated in FtGF cells to goldfish

The goldfish challenged with 1.4 X 10^7 $TCID_{50}$/mL CyHV-2 (passage 10) produced in the FtGF cell line began to die at 5 days post inoculation (dpi) and the mortality reached 100% at 12 dpi (Fig.5). The dead fish from infected groups exhibited similar clinical signs seen in naturally infected fish. The clinical signs of challenged goldfish were haemorrhages on the body surface, exophthalmia, pale gills and a swollen abdomen. However, there was no mortality in the mock-infected group. The tissues from randomly selected fish from the infected groups were positive by PCR and produced CPE in FtGF, while fish from control group were negative. The CyHV-2 recovered from experimentally infected fish was also found to be virulent to goldfish and caused similar symptoms (unpublished data).

Confirmation of the CyHV-2 virus

Polymerase chain reaction

The PCR product using the primers CyHVpolFOR and CyHVpolREV were of 362 bp. Tissue samples (gills, spleen and kidney) collected from naturally infected tissues, FtGF cell culture suspension, and experimentally challenged fish were found to be positive for CyHV-2 in PCR

293 assay. A GenBank BLAST search on the sequence revealed a high identity to CyHV-2 isolate
294 SYC1 strain (KM200722, 99.9%) and CyHV-2 isolate STJ1 strain (JQ815364, 99.9%). The
295 sequence of the DNA polymerase gene of CyHV-2 isolated from goldfish in this study was
296 deposited in GenBank (GenBank accession nos. KU527548 and KU527549). The nucleotide
297 sequences of fragment DNA polymerase gene of CyHV-2 were submitted to NCBI GenBank
298 (GenBank accession nos. KU527548 and KU527549).

299 **Histopathology**

300 The gill lamellae of experimentally infected fish samples demonstrated necrosis and sloughing of
301 epithelial cells, and the haematopoietic tissue of kidney and spleen showed diffuse necrosis.
302 Hypertrophied nuclei with marginated chromatin material were observed in sections of spleen
303 and kidney in goldfish experimentally infected with CyHV-2 (Fig. 6A & B) and lesions were
304 compared with the sections of spleen and kidney in naturally CyHV-2 infected goldfish (Fig. 6C
305 & D).

306 **Transmission electron microscopy**

307 The mature virus particles were observed ultra structurally in cytoplasm of cells in gills as well
308 as spleen tissue of experimentally challenged fish that were about 170–180 nm in diameter. The
309 virions were icosahedral in shape and had an electron dense core surrounded by a capsid (Fig.
310 7A & B). Supernatant of FtGF cells with CPE were examined by TEM, and viral particles
311 morphologically similar to a herpesvirus were observed (Fig. 7C).

312 **Discussion**

313 The ease of detecting and isolating the virus using cell lines provides a remarkable advantage in
314 viral disease management. Isolation of virus in cell culture has been considered as the “gold
315 standard” for the viral disease diagnosis for decades. It would be a valuable approach when a

316 viable virus isolate is needed and also to differentiate nonviable virus in a clinical specimen.
317 Over the last few years, a number of fish viruses *viz*., viral nervous necrosis virus (Azad et al.,
318 2005), iridovirus (George et al., 2015), cyprinid herpesvirus-2 (Sahoo et al., 2016), and carp
319 edema virus (Swaminathan et al., 2016a), have been reported from India and some of these have
320 also been isolated using fish cell lines. As CyHV-2 mainly affects goldfish, development of
321 species-specific cell line will be appropriate for the propagation of the virus in disease health
322 management. Several cell lines have been employed for isolation of CyHV-2 (Rougée et al.,
323 2007; Yan et al., 2011; Ito et al., 2013; Ma et al., 2015; Jing et al., 2016). In India, Sahoo et al.
324 (2016) isolated CyHV-2 using a koi carp cell line, CCKF but could not propagate the virus
325 beyond 4th passage. The continuous culture of CyHV-2 has been challenging due to the lack of
326 permissive cell lines in India. In this paper, we have demonstrated the continuous propagation of
327 CyHV-2 using FtGF cell line as well as successful experimental infection of goldfish by
328 intraperitoneal injection with the CyHV-2 propagated in FtGF cells.

329 In our study, a new cell line, FtGF, which consists of predominantly epithelial cells with
330 diploid number of chromosome (2n=104) and grows in L-15 medium with 10% FBS at growth
331 temperature of 28 °C, was developed from caudal fin of fantail goldfish. The caudal fin has the
332 feature of natural regeneration capacity (Akimenko et al., 2003) accounting for a high *in vitro*
333 cell proliferation potential (Santos-Ruiz, Santamaria & Becerra, 2005). The FtGF cell line
334 exhibited stable growth over about 55 passages over the past one year. Using the new cell line,
335 we isolated CyHV-2 using filtered tissue homogenate from infected goldfish. Morphological
336 changes in FtGF cells infected with CyHV-2 included cytoplasmic vacuolation, rounding and
337 detachment of cells, as reported previously in other CyHV-2 infected cells. Moreover, CyHV-2
338 could be continuously propagated over 20 times using FtGF cell line and also consistently

produced the similar CPE as the initial passage. Similar to our results, Ma et al. (2015) could also propagate the virus for over 50 passages in GiCB cell line. However, in contrast to our results, Jung & Miyazaki (1995) reported that using epithelioma papulosum cyprini and FHM cell cultures; CyHV-2 could not be sub-cultured beyond the fourth passage. Previously, several attempts have been made to propagate CyHV-2 in different fish cell lines (Li & Fukuda, 2003; Waltzek et al., 2005; Jeffery et al., 2007; Ito et al., 2013; Xu et al., 2013; Jing et al., 2017) but it has not been possible to propagate CyHV-2 beyond 4-6 passages and virus titre from these cells was very low.

The virus titer was estimated at $10^{7.8\pm0.26}$ $TCID_{50}$/mL, which is much higher than any earlier reported titer for CyHV-2 in other studies. In this study, the removal of cell culture medium from the flask before inoculating the tissue homogenate in FtGF cells, incubation temperature and shaking during adsorption of the viral inoculum, might have increased the viral titre of CyHV-2 in FtGF cell line. Ma et al. (2015) reported that the CyHV-2 titer reached $10^{7.5\pm0.37}$ $TCID_{50}$/mL and has been effectively cultured over 50 times in the GiCB cell line. Ito et al. (2013) reported that incubation temperature of 25 °C is considered to be highly tolerant for isolation of CyHV-2 in GFF cells. But, in our study, we got high CyHV-2 tire of $10^{7.8\pm0.26}$ $TCID_{50}$/mL at 28 °C which is a high incubation temperature. In accordance with our results, Piaskoski, Plumb & Roberts (1999) and McClenahan, Beck & Grizzle (2005) found that incubating the cells inoculated with the largemouth bass virus (LMBV) samples at 30 °C resulted in a higher number of LMBV plaques than incubation at 25 °C or 32 °C. Similarly, Chi et al., (1999) also found that the optimum temperature range for grouper nervous necrosis virus (GNNV) infection in GF-1 cells was 24–32 °C and virus titer increased with an increase of the temperature. The removal of tissue culture medium might have made the virus concentration

higher during adsorption and agitation resulting in a more uniform distribution of the viral inoculum among all the FtGF cells in the flasks. The present isolate of CyHV-2 in this study showed a different temperature range for its culture compared to other published reports by various workers discussed earlier. One of the possible explanations could be that the present Indian CyHV-2 might belongs to a different strain or genotype or it could be due to an adaptation of the host fish species to the native environmental temperature (Ciulli et al., 2006).

An experimental infection in goldfish was carried out with the virus passaged 10 times in FtGF cells. In this experiment, fish injected IP with the cell culture grown virus, began to die at 5 dpi and the cumulative mortality reached 100% at 12 dpi. This finding is in accordance with a previous report (Ito et al., 2013), where cumulative mortality of 90 (at 16 dpi) and 100% (at 18 dpi) were observed in Edonishiki and Ryukin goldfish varieties, respectively. In addition, Ma et al. (2015) reported cumulative mortality of 100% at 14 dpi in healthy gibel carp challenged with CyHV-2 (passage 9) produced in the GiCB. The CyHV-2 virus, re-isolated from experimentally infected fish in FtGF cells, was also pathogenic to goldfish and caused disease in goldfish. In the present study, the electron microscopic observations demonstrated mature virus particles similar to herpesvirus in affected gill and spleen tissues from the experimentally challenged goldfish and these findings are consistent with previous reports (Jeffery et al., 2007; Wu et al., 2013; Ma et al., 2015). CyHV-2 cultured in FtGF passages has been stored in liquid nitrogen. The recovered viruses from liquid nitrogen maintained high infection activity to cells (data not shown). The results indicated that the FtGF cell line is capable of producing high concentrations of CyHV-2 *in vitro* and a highly permissive to the propagation of CyHV-2.

Conclusion

In conclusion, the newly established FtGF cell line is highly susceptible to CyHV-2 culture *in vitro*. The newly developed FtGF cell line would play a crucial role in future research on CyHV-2, including studying the molecular pathogenesis of HVHN disease and development of strategies for the prevention and control of the disease in the country. The FtGF cell line (NRFC accession number: NRFC058; http://mail.nbfgr.res.in/nrfc/cellline-available.php) was deposited in the National Repository of Fish Cell Line (NRFC) (the largest fish cell line repository), ICAR National Bureau of Fish Genetic Resources, India for further dissemination to scientists for carrying out research in developing CyHV-2 management strategies in this field.

Ethical statement: All the experimental challenge procedures in this study (Proposal number: NBFGR/IAEC/2019/0014) were evaluated and approved by Institute Animal ethics Committee (IAEC) of ICAR National Bureau of Fish Genetic Resources (NBFGR) (CPCESA Registration No: 909/GO/Re/S/05/CPCSEA dated 09.09.2005 and CPCSEA Ref file No. 25/111/2014-CPCESA dated 05th December 2018) vide approval Number G/IAEC/2019/1 dated 04th October 2019.

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Government of India. We thank the Department of Gastroenterology, Christian Medical College, Vellore, India and High Resolution Transmission Electron Microscope Facility, Vellore Institute of Technology, Vellore, India for the TEM analysis of the samples.

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Legend to the figures

Fig. 1 Photomicrographs of Fantail Goldfish (FtGF) cell line. (A) Cells emerging from the explant of caudal fin; (B) morphology of FtGF cells at 10th passage; (C) morphology of FtGF cells at 25th passage; (D) morphology of FtGF cells at 56th passage.

Fig. 2 Growth rate of newly established FtGF cell line. (A) Growth at different temperatures; (B) Growth at different FBS concentration.

Fig. 3 Morphological characteristics and frequency distribution of the chromosomes of the FtGF cell line at passage 30. (A) Phase-contrast photomicrograph of chromosome spread arrested in metaphase (original magnification: X400); (B) Frequency distribution of chromosomes in 100 cells.

Fig. 4 The cytopathic effect (CPE) in FtGF cells following infection with CyHV-2 at 28^{o} C. (A) Uninfected FtGF cells; (B) FtGF cells infected with CyHV-2 at passage 15 at 2 dpi; (C) FtGF cells infected with CyHV-2 at passage 15 at 4 dpi; (D) FtGF cells infected with CyHV-2 at passage 15 at 6 dpi.

