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Establishment and characterization of fin-derived cell line from ornamental carp, *Cyprinus carpio* koi, for virus isolation in India

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Abstract *Cyprinus carpio* koi fin (CCKF) cell line was established and characterized from the caudal fin tissue of ornamental common carp, *C. carpio* koi. This cell line has been maintained in L-15 medium supplemented with 15% foetal bovine serum (FBS) and subcultured more than 52 times over a period of 24 mo. The CCKF cell line consisted of epithelial cells and was able to grow at temperatures between 22 and 35°C with an optimum temperature of 28°C. The growth rate of these cells increased as the proportion of FBS increased from 2 to 20% with optimum growth at the concentrations of 15% FBS. Karyotype analysis revealed that the modal chromosome number of CCKF cells was $2n=100$. Partial amplification and sequencing of fragments of two mitochondrial genes 16S rRNA and COI confirmed that CCKF cell line originated from ornamental common carp. The CCKF cells showed strong reaction to the cytokeratin marker, indicating that it was epithelial in nature. The extracellular products of *Vibrio cholerae* MTCC 3904 and *Aeromonas hydrophila* were toxic to the CCKF cells and not susceptible to viral nervous necrosis virus (VNNV). These CCKF cells were confirmed for the absence of *Mycoplasma* sp. by polymerase chain reaction. Furthermore, 90% of viable cells could be effectively revived 4 mo after cryopreservation from CCKF cell population suggesting the possibility of long-term storage of the cells.

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Introduction

Ornamental carp, *Cyprinus carpio* koi, is being reared as food fish and also as ornamental fish in many countries. The growth of ornamental industry has also been accompanied by an increased awareness of the adverse impact of diseases, particularly those caused by pathogenic viruses. A viral disease caused by cyprinid herpesvirus (CyHV-3) was first recorded from Israel and the USA in 1998 (Hedrick et al. 1999) in common carp, *C. carpio*, and ornamental common carp, *C. carpio* koi, and the virus was designated as koi herpesvirus (KHV) (Hedrick et al. 2000). CyHV-3 has become a globally distributed pathogen and affecting koi and common carps from at least 26 different countries of the world (Michel et al. 2010; Maya et al. 2011). Incidence of KHV disease has so far not been reported from India (Rathore et al. 2012; Swaminathan et al. 2013), in spite of KHV disease outbreaks happening in neighbouring Southeast Asian countries (Lio-Po et al. 2009). However, unregulated and illegal international trade of ornamental fish, particularly koi carp can lead to introduction of KHV into India. Common carp is one of the important fish species contributing to aquaculture production in India and KHV can severely affect this species causing huge economic losses.

The establishment of permissive cell lines from host animals is essential for the isolation, identification and study of pathogenic viruses. Species specific cell lines from *C. carpio* koi viz., KF-1 (Hedrick et al. 2000) and KCF-1 (Dong et al. 2011) were established for KHV isolation affecting koi carp and related fish species. Currently in India, there is no cell line available from *C. carpio* koi. In the present study, the

establishment and characterization of a new cell line derived from a koi carp, an important fish species with high demand in the international ornamental fish market is discussed.

Material and Methods

Development of primary cell culture from caudal fin of koi carp. Healthy *C. carpio* koi, koi carp having an average body weight of 10–15 g were procured from local ornamental fish farm in Kochi, Kerala, India. To begin primary cell culture, the caudal fin was clipped with sterile scissors and washed with antibiotic phosphate-buffered saline (aPBS) containing PBS (Life Technologies, Grand island, NY; catalogue number: 14190-250), 200 μg streptomycin per ml, 200 IU penicillin per ml (Sigma-Aldrich, St. Louis, MO; catalogue number: P0781), and 2.5 μg amphotericin B per ml (Life technologies; catalogue number: 15290-018), the caudal fin tissue was placed into chilled aPBS and incubated at room temperature (RT) for 2 h. Following two washes with aPBS (30 min per wash), fin tissues were minced into pieces and then washed twice with aPBS. Approximately 25 fin clips (1 mm^3) were seeded uniformly into 25 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark; catalogue number: 156367) with 100 μl of heat-inactivated defined foetal bovine serum (FBS) (Life technologies; catalogue number: 10082147) for facilitating the attachment of fin explants to the surface of the flask. After a 30-min attachment at RT, excess FBS was drained from the flasks to allow the fin clips to dry and incubated in a 28°C incubator with growth medium, Leibovitz's-15 (L-15) medium (Life technologies; catalogue number: 11415-064) supplemented with 15% heat-inactivated defined FBS and antibiotics (200 IU penicillin per ml, 200 μg streptomycin per ml and 1 μg amphotericin B per ml).

Exchange of the 50% of the existing growth medium was carried out every 3 d with new growth medium. After the confluence growth of the primary cells, the medium was removed and the cells were trypsinized with 0.25% trypsin-EDTA solution (Life technologies; catalogue number: 15400-054) and subcultured at a split ratio of 1:2–1:3. After 15 passages, the amount of FBS in the growth medium was decreased from 15 to 10%, and the antibiotics were decreased to normal concentrations (100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B).

Effects of temperature and FBS concentration. The effects of temperature and FBS concentration on cell growth were carried out with the cell line at its 25th and 40th passages with modification as described by Huