Establishment and characterisation of fantail goldfish fin (FtGF) cell line from goldfish, *Carassius auratus* for *in vitro* 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 the aquaculture of goldfish, *Carassius auratus.* Permissive cell lines for the isolation and propagation of CyHV-2 have been established from various goldfish tissues by sacrificing the fish. Here, we report the development of a cell line, FtGF (Fantail Goldfish Fin) from caudal fin of goldfish using non-lethal sampling. We also describe a simple protocol for successful establishment and characterization of a permissive cell line through explant method and continuous propagation of CyHV-2 with high viral titre using this cell line. **Methods:** Caudal fin tissue samples were collected from goldfish without killing the fish. 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, incubated at 28 °C. Cells were characterized and origin of the cells was confirmed by sequencing fragments of the 16S rRNA and COI genes. CyHV-2 was grown in the FtGF cells and passaged continuously 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 56 times in L-15 with 10% FBS. Karyotyping of FtGF cells at 30[th,] 40th and 56th passage indicated that modal chromosome number was 2n = 104. Species authentication of FtGF was performed by sequencing of the 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 CyHV-2 positive tissue homogenate, FtGF cells showed cytopathic effect by 2nd day post-inoculation (dpi) and complete destruction of cells was observed by the 10th 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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10 **Running page head**: CyHV-2 isolation in India

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Abstract

Background:

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Methods:

Caudal fin tissue samples were collected from goldfish without killing the fish. 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, incubated at 28 °C. Cells were characterized and origin of the cells was confirmed by sequencing fragments of the 16S rRNA and COI genes. CyHV-2 was grown in the FtGF cells and passaged continuously 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 56 times in L-15 with 10% FBS. Karyotyping of FtGF cells at 30th, 40th and 56th passage indicated that modal chromosome number was 2n = 104. Species authentication of FtGF was performed by sequencing of the 16S rRNA and COI genes. The cell line was used for continuous propagation of CyHV-2 over 20 passages with high viral titer of

42    $10^{7.8\pm0.26}$ TCID$_{50}$/mL. Following inoculation of CyHV-2 positive tissue homogenate, FtGF cells

43   showed cytopathic effect by 2nd day post-inoculation (dpi) and complete destruction of cells was

44   observed by the 10th dpi. An experimental infection of naïve goldfish using supernatant from

45   infected FtGF cells caused 100% mortality and CyHV-2 infection in the challenged fish was

46   confirmed by the amplification of DNA polymerase gene, histopathology and transmission

47   electron microscopy. These findings provide confirmation that the FtGF cell line is highly

48   permissive to the propagation of CyHV-2.

49   **Introduction**

50   Cyprinid herpesvirus-2 (CyHV-2), the etiological agent of herpesviral haematopoietic necrosis

51   (HVHN) disease, is a pathogen of goldfish that has been associated with disease outbreaks in

52   goldfish culture (Jeffery et al., 2007). The pathogen first reported as the cause of outbreaks in

53   juvenile goldfish in 1992 from Japan (Jung & Miyazaki, 1995), has subsequently been reported

54   from more than 13 countries (Adamek et al., 2018). In India, CyHV-2 infection was associated

55   with large-scale mortalities of goldfish in West Bengal (Sahoo et al., 2016). As the surviving fish

56   become lifelong carriers of latent infection without exhibiting any clinical signs of the disease

57   (Wang et al., 2012) and goldfish are one of the most traded ornamental fish globally, the

58   international trade of such apparently healthy but latently infected goldfish is suspected to be

59   responsible for the global spread of CyHV-2 (Ito et al., 2013; Adamek et al., 2018).

60   Cell lines are considered the gold standard for isolation and identification of viruses

61   (Landry, 2009). Earlier, a number of cell lines from other fish species, namely Epithelioma

62   papulosum cyprini cells (EPC), fathead minnow cells (FHM) and koi fin cell lines (KF-1) have

63   been used for CyHV-2 cultivation, but these cell lines did not support its continuous propagation

64   beyond 5th passage (Jeffery et al., 2007; Wang et al., 2012). In an earlier study from India, the

65 virus could not be propagated beyond 4th passage, despite producing cytopathic effects in CCKF

66 cell line (Sahoo et al., 2016),. It has been reported that cell lines derived from homologous

67 species provide sensitive tools for virus propagation (Fryer & Lannan, 1994) and successful

68 cultivation of CyHV-2 has been reported in cell lines developed from goldfish, namely GFTF

69 (Yan, Nie & Lu, 2011), GFF and SRTF (Ito et al., 2013) and GiCB (Ma et al., 2015). More than

70 15 cell lines have been developed globally from different goldfish tissues since 1964 (Table.1).

71 However, no cell line from goldfish is available in India, therefore, the need of establishing a

72 homologous cell line from goldfish was deemed necessary for the continuous propagation and

73 study of CyHV-2.

74   In this paper, we report the development of a cell line from the caudal fin of fantail

75 goldfish (FtGF). The cell line has been successfully employed for propagation of CyHV-2, and

76 experimental reproduction of HVHN using supernatant from the CyHV-2 infected cell line (at

77 10th passage). The newly developed FtGF cell line will play significant role in future research on

78 CyHV-2, including the development of strategies for the prevention and control of the disease

79 caused by CyHV-2 in India.

80 **Materials and methods**

81   **Generation of the primary cell culture**

82   A healthy fantail goldfish weighing 20 g was purchased from a local commercial aquarium shop

83 and kept in properly aerated and filtered fish tank. The fish was fed daily and water changes were

84 performed on alternate days. The goldfish was anaesthetized using 3- aminobenzoic acid ethyl

85 ester methanesulphonate (MS-222, Sigma-Aldrich, St. Louis, MO) at the dose rate of 150 mg/L

86 of water and the caudal fin was excised after wiping with 70% ethanol. The excised tissue was

87 washed three times in Phosphate Buffer Saline (PBS) (Life Technologies, Carlsbad, CA; Lot

88 number – 1967526; Catalogue number – 14190-144 500mL) containing antibiotic-antimycotic

89 solution (200 IU/mL penicillin, 200 μg/mL streptomycin and 0·5 μg/mL amphotericin B) (Life

90 Technologies, Carlsbad, CA; Lot number – 2112695; Catalogue number - 15240-062 100 mL).

91 The fin tissue was mechanically cut with a fine scalpel into smaller pieces and then seeded in a

92 25 cm² flask. The PBS was removed and attachment of the tissues to the surface was facilitated

93 by adding 200 µl of fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA; Lot number –

94 42G829K; Catalogue number – 10270-106 500 mL). After 2 h, about 7 mL of Leibovitz's L-15

95 (L-15) medium (Life Technologies, Carlsbad, CA; Lot number – 2085192; Catalogue number –

96 11415-064 500 mL) containing 20% FBS and 1X concentration of antibiotic antimycotic

97 solution was added to the flask, which was then incubated at 28 °C. The flask was observed daily

98 under an inverted light microscope (Nikon Corporation, Tokyo, Japan).

99 **Subculture and maintenance**

100 After formation of monolayer, the cells were washed with PBS followed by trypsinization with

101 0.25% trypsin EDTA solution (Life Technologies, Carlsbad, CA; Lot number – 2053183;

102 Catalogue number – 15400-054 100 mL). The cells were observed under an inverted light

103 microscope. The trypsin EDTA solution was discarded immediately on observing cell

104 detachment. The flasks were gently tapped to release the cells from the surface and L-15 medium

105 with 20% FBS was added to stop the action of trypsin. The cells were subcultured at a split ratio

106 of 1:2. The flasks were observed regularly and the cells were subcultured after attaining 80-90%

107 confluence. After 10 subcultures, the concentration of FBS in medium was reduced to 10%.

108 **Cell growth characteristics**

109 Cell growth studies were conducted at different temperatures and FBS concentrations in L-15

110 medium. The growth rate assays were conducted at temperatures ranging from 20 °C to 37 °C

111 and FBS concentrations from 5% - 20%. A monolayer flask at 20th passage was trypsinized and

112 the cells were seeded into different 6 well plates. The plates were incubated overnight at 28 °C.

113 Thereafter, the plates were incubated at different temperatures of 20 °C, 24 °C, 28 °C, 35 °C and

114 37 °C and the growth rate was observed. Cells were harvested from the duplicate wells daily and

115 counted with a Neubauer haemocytometer. A similar study was conducted using different 6 well

116 plates incubated at 28 °C having different FBS concentrations of 5%, 7.5%, 10%, 15% and 20%.

117 The cells at 25th passage at the concentration of 1×10^5 cells per ml were seeded into 24-well

118 plates and cultured for 7 d at 28° C. The cells were harvested, the number of cells in each well

119 counted with a hemocytometer, and the average value of 3 wells at each time was used to plot

120 the growth curve. The growth curve experiment of FtGF cells was repeated two times. Cell

121 growth curves were plotted, and the population doubling time (PDT) was calculated based on the

122 growth curve (Weingartl et al., 2002). Growth characteristic experiment assays were carried out

123 for 8 d, and cell number was represented as mean± standard deviation (SD).

124 **Chromosome analysis**

125 The chromosomal counts for FtGF were undertaken from the 30th, 40th and 56th passage flasks. A

126 flask with 80% confluence was treated with 1 μg/mL colchicine (Sigma, St Louis, MO, USA) for

127 4 h at 28 °C and then the supernatant was removed, the cells harvested and resuspended in a

128 hypotonic 0.5% KCl solution for 10 min, and then fixed in a 3:1 methanol : acetic acid solution.

129 The cells were pelleted at 367 g at 4 °C for 10 min and washed four times with methanol and

130 acetic acid solution. Smears were prepared following a conventional drop-splash technique

131 (Freshney, 2010), the slides were stained with 10 % Giemsa solution and air dried for 30 min. A

132 total of 100 chromosome spreads were observed and counted under a light microscope (Leica,

133 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. at a density of 5 x 10^6 cells/mL, Briefly, the flask was examined using an inverted microscope to monitor the confluence and to check for microbial contamination if any. The spent cell culture medium was removed. The cell monolayer was washed with a balanced PBS salt solution without calcium and magnesium three times and ensuring the adherence of the cells. One ml of 0.25% trypsin EDTA was added to the 25 cm^2 surface area flask to cover the monolayer. Excess solution was discarded and the flask was incubated at 37°C for 4 min. with gentle rocking. The flask was examined for the detachment of cells, the remaining cells were released with gentle tapping and cells were re-suspended in L-15 medium with 20% FBS to deactivate the trypsin. The harvested cells were dispersed by repeated mild pipetting and cells were counted (Freshney, 2010). The cells were suspended in recovery cell-culture freezing medium (Life Technologies, Carlsbad, CA; Lot number – 492531; Catalogue number – 12648-010 50 mL) aliquoted into 2 mL sterile cryovials (Nunc), kept at 4 °C for 2 h, -20 °C for 1 h, -80 °C 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.

Transfection assays

157   The FtGF cells at the 25th passage (revived from cryopreserved cells) were harvested and

158   grown on coverslip for determining the nature of the cells and to know the transfection efficiency

159   of the cells. The protocols earlier described by Swaminathan et al (2016) were followed for

160   immunophenotyping of cells and transfection studies. FtGF cells in 20 fields were counted

161   randomly and the percentage of positive green fluorescent protein (GFP) cells was calculated.

162   **Molecular characterization of the cell line**

163   DNA isolation was carried out by salting out method following Miller, Dykes & Polesky (1988).

164   The DNA was extracted from FtGF cells at 40th passage and muscle tissue of goldfish. PCR was

165   carried out as per Swaminathan et al., (2016b). PCR products of 562 bp and 642 bp were

166   amplified for mitochondrial 16S rRNA and cytochrome c oxidase subunit I (COI) genes using

167   universal primers ((F 5′CGC CTG TTT ATC AAA AAC AT 3′and H 5′CCG GTC TGA ACT

168   CAG ATC ACG T 3′ (Ward, Zemlak, Innes, Last & Hebert, 2005) and F 5′TCA ACC AAC

169   CAC AAA GAC ATT GGC AC 3′and R 5′TAG ACT TCT GGG TGG CCA AAG AAT CA3′

170   (Palumbi et al., 1991) respectively. The PCR products of both the fragments were sequenced in

171   an ABI 3730 DNA analyser (Applied Biosystems). The sequences of both the mt DNA gene

172   PCR fragments were compared with the published and known sequences in the National Centre

173   for Biotechnology Information (NCBI) database by the basic local alignment search tool

174   (BLAST) (Altschul et al., 1990).

175   **Detection of *Mycoplasma* contamination in FtGF cell line**

176   *Mycoplasma* contamination was tested by PCR with 15th and 40th passage FtGF cells grown for 4

177   days in L-15 medium without antibiotics. Briefly, the harvested cells were centrifuged at 200 *g*

178   for 10 min and the supernatant was transferred into micro-centrifuge tubes and centrifuged at 250

179   *g* to remove debris. The *Mycoplasma* contamination was checked using EZdetect PCR Kit

180 (HiMedia) based on amplification of spacer region between 16S rRNA and 23S rRNA. The

181 amplification products were analysed in 1.5% agarose gel.

182 **CyHV-2 isolation on FtGF cells and viral titer determination**

183 Diseased goldfish obtained from a farm in Kerala, India were confirmed to be infected with

184 CyHV-2 by PCR as per Jeffery et al. (2007). External examination of moribund goldfish

185 revealed clinical signs including simple loss of scales, pale mucous covered gills. Internally, all

186 visceral organs of affected fish were congested and enlarged and white nodules were found on

187 the spleen and the kidney. Gill, kidney and spleen tissues from diseased fish were collected

188 aseptically, and then homogenized in Dulbecco's Phosphate Buffered Saline (DPBS), freeze-

189 thawed for 3 cycles and the tissue homogenate was centrifuged at 3000 g for 30 min at 4 °C. The

190 supernatant was then filtered through a 0.22 μm filter (Millipore) and checked for bacterial

191 contamination before use. Thereafter, 500 μl of the filtered tissue homogenate was inoculated

192 onto 25 cm^2 flasks after removing the medium and incubated in a shaking incubator (45 rpm) at

193 28 °C. In control flasks, maintenance medium was used in place of tissue homogenate. After 1 h

194 adsorption, 6.5 mL of the maintenance medium (L-15 medium with 2% FBS) was added to the

195 FtGF flasks, which were incubated at 28 °C. The inoculated flasks were checked daily for

196 cytopathic effects (CPE). The cells from flasks exhibiting 80 - 90% CPE were harvested along

197 with supernatant. This cell suspension was frozen at -80 °C for further use. Further, to know the

198 temperature range for CyHV-2 replication, FtGF cells were infected with CyHV-2 and incubated

199 at 18, 20 and 25 °C.For titration, FtGF cells were grown in a 96-well plate with a confluence of

200 70% to 80%. After removing the medium, filtered tissue homogenate was diluted ten-fold (10^{-1}

201 to 10^{-9}), and 0.1 mL of diluted filtrate was inoculated in triplicate and allowed to adsorb for 1 h.

202 In control wells, DPBS was added in place of filtered tissue homogenate. Thereafter, 0.2 mL of

203   maintenance medium was added to each well and the plate was incubated at 28 °C. The wells

204   were examined daily for the appearance of CPE up to 2 weeks. The virus titer was determined by

205   50% tissue culture infective dose ($TCID_{50}$) assay using Reed & Muench (1938) calculations. The

206   viral susceptibility and titration assays of FtGF cells were determined based on three independent

207   experiments.

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

209   Clinically healthy goldfish (12-15 cm; 16-23 gm), procured from a local ornamental fish farm,

210   were divided into two groups of 30 fish each and acclimatized in the laboratory aquarium tanks

211   for a week. The tissues from randomly collected goldfish (n=5) were screened for CyHV-2 using

212   PCR following Jeffery et al. (2007). The fish were anaesthetized with MS-222 (Sigma-Aldrich).

213   Fishes in the infected group were challenged with an intraperitoneal (IP) injection of 0.5 mL of

214   10th passage CyHV-2 FtGF cell culture supernatant, whereas fish in the control group were

215   injected with 0.5 mL maintenance medium. The water temperature was maintained at 28 °C

216   during the experiment and fish were observed daily for clinical signs and mortality. Three

217   goldfish each in the infected and control group were selected randomly after 7 days post-

218   injection (dpi) and screened for CyHV-2 by PCR assay. The experimental challenge study in fish

219   was carried out following ARRIVE guidelines and carried out in accordance with the National

220   Institutes of Health guide for the care and use of Laboratory animals. The experimental challenge

221   trials were evaluated and approved by Institute Animal ethics Committee (IAEC) of ICAR

222   National Bureau of Fish Genetic Resources (NBFGR) vide approval Number G/IAEC/2019/1

223   dated 04th October 2019.

224   **Confirmation of the CyHV-2 virus**

225   **Polymerase chain reaction**

226 The naturally infected tissues from goldfish, supernatant along with cells collected from FtGF

227 flask and infected tissues from experimentally infected goldfish were processed for confirmation

228 by PCR. The harvested FtGF cells were centrifuged and DNA was isolated from cell pellet using

229 DNeasy blood and tissue kit (Qiagen). Concentration and purity of the extracted DNA was

230 determined by measuring OD at 260 and 280 nm using a NanoDrop ND1000 spectrophotometer

231 (Nano Drop Technologies Inc). The samples were stored at -20 °C for further analysis. PCR was

232 performed using published oligonucleotide primers CyHVpol-FOR and CyHVpol-REV primers

233 (Jeffery et al., 2007) for confirmation. Briefly, amplification was performed in 25 µl reaction

234 mixture containing 2.5 µl of 10X Taq buffer, 1.5 µl (10 pmol) of each primer, 0.5 µl of dNTPs (2

235 mM), 0.25 µl of Taq DNA polymerase (5 U µl^{-1}), 1 µl of total DNA and ddH$_2$O to make final

236 volume to 25 µl. The reaction mixture was pre-heated at 95° C for 3 min followed by 40 cycles

237 of 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension

238 at 72 °C for 10 min. The PCR products were visualized following electrophoresis in 1.5%

239 agarose gel. Representative PCR amplicons from each primer set were purified and sequenced by

240 Sanger sequencing facility (Scigenom Pvt. Ltd). These sequences of amplified PCR products

241 from all the cases (natural infected goldfish, experimentally infected goldfish and infected FtGF

242 cells) were confirmed by BLAST analysis.

243 **Histopathology**

244 For histopathological examination, tissues including gills, kidney and spleen were collected from

245 moribund fish at 7 dpi and fixed in 10% neutral buffered formalin. Tissues were dehydrated in

246 ascending grades of ethanol, cleared in chloroform and embedded in paraffin wax. Thin tissue

247 sections, 4-5 µm thick were cut and stained with haematoxylin-eosin for examination under a

248 compound microscope.

249 **Transmission electron microscopy**

250 The experimentally infected fish tissues and CyHV-2 infected FtGF cells were fixed with 2.5%

251 glutaraldehyde, post fixed with osmium tetroxide for 1 h at 4 °C, then dehydrated and embedded.

252 A microtome (Leica ultracut UCT) was used to cut thin sections of about 60–70 nm. Sections

253 were stained with uranyl acetate and alkaline lead citrate after mounting on copper grids.

254 Sections were observed and photographed under a Tecnai T12 Spirit transmission electron

255 microscope at 60 kV. Supernatant from CyHV-2 infected FtGF cells displaying CPE were

256 studied by electron microscopy. Subcultured samples were negatively stained with

257 phosphotungstic acid and examined using a High Resolution Transmission Electron Microscope,

258 (FEI-TECNAI-G2 20 TWIN) at 7800× magnification and an accelerating voltage of 120 kV.

259 **Statistical analysis**

260 In the present study, all statistical analysis of the data was performed with SPSS version 13.0

261 (IBM; www.ibm.com). All results are expressed as mean ± SD. *P* values<0·05 were considered

262 statistically significant.

263 **Results**

264 **Primary cell culture and maintenance**

265 The primary culture of fin cells from goldfish was achieved using L-15 medium supplemented

266 with 20% FBS at 28 °C. The cells were seen emerging from the sides and edges of caudal fin

267 tissues (Fig. 1A) and they readily attached to the bottom of the flask. A monolayer culture was

268 obtained within 10 days at 28 °C. The monolayer was passaged at 1:2 ratio every 6-7 days. The

269 growth pattern of lag phase (1-3 days), log phase (3-5 days) and stationary phase (5-7 days) was

270 observed. During the initial passages, mixed fibroblast-like and epithelial-like cells were

271 observed and after 10 passages, epithelial cells dominated over fibroblastic cells (Fig. 1B). The

272   FtGF cells have been subcultured for over 55 passages and the cell line has been designated as

273   Fantail Goldfish Fin (FtGF) cell line. The morphology of FtGF cells at 25th passage and at 56th

274   passage are shown in Fig.1 C & D.

275   **Characterization of FtGF cell line**

276   The FtGF cells were characterized to determine the ability to grow at different temperatures and

277   different concentrations of FBS, to check the chromosome number of the cells, to authenticate

278   the species origin by DNA barcoding, to examine for the *Mycoplasma* contamination, to confirm

279   the nature of the cells by immunofluorescence test and to check the transfection efficiency of the

280   cells. The most favourable conditions for culturing of FtGF cells were tested at different

281   incubation temperatures including 20, 24, 28, 35 and 37 °C as well as at different concentrations

282   of FBS viz., 5, 7.5, 10, 15 and 20%. The cells incubated at 20 and 37 °C showed rounding and

283   started detaching on 2nd day. Cells grew in the flask at 24 °C, but the growth rate was slow.

284   Maximum growth of the cells was observed at 28 °C. At 35 °C, FtGF cells proliferated very fast

285   initially, but became puffy and were dying by the 2nd day. Culture of FtGF cells grew best in L-

286   15 with 20% FBS and a slower growth was recorded with decreasing concentration of FBS. The

287   growth kinetics of FtGF cells did not show much difference in 10, 15 and 20% FBS, but cells in

288   7.5 and 5% FBS had slower growth. The suitable temperature and FBS concentration for the

289   culture of FtGF cells were found to be 28 °C and 10% FBS, respectively (Fig. 2A & B). The

290   growth curve of FtGF cells at passage 25 (Fig. 2C) showed as typical "S" shape and the cells

291   were at latent stage before day 1 after seeding and then the cells proliferated rapidly and went

292   into logarithmic stage from day 1 to day 5. The cell number remained stationary between day 5

293   and day 8, but began to decline after day 9. The FtGF cells grew and proliferated at a steady rate,

294   and their doubling time was calculated to be 33.9 h at passage 25. The FtGF cell growth was

295   arrested in metaphase using Colchicine at final concentration of 1 µg/mL. Karyotyping results

296   obtained by counting 100 spreads at metaphase showed 82–114 chromosomes, with a distinct

297   peak for the cell line at 104 diploid chromosomes at 30th, 40th and 56th passage. The majority of

298   the cells (60%) had a diploid chromosome number (2n=104) at all three passage levels. The

299   diploid karyotype of FtGF cells and its frequency distribution at passage 30 are shown (Fig. 3A

300   & 3B) respectively. The FtGF cells at 20th and 40th passage were revived and these exhibited 70-

301   75% viability. The cells were incubated at 28 °C and a monolayer was established within 15

302   days. Green fluorescent signals were observed in FtGF cells transfected with 2 *µ*g of pAcGFP1-

303   N1 expression vector and strong green fluorescent signals were detected at 52 h post transfection

304   and transfection efficiency was calculated about 28% thus confirming their potential to be used

305   in gene expression studies. (Fig.3C). The origin of FtGF cell line was confirmed by partial

306   amplification and sequencing of 16S rRNA and COI genes from FtGF cells and goldfish muscle

307   and comparison with available COI and 16S rRNA sequences in GenBank. Nucleotide sequence

308   analysis revealed 100% similarity with sequences from goldfish muscle and maximum similarity

309   (99%) with COI (Accession number KX145542 and KX145499) and 16S rRNA sequences

310   (Accession number AJ247070, KY231826) of goldfish in GenBank. These data confirm that the

311   origin of the developed FtGF cell line is from goldfish. No target band of spacer region between

312   16S and 23S rRNA of *Mycoplasma* was observed indicating that the FtGF cells were free of

313   *Mycoplasma* contamination.

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

315   FtGF cells were infected with CyHV-2 and evaluated by significant CPEs observed. In this

316   study, CyHV-2 was propagated with high viral titre in the FtGF cell line. The FtGF cell line (Fig.

317   4A) infected with CyHV-2 began to show morphological changes from the 2nd dpi (Fig. 4B) and

318   the CPE such as cell elongation, rounding, and cell fusion with cytoplasmic vacuolation were

319   observed from 4th dpi (Fig. 4C) at 28 °C. Cell death started from the 6th dpi (Fig. 4D) and

320   complete detachment was observed by 10 days. The appearance of typical CPE in FtGF cells

321   infected with Indian CyHV-2 was observed at 8 dpi, 6 dpi and 5 dpi when the cells were

322   incubated at 18, 20 and 25 °C respectively after inoculation of the virus and it was observed that

323   the efficient propagation of Indian CyHV-2 was most rapid (2 dpi) at 28 °C. The infected FtGF

324   cells and the supernatant were confirmed for the presence of CyHV-2 by PCR and sequencing.

325   No CPE was observed in the control cells inoculated with maintenance medium. The $TCID_{50}$ of

326   the culture suspension harvested from infected FtGF was found to be $10^{7.8\pm0.26}$ $TCID_{50}$/mL

327   (Fig.5A). The CyHV-2 could be propagated in FtGF flasks for 20 passages.

328   **Virulence of CyHV-2 propagated in FtGF cells to goldfish**

329   To confirm the infectivity of CyHV-2 propagated on FtGF cell line, an experimental challenge

330   study was carried out in healthy goldfish. The goldfish challenged with 1.4 X 10^7 $TCID_{50}$/mL

331   CyHV-2 (passage 10) produced in the FtGF cell line began to die at 5 days post inoculation (dpi)

332   and the mortality reached 100% at 12 dpi (Fig.5B). The dead fish from infected groups exhibited

333   similar clinical signs seen in naturally infected fish. The clinical signs of challenged goldfish

334   were haemorrhages on the body surface, exophthalmia, pale gills and a swollen abdomen (Fig.

335   5C and D). There was no mortality in the mock-infected group. The tissues from randomly

336   selected fish from the infected groups tested positive for CyHV-2 by PCR and produced CPE in

337   FtGF, while fish from control group were negative. The CyHV-2 recovered from experimentally

338   infected fish was also found to be virulent to goldfish and caused similar symptoms (unpublished

339   data).

340   **Confirmation of the CyHV-2 virus**

341   The confirmation of the CyHV-2 infection in infected tissues, FtGF cell culture suspension, and

342   experimentally challenged fish was carried out by amplification of fragment of DNA polymerase

343   gene using PCR, histopathological investigation and transmission electron microscopy.

344   **Polymerase chain reaction**

345   Tissue samples (gills, spleen and kidney) collected from naturally infected tissues, FtGF cell

346   culture suspension, and experimentally challenged fish were found to be positive for CyHV-2 in

347   PCR assay. The expected PCR product of 362 bp was obtained in all CyHV-2 positive samples.

348   A GenBank BLAST search on the sequence revealed a high identity to CyHV-2 isolate SYC1

349   strain (KM200722, 99.9%) and CyHV-2 isolate STJ1 strain (JQ815364, 99.9%). The sequence

350   of the DNA polymerase gene of CyHV-2 isolated from goldfish in this study was deposited in

351   GenBank (GenBank accession nos. KU527548 and KU527549).

352   **Histopathology**

353   Hypertrophied nuclei with marinated chromatin material in sections of gills, spleen and kidney

354   from goldfish experimentally infected with CyHV-2 (Fig. 6A, B & C) and inflammatory exudate

355   between the secondary lamellae were observed in sections of gills. The lesions of the

356   experimentally challenged fish were compared with the sections of gills, spleen and kidney in

357   naturally CyHV-2 infected goldfish.

358   **Transmission electron microscopy**

359   The mature virus particles were observed ultra structurally in cytoplasm of cells in gills as well

360   as spleen tissue of experimentally challenged fish (Fig. 7A & B) and CyHV-2 infected FtGF

361   cells that were about 170–180 nm in diameter (Fig. 7C). Supernatant of FtGF cells with CPE

362   were examined by TEM, and viral particles morphologically similar to a herpesvirus were

363   observed (Fig. 7D).

364   **Discussion**

365   The ease of detecting and isolating the virus using cell lines provides a remarkable advantage in

366   viral disease management. Isolation of virus in cell culture has been considered as the "gold

367   standard" for the viral disease diagnosis for decades. It would be a valuable approach when a

368   viable virus isolate is needed and also to differentiate nonviable virus in a clinical specimen.

369   Over the past few years, a number of fish viruses *viz*., viral nervous necrosis virus (Azad et al.,

370   2005), iridovirus (George et al., 2015), cyprinid herpesvirus-2 (Sahoo et al., 2016), and carp

371   edema virus (Swaminathan et al., 2016a), have been reported from India and some of these have

372   also been isolated using fish cell lines. As CyHV-2 mainly affects goldfish, development of

373   species-specific cell line will greatly aid in disease health management. Several cell lines have

374   been employed for isolation of CyHV-2 (Rougée et al., 2007; Yan et al., 2011; Ito et al., 2013;

375   Ma et al., 2015; Jing et al., 2016). In India, Sahoo et al. (2016) isolated CyHV-2 using a koi carp

376   cell line, CCKF but could not propagate the virus beyond 4th passage. The continuous culture of

377   CyHV-2 has been challenging due to the lack of permissive cell lines in India. In this paper, we

378   have demonstrated the continuous propagation of CyHV-2 using FtGF cell line as well as

379   successful experimental infection of goldfish by intraperitoneal injection with the CyHV-2

380   propagated in FtGF cells.

381   The caudal fin has the feature of natural regeneration capacity (Akimenko et al., 2003)

382   accounting for a high *in vitro* cell proliferation potential (Santos-Ruiz, Santamaria & Becerra,

383   2005). The FtGF cell line exhibited stable growth over about 55 passages over the past one year.

384   Using the new cell line, we isolated CyHV-2 using filtered tissue homogenate from infected

385   goldfish. Morphological changes in FtGF cells infected with CyHV-2 included cytoplasmic

386   vacuolation, rounding and detachment of cells, as reported previously in other CyHV-2 infected

387   cells. Moreover, CyHV-2 could be continuously propagated over 20 times using FtGF cell line

388   and also consistently produced the similar CPE as the initial passage. Similar to our results, Ma

389   et al. (2015) could also propagate the virus for over 50 passages in GiCB cell line. However, in

390   contrast to our results, Jung & Miyazaki (1995) reported that using epithelioma papulosum

391   cyprini and FHM cell cultures; CyHV-2 could not be sub-cultured beyond the fourth passage.

392   Previously, several attempts have been made to propagate CyHV-2 in different fish cell lines (Li

393   & Fukuda, 2003; Waltzek et al., 2005; Jeffery et al., 2007; Ito et al., 2013; Xu et al., 2013; Jing

394   et al., 2017) but it has not been possible to propagate CyHV-2 beyond 4-6 passages and virus

395   titre from these cells was very low.

396   In the present study, efficient propagation of CyHV-2 with high viral titre was achieved when the

397   infected FtGF cells was incubated at 28 °C. Jeffery et al. (2007) isolated CyHV-2 from extracts

398   of gill tissue in KF-1 cells at 20 °C, whereas, Xu et al. (2013) observed CPE by CyHV-2 at 10

399   dpi on the KF cell line when incubated at 25°C. Similarly, CPE by CyHV-2 was observed at 6

400   dpi in GFF and SRTF cell cultures (Ito et al., 2013) and GiCB cells (Ma et al., 2015) when

401   incubated at 25 °C.The virus titer was estimated at $10^{7.8\pm0.26}$ TCID$_{50}$/mL, which is much higher

402   than any earlier reported titer for CyHV-2 in other studies. In this study, the removal of cell

403   culture medium from the flask before inoculating the tissue homogenate in FtGF cells,

404   incubation temperature and shaking during adsorption of the viral inoculum, might have

405   increased the viral titre of CyHV-2 in FtGF cell line. Ma et al. (2015) reported that the CyHV-2

406   titer reached $10^{7.5\pm0.37}$ TCID$_{50}$/mL and has been effectively cultured over 50 times in the GiCB

407   cell line. Ito et al. (2013) reported that incubation temperature of 25 °C is considered to be highly

408   tolerant for isolation of CyHV-2 in GFF cells. But, in our study, we got high CyHV-2 tire of

409     $10^{7.8\pm0.26}$ TCID$_{50}$/mL at 28 °C which is a high incubation temperature. In accordance with our

410   results, Piaskoski, Plumb & Roberts (1999) and McClenahan, Beck & Grizzle (2005) found that

411   incubating the cells inoculated with the largemouth bass virus (LMBV) samples at 30 °C resulted

412   in a higher number of LMBV plaques than incubation at 25 °C or 32 °C. Similarly, Chi et al.,

413   (1999) also found that the optimum temperature range for grouper nervous necrosis virus

414   (GNNV) infection in GF-1 cells was 24–32 °C and virus titer increased with an increase of the

415   temperature. The removal of tissue culture medium might have made the virus concentration

416   higher during adsorption and agitation resulting in a more uniform distribution of the viral

417   inoculum among all the FtGF cells in the flasks. The isolate of CyHV-2 in this study showed a

418   different temperature range for its culture compared to other published reports by various

419   workers discussed earlier. One of the possible explanations could be that the present Indian

420   CyHV-2 might belongs to a different strain or genotype or it could be due to an adaptation of the

421   host fish species to the native environmental temperature (Ciulli et al., 2006).

422   An experimental infection in goldfish was carried out with the virus passaged 10 times in FtGF

423   cells. In this experiment, fish injected IP with the cell culture grown virus, began to die at 5 dpi

424   and the cumulative mortality reached 100% at 12 dpi. This finding is in accordance with a

425   previous report (Ito et al., 2013), where cumulative mortality of 90 (at 16 dpi) and 100% (at 18

426   dpi) were observed in Edonishiki and Ryukin goldfish varieties, respectively. In addition, Ma et

427   al. (2015) reported cumulative mortality of 100% at 14 dpi in healthy gibel carp challenged with

428   CyHV-2 (passage 9) produced in the GiCB. The CyHV-2 virus re-isolated from experimentally

429   infected fish in FtGF cells was also pathogenic to goldfish and caused disease in goldfish. In the

430   present study, the electron microscopic observations demonstrated mature virus particles similar

431   to herpesvirus in affected gill and spleen tissues from the experimentally challenged goldfish and

432   these findings are consistent with previous reports (Jeffery et al., 2007; Wu et al., 2013; Ma et

433   al., 2015). CyHV-2 cultured in FtGF passages has been stored in liquid nitrogen. The recovered

434   viruses from liquid nitrogen maintained high infection activity to cells (data not shown). The

435   results indicated that the FtGF cell line is capable of producing high concentrations of CyHV-2

436   *in vitro* and a highly permissive to the propagation of CyHV-2.

437   **Conclusion**

438   In conclusion, the newly established FtGF cell line is highly susceptible to CyHV-2 culture *in*

439   *vitro*. The newly developed FtGF cell line would play a crucial role in future research on CyHV-

440   2, including studying the molecular pathogenesis of HVHN disease and development of

441   strategies for the prevention and control of the disease in the country. The FtGF cell line (NRFC

442   accession number: NRFC058; http://mail.nbfgr.res.in/nrfc/cellline-available.php) was deposited

443   in the National Repository of Fish Cell Line (NRFC) (the largest fish cell line repository), ICAR

444   National Bureau of Fish Genetic Resources, India for further dissemination to scientists for

445   carrying out research in developing CyHV-2 management strategies in this field.

446   **Ethical statement:** All the experimental challenge procedures in this study (Proposal number:

447   NBFGR/IAEC/2019/0014) were evaluated and approved by Institute Animal ethics Committee

448   (IAEC) of ICAR National Bureau of Fish Genetic Resources (NBFGR) (CPCESA Registration

449   No: 909/GO/Re/S/05/CPCSEA dated 09.09.2005 and CPCSEA Ref file No. 25/111/2014-

450   CPCESA dated 05th December 2018) vide approval Number G/IAEC/2019/1 dated 04th October

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List of goldfish cell lines

1   **Table.1. List of goldfish Cell line**

S. No	Designation of cell line	Tissue	Morphology	Growth Medium	Passage level	Chromosome	Revival rate	Viral susceptibility	Country	Reference
1.	-	Whole fish	-	At 25 °C TC medium 199 with 20% bovine serum	27	-	-	-	United states of America	Kroeker, 1964
2.	-	Muscle	-	At 25 °C TC 199 with 20% bovine serum	18	-	-	-	United states of America	Kroeker, 1964
3.	-	Heart	-	At 25 °C TC 199 with 20% bovine serum	18	-	-	-	United states of America	Kroeker, 1964
4.	SJU-1	Skin tissue	fibroblast-like	At 20°C Earle's basal	110	94	-	IPNV	United states of	Rio et al., 1973

									America	
5.	GFSk-S1	Skin	fibroblastic	At 25 °C in L-15 with 10% FBS	over a 1-year period	-	-	-	Canada	Lee et al 1997
6.	GAKS	Scales	epithelial type	DM 160 with 10% FBS	-	-	-	-	Japan	Akimoto et al., 2000
7.	GFF	Fin	-	-	-	-	-	CyHV-2	China	Li and Fukuda 2003
8.	GFM	Muscle	epithelial	At 30°C in L-15 medium with 20% FBS	35	104	92%	IHNV, SHRV, SVCV, CCV and IPNV	United States of America	Rougée et al., 2007
9.	GFSB	Swim bladder	fibroblastic	At 30°C in L-15 with 20% FBS	35	104	93%	-	United States of America	Rougée et al., 2007
10.	GFTF	Tail fin	fibroblast-like	At 25°C in L-15 with	50	104	90%	SHRV, SVCV	China	Yan et al., 2011

				20% FBS				and CCV		
11.	SRTF	Fin of standard Ryukin Takafumi	-	At 25 °C MEM with 10% FBS	-	-	-	CyHV-2	Japan	Ito et al 2013
12.	RKF	Fin of Ryukin	-	At 25 °C MEM with 10% FBS	-	-	-	CyHV-2	Japan	Ito et al 2013
13.	GFSe	Snout tissue	epithelial-like cells	At 25 °C in M199 with 15–20 % FBS	80	100	-	SVCV, GCRV, IPNV-SP, and CyHV-2	China	Jing et al 2016
14.	GFKf	Kidney	fibroblast-like cells	At 25 °C in M199 with 15–20 % FBS	80	100	-	SVCV, GCRV, IPNV-SP, and CyHV-2	China	Jing et al 2016
15.	GH	Heart	Fibroblast-like and	At 25 °C in M199	95	100	90–95%	EHNV, ADIV	China	Jing et al 2017

			epithelioid cells	with 10–20% FBS				and BIV		
16.	GFB	Brain	fibroblastic	At 30°C in199 with 20%f FBS	40	110	90%	CyHV-2	China	Xu et al., 2019

3 IHNV - Infectious hematopoietic necrosis virus; SHRV - snakehead rhabdoviruses; SVCV- spring viraemia carp virus; CCV - channel

4 catfish virus; IPNV - Infectious pancreatic necrosis virus; CyHV-2 - cyprinid herpesvirus 2; EHNV - epizootic hematopoietic necrosis

5 virus; ADIV - Andrias davidianus iridovirus; BIV - Bohle iridovirus; L-15 - Leibovitz-15 medium; FBS – Foetal bovine serum.

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.

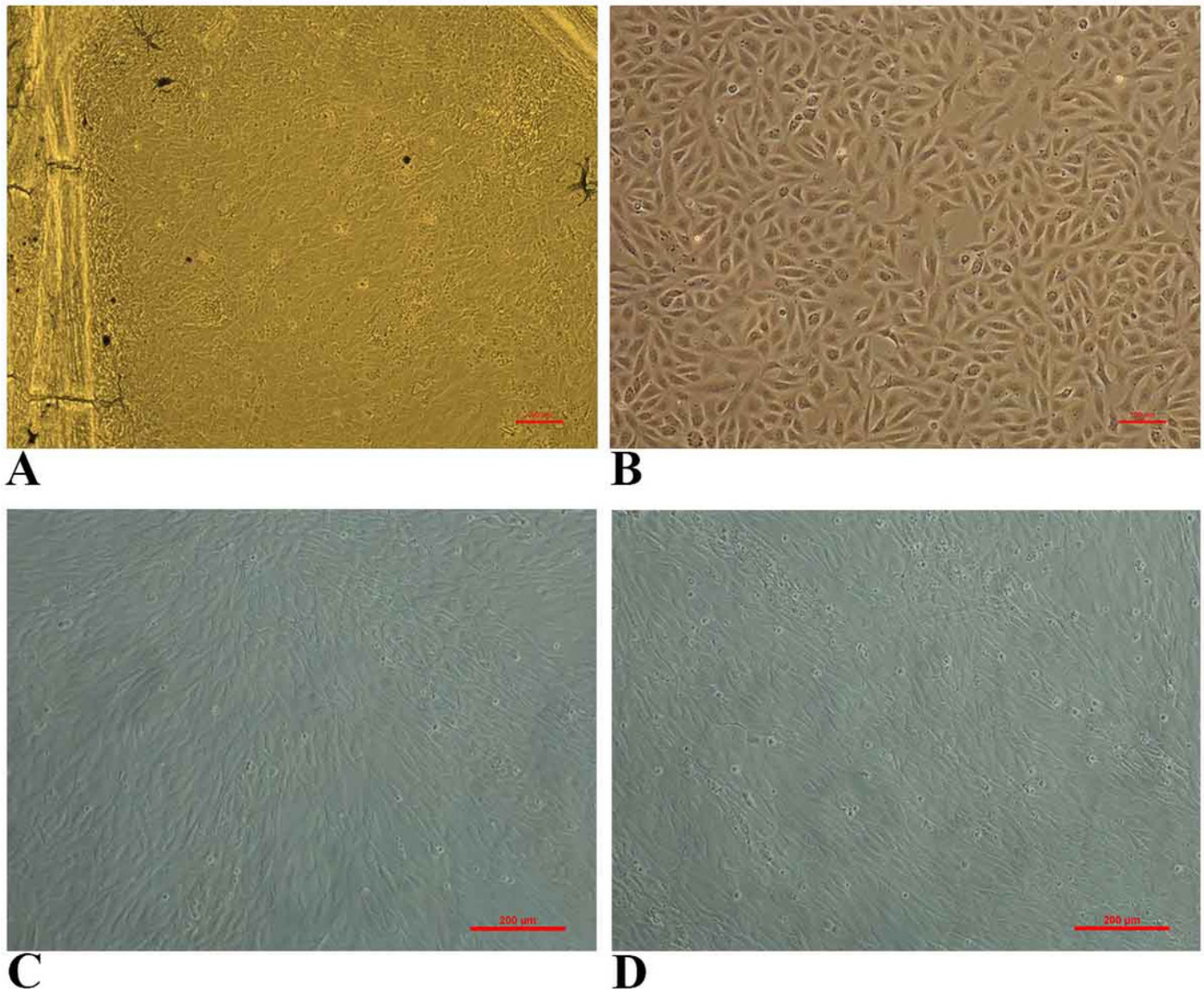



Figure 2

Mean ± SD (n=2) growth rate of newly established FtGF cell line.

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

curve of FtGF cell at 25th passage with four phases including latency, exponential growth, and

stationary phases. Values plotted are means ±SD of the measurements.

A

— 20ºC   — 24ºC   — 28ºC   — 35ºC   — 37ºC

B

— 5%   — 7.50%   — 10%   — 15%   — 20%

C

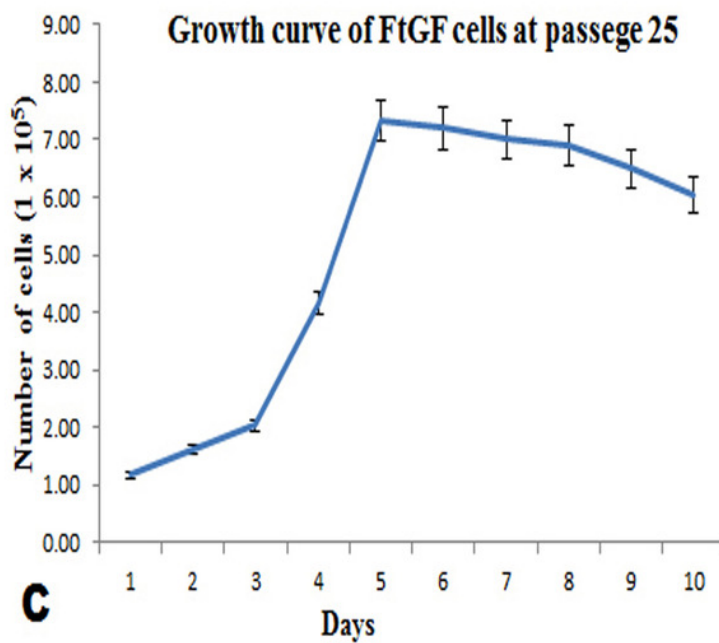
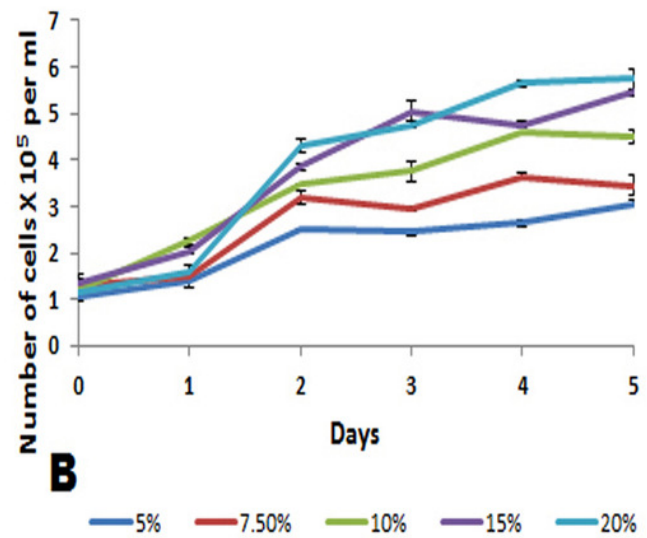
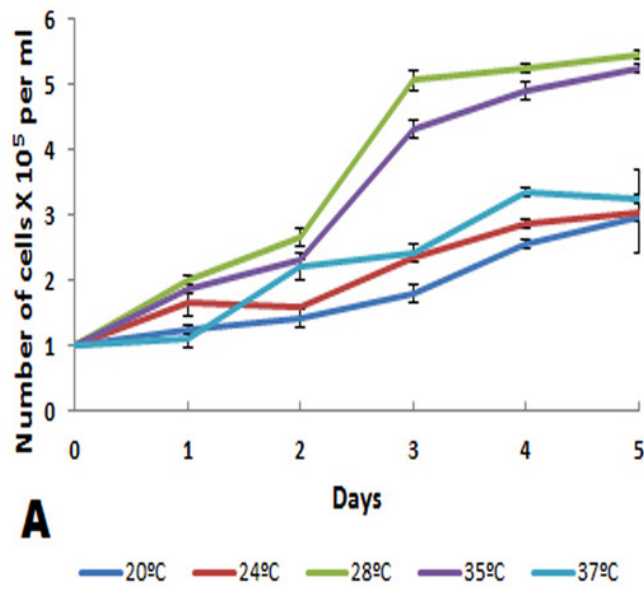



Figure 3

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

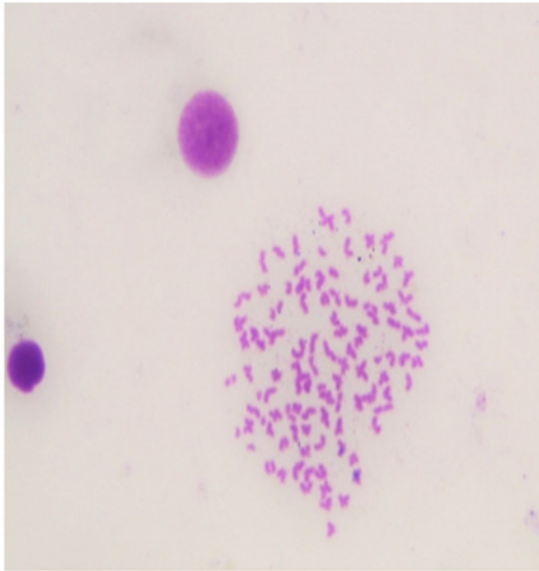
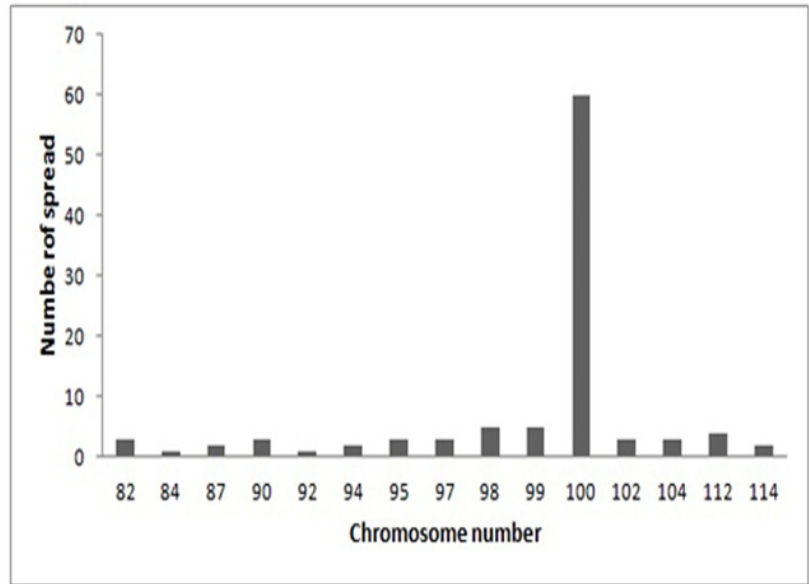
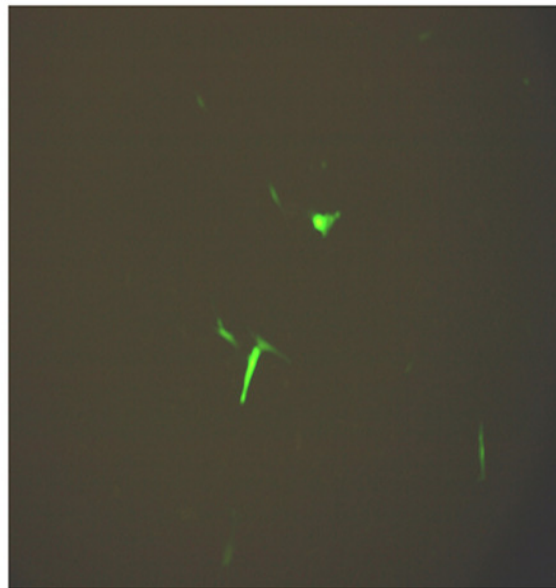
(A) Phase-contrast photomicrograph of chromosome spread arrested in metaphase (original
magnification: X400); (B) Frequency distribution of chromosomes in 100 cells; (C) Expression
of green fluorescent protein (GFP) in FtGF cells at 25h passage (200X).

A

B

C

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.

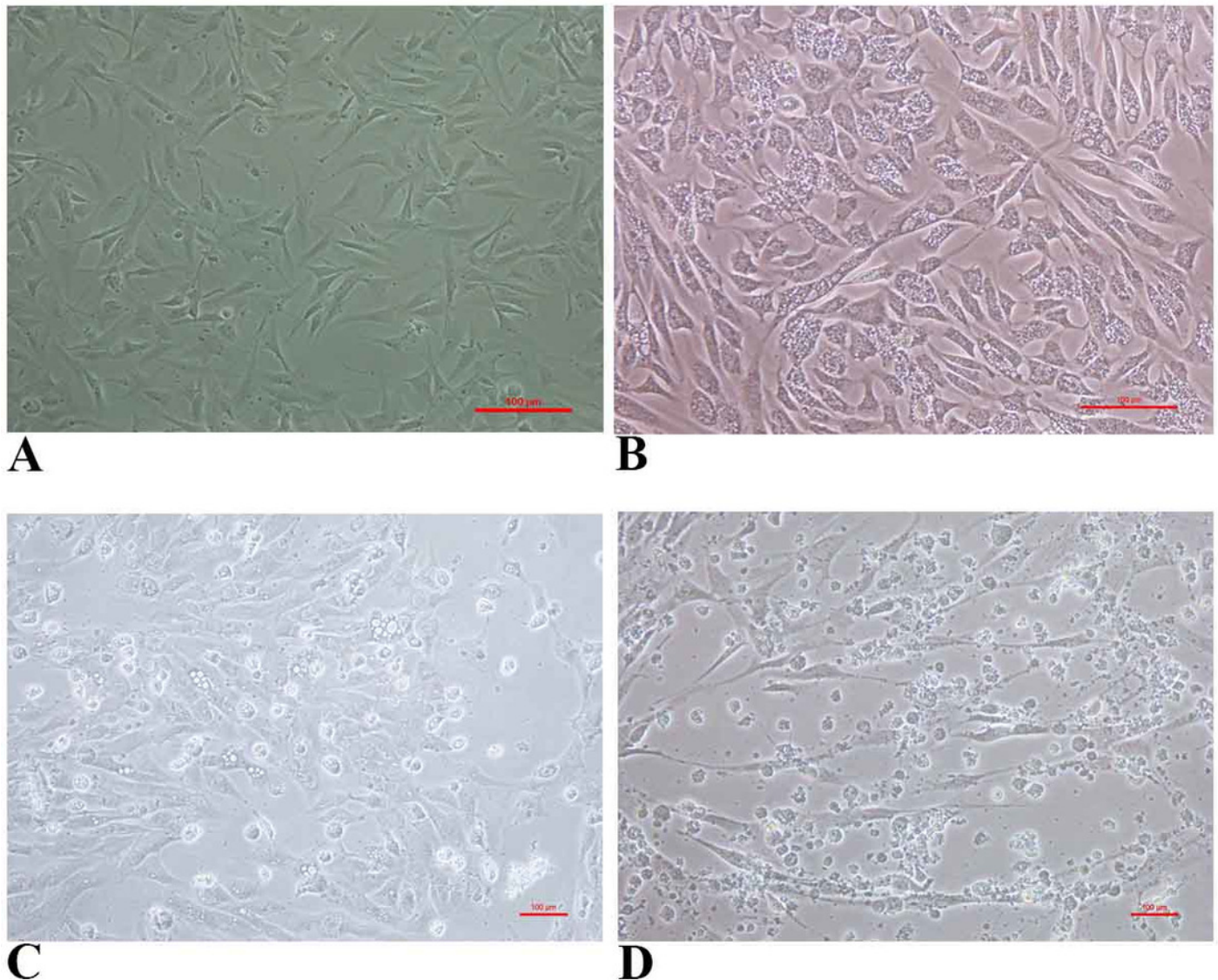



Figure 5

Mean ± SD (n=2) growth curve of CyHV-2 in FtGF cell line and mortality curve of experimentally infected goldfish and gross pathology of infected goldfish.

(A) Growth curve of CyHV-2 (at passage 20) in the FtGF cell line. (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. Values plotted are means ±SD of the measurements. (C) Experimentally challenged goldfish showing swollen body and (D) severe inflammation of gills.

Growth curve of CyHV-2 in FtGF cell line

y-axis: CyHV-2 titre Log TCID $_{50}$/ml

x-axis: Days post Inoculation

A

Mortality curve of experimentally infected

y-axis: Cumulative mortality (%)

x-axis: Days post inoculation

Legend: Infected, Control

B

C

D

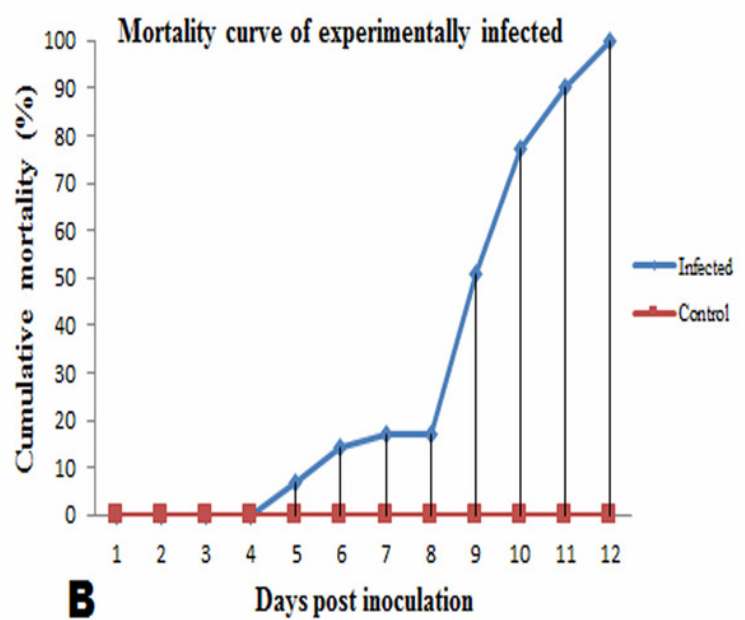
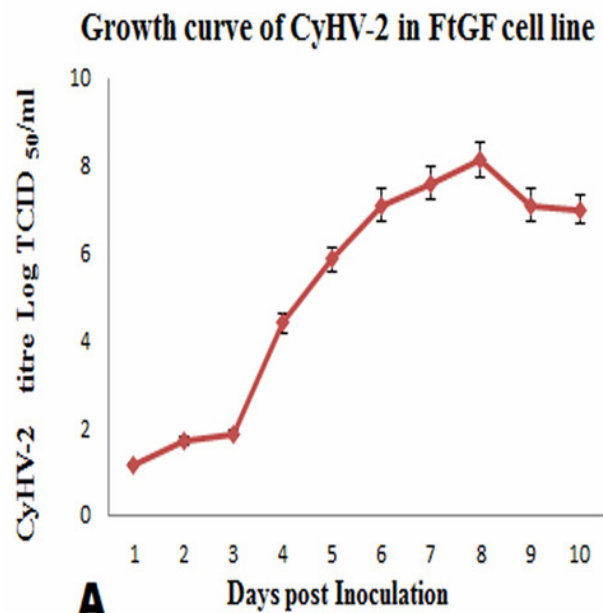



Figure 6

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

(A) gills; (B) spleen and (C) kidney showing margination of chromatin material (arrows).

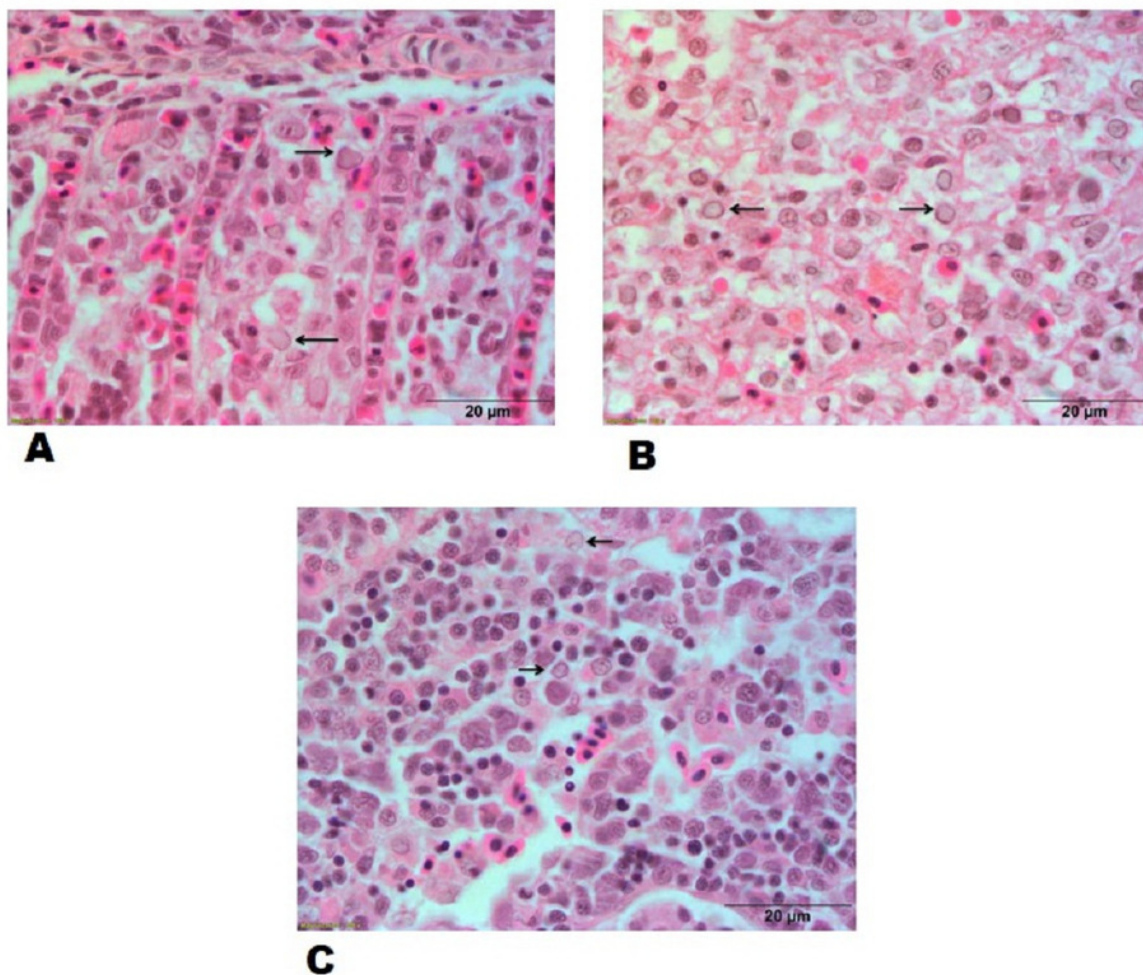



Figure 7

Transmission electron micrograph showing enveloped mature CyHV-2 virions in the ultrathin sections of tissues and infeced FtGF cells

(A) gill; (B) spleen cells of experimentally challenged of goldfish and mature virus particles;

(C) CyHV-2 infected FtGF cell; (D) purified from CyHV-2 infected FtGF cells.