Fig. 5 Growth curve of CyHV-2 in FtGF cell line and mortality curve of experimentally infected goldfish. (A) Growth curve of CyHV-2 (at passage 20) in the FtGF cell line. Values plotted are means ±SD of the measurements. (B) Cumulative mortality curve of the experimentally infected goldfish (12-15 cm; 16-23 gm), using challenged with $10^{7.8\pm0.26}$ $TCID_{50}$/mL CyHV-2 (passage 10) propagated in FtGF cells.

Fig. 6 Histopathological lesions in kidney, spleen and gills of a goldfish with Goldfish Herpesviral Hematopoietic Necrosis Disease. The nuclei of cells with marginated chromatin (arrows) in goldfish experimentally infected with CyHV-2 (A) Kidney and (B) Spleen. Nuclei of

infected cells with marginated chromatin (arrows) in naturally CyHV-2 infected goldfish (C) Kidney and (D) Spleen.

Fig. 7 Transmission electron micrograph showing enveloped mature CyHV-2 virions in the ultrathin sections of gill (A), spleen (B) cells of experimentally challenged of goldfish and mature virus particles (C) purified from CyHV-2 infected FtGF cells. Viral capsid in cytoplasm with electron-dense cores and capsid surrounded by electron-dense material. Scale bar = 200 nm.

Figure 1

Photomicrographs of Fantail Goldfish (FtGF) cell line.

(A) Cells emerging from the explant of caudal fin; (B) morphology of FtGF cells at 10th passage; (C) morphology of FtGF cells at 25th passage; (D) morphology of FtGF cells at 56th passage.

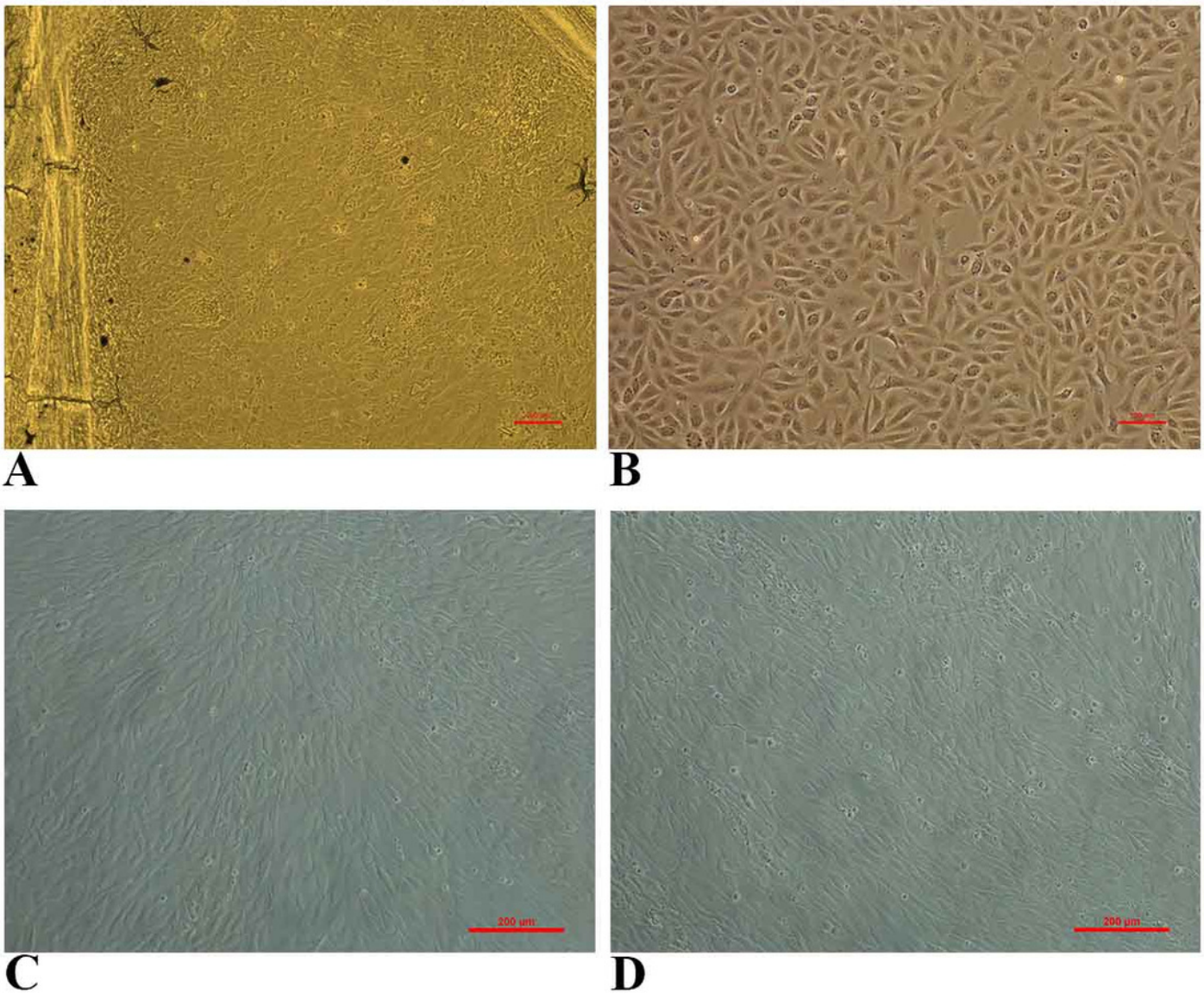
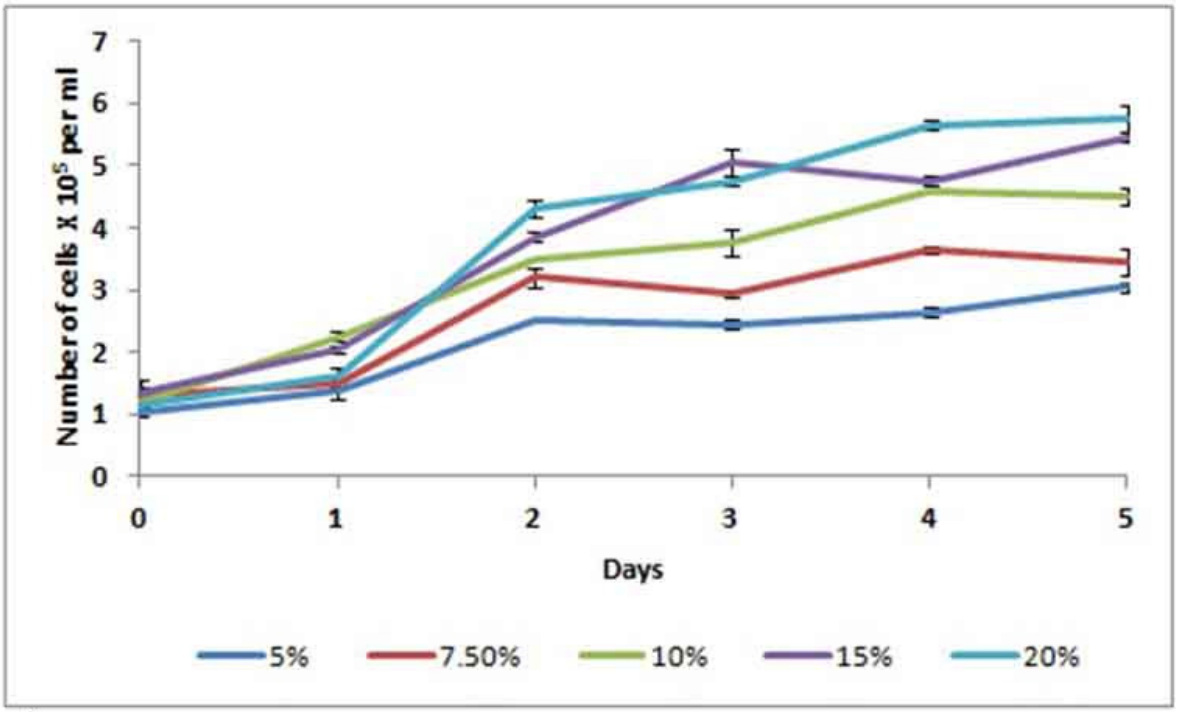


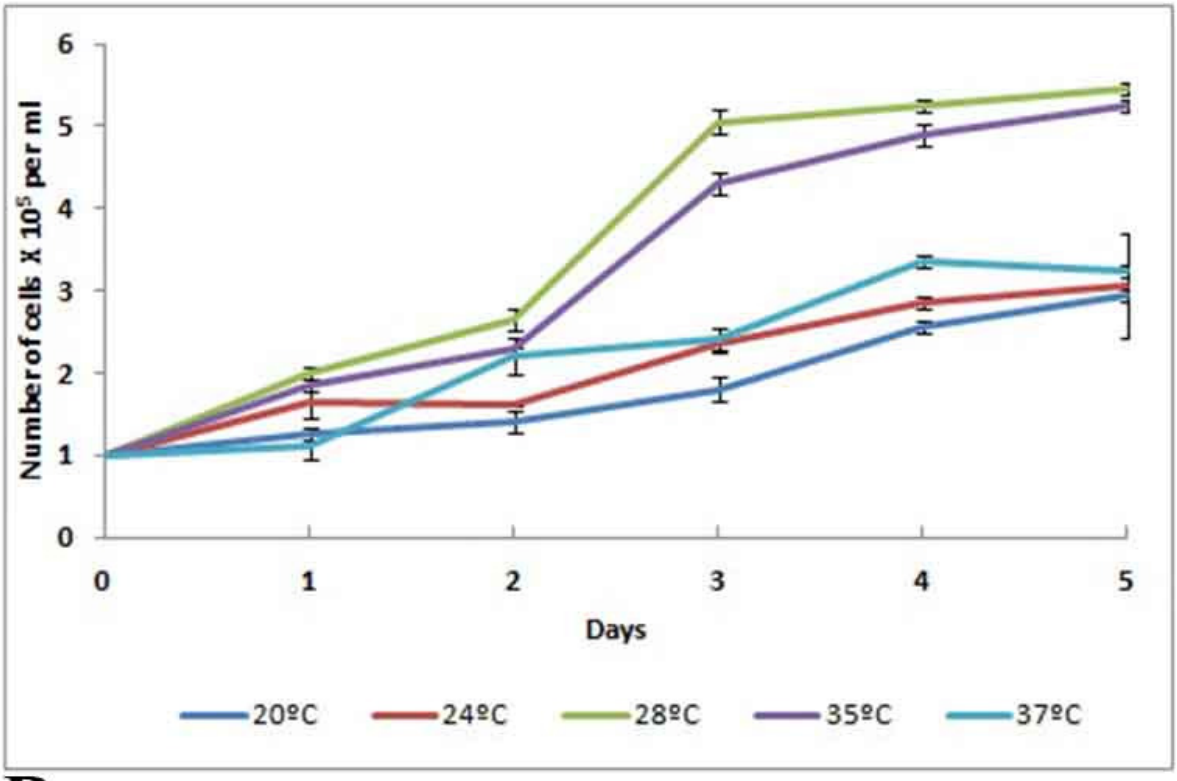
Figure 2

Growth rate of newly established FtGF cell line.

(A) Growth at different temperatures; (B) Growth at different FBS concentration.


Number of cells X 10^5 per ml
0
1
2
3
4
5
6
7
Days
0
1
2
3
4
5
5%
7.50%
10%
15%
20%

A

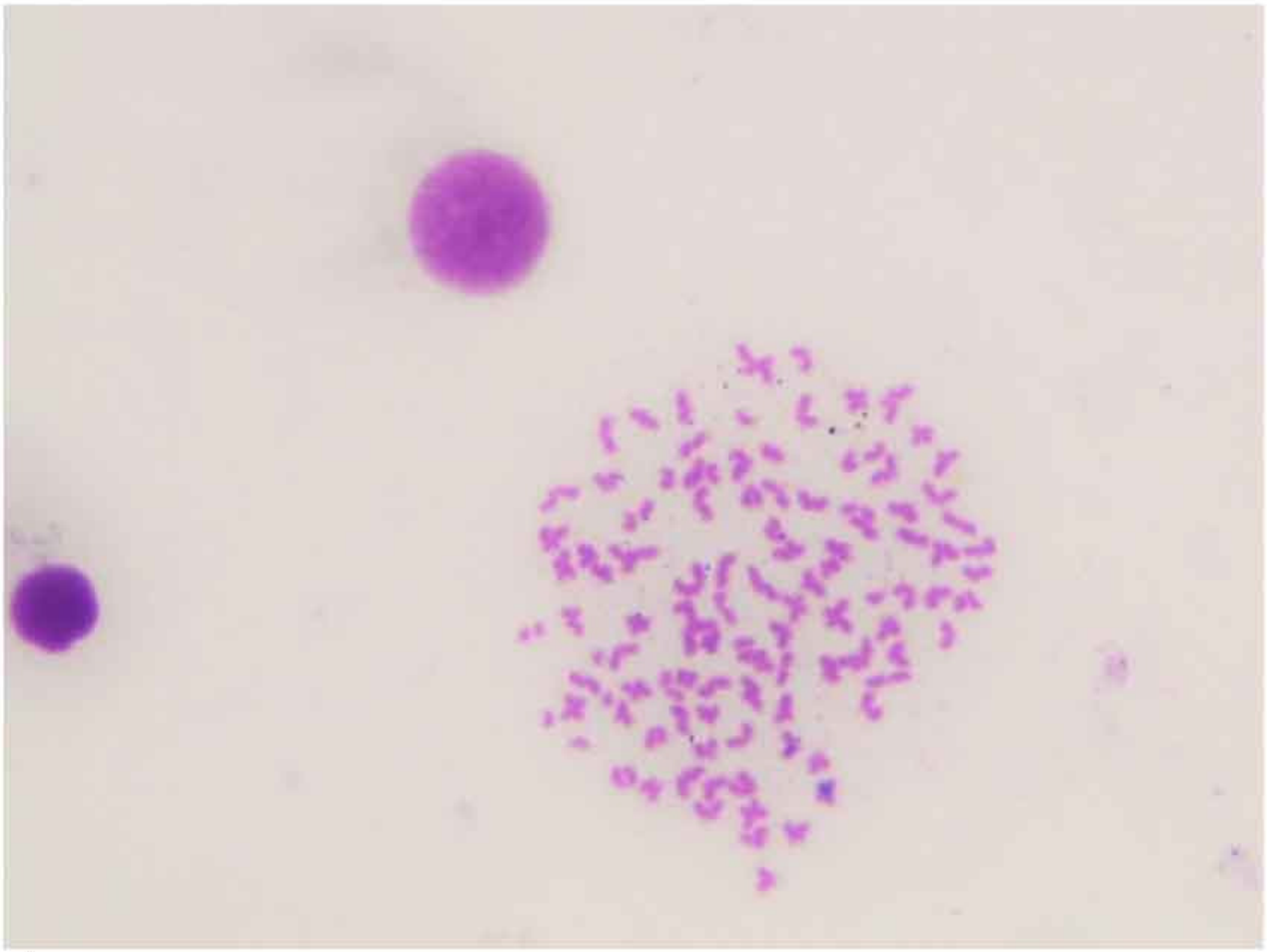

Number of cells X 10^5 per ml
0
1
2
3
4
5
6
Days
0
1
2
3
4
5
20ºC
24ºC
28ºC
35ºC
37ºC

B

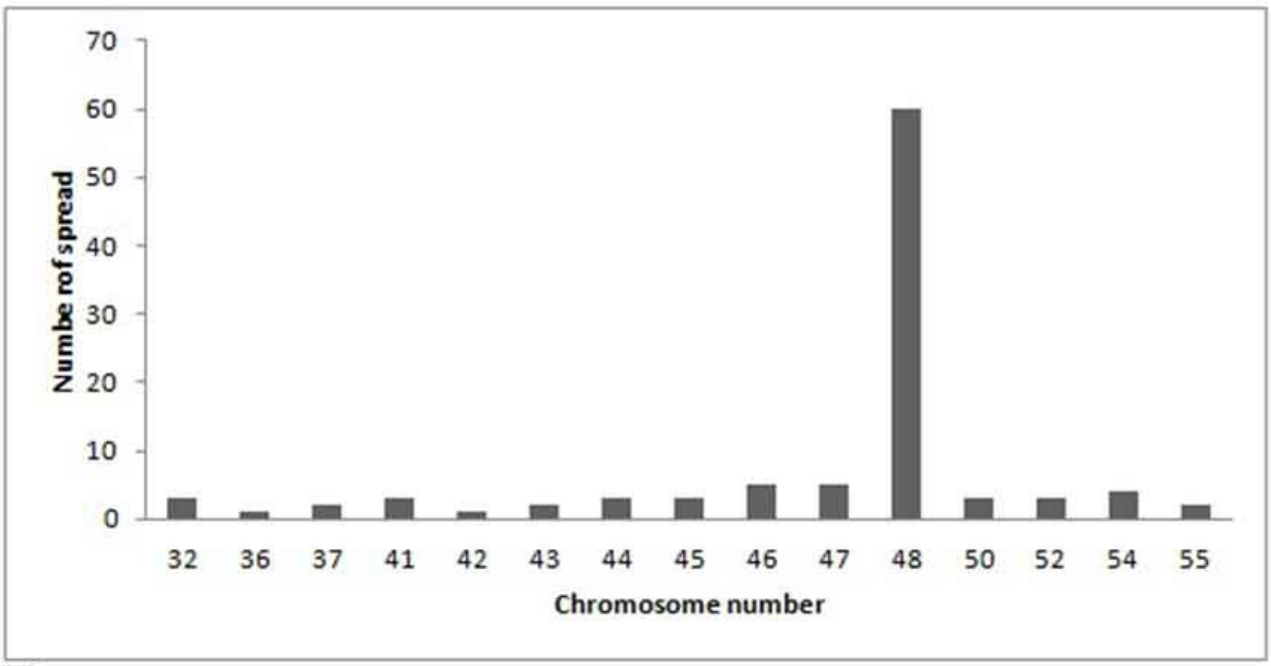
Figure 3

Morphological characteristics and frequency distribution of the chromosomes of the FtGF cell line at passage 30.

(A) Phase-contrast photomicrograph of chromosome spread arrested in metaphase (original magnification: X400); (B) Frequency distribution of chromosomes in 100 cells.



A


70
60
50
40
30
20
10
0
Numbe rof spread
32
36
37
41
42
43
44
45
46
47
48
50
52
54
55
Chromosome number

B

Figure 4

The cytopathic effect (CPE) in FtGF cells following infection with CyHV-2 at 28° C.

(A) Uninfected FtGF cells; (B) FtGF cells infected with CyHV-2 at passage 15 at 2 dpi; (C) FtGF cells infected with CyHV-2 at passage 15 at 4 dpi; (D) FtGF cells infected with CyHV-2 at passage 15 at 6 dpi.

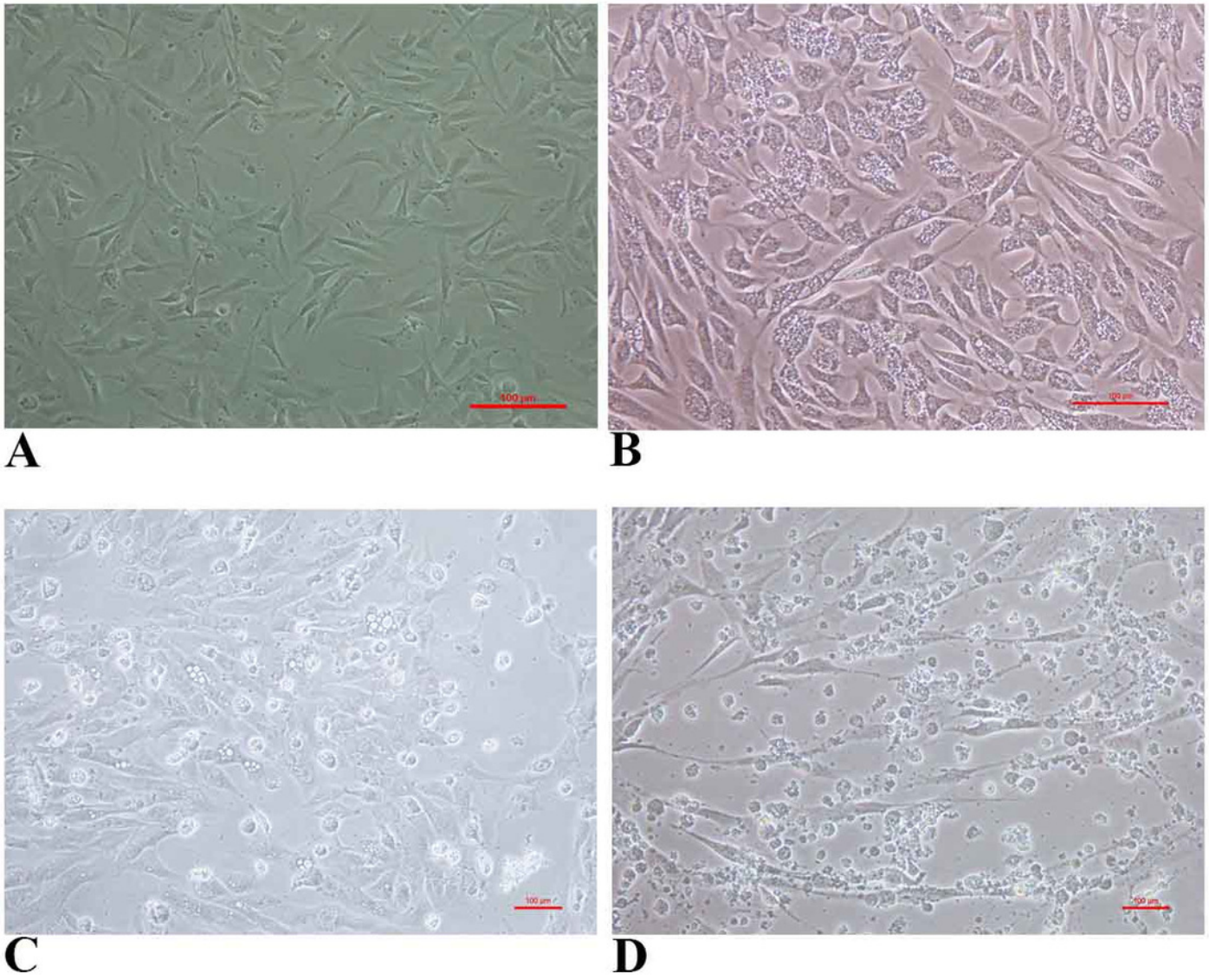
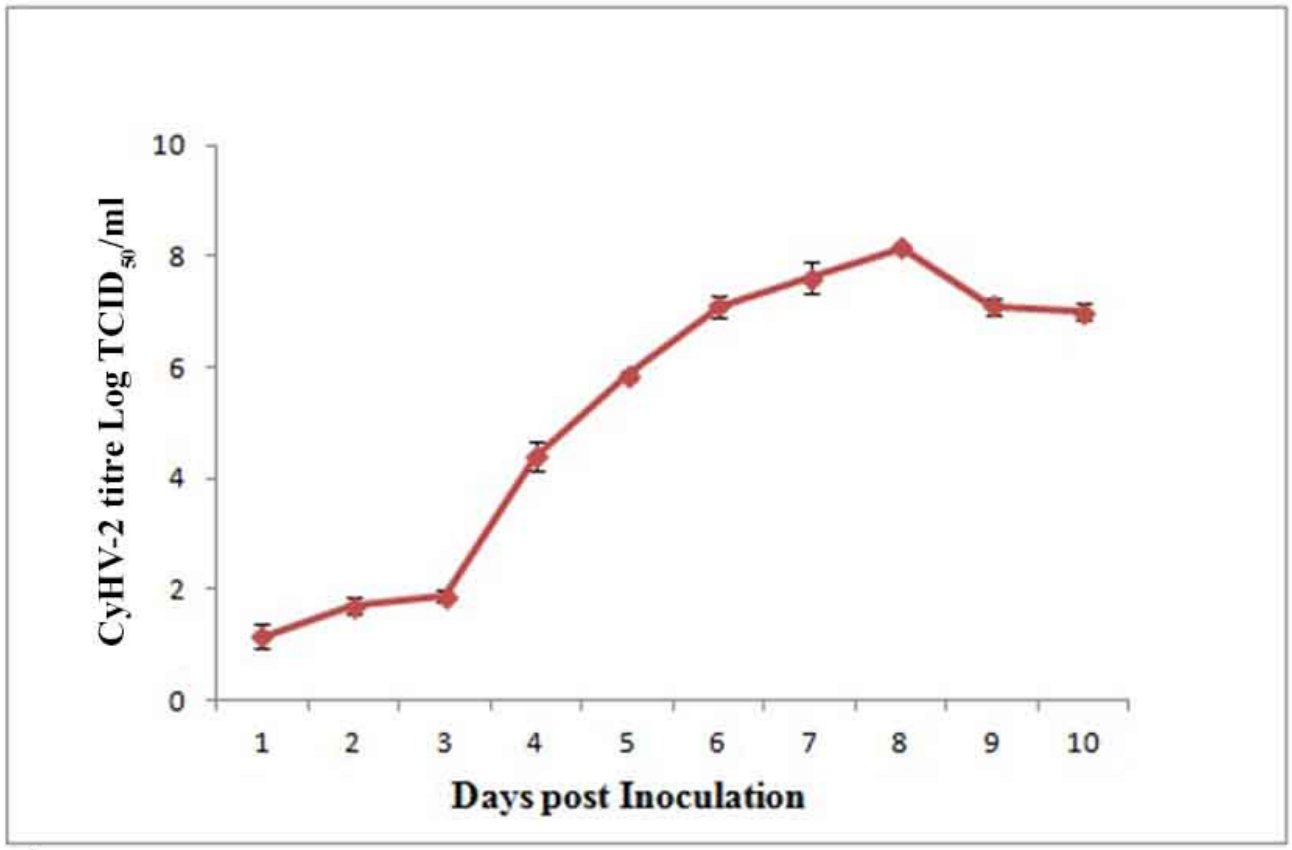


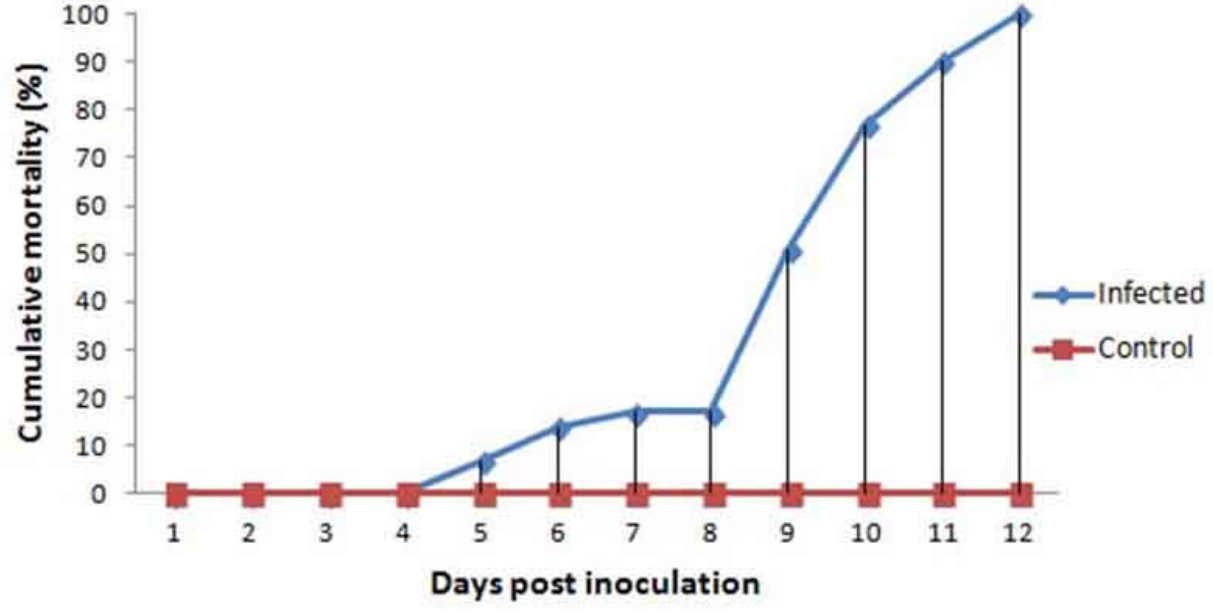
Figure 5

Growth curve of CyHV-2 in FtGF cell line and mortality curve of experimentally infected goldfish.

(A) Growth curve of CyHV-2 (at passage 20) in the FtGF cell line. Values plotted are means ±SD of the measurements. (B) Cumulative mortality curve of the experimentally infected goldfish (12-15 cm; 16-23 gm), using challenged with $10^{7.8\pm0.26}$ $TCID_{50}$/mL CyHV-2 (passage 10) propagated in FtGF cells.


CyHV-2 titre Log TCID50/ml
0
2
4
6
8
10
1
2
3
4
5
6
7
8
9
10
Days post Inoculation

A


Cumulative mortality (%)
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8
9
10
11
12
Days post inoculation
Infected
Control

B

Figure 6

Histopathological lesions in kidney, spleen and gills of a goldfish with Goldfish Herpesviral Hematopoietic Necrosis Disease.

The nuclei of cells with marginated chromatin (arrows) in goldfish experimentally infected with CyHV-2 (A) Kidney and (B) Spleen. Nuclei of infected cells with marginated chromatin (arrows) in naturally CyHV-2 infected goldfish (C) Kidney and (D) Spleen.

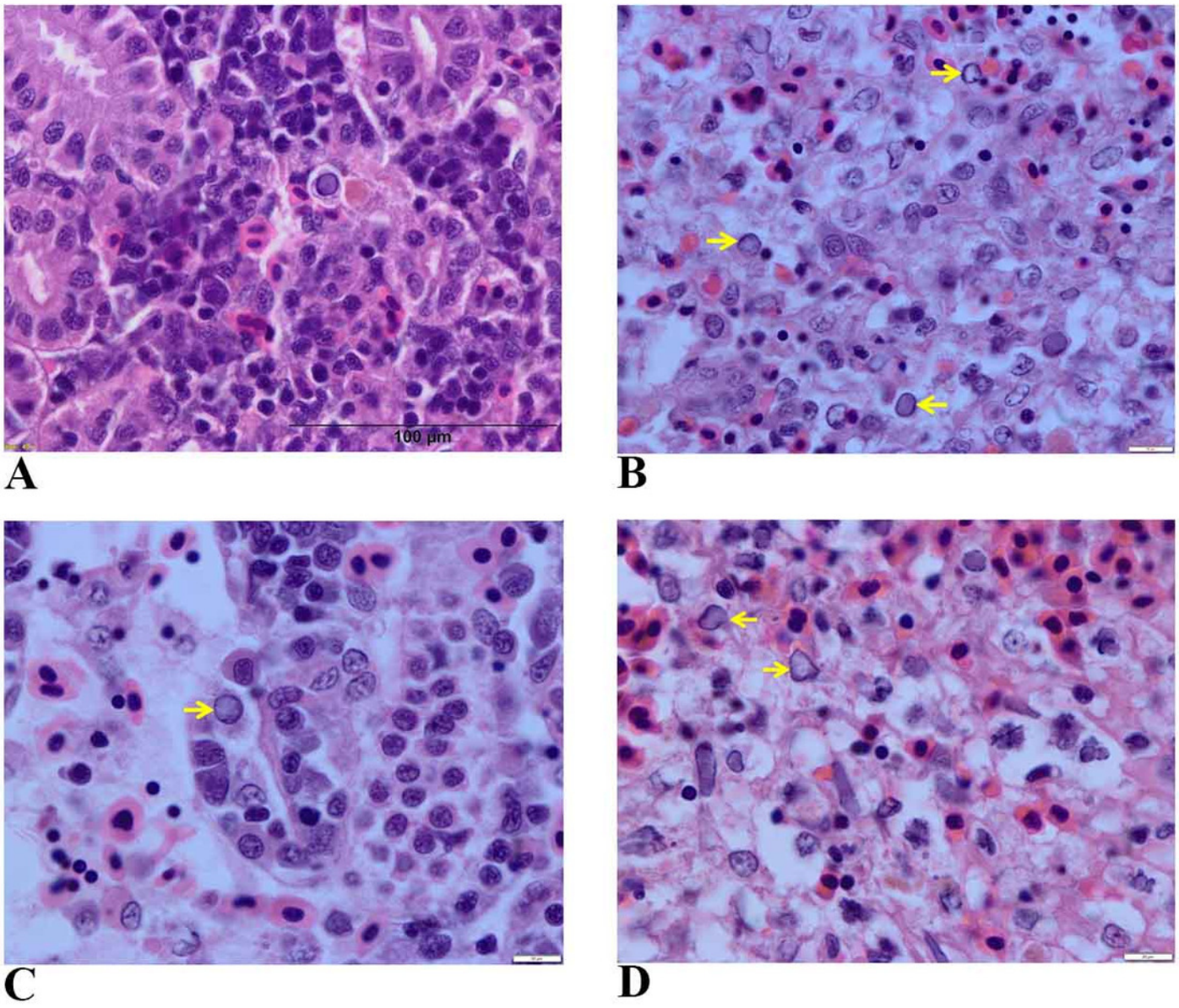


Figure 7

Transmission electron micrograph

Enveloped mature CyHV-2 virions in the ultrathin sections of gill (A), spleen (B) cells of experimentally challenged of goldfish and mature virus particles (C) purified from CyHV-2 infected FtGF cells. Viral capsid in cytoplasm with electron-dense cores and capsid surrounded by electron-dense material. Scale bar = 200 nm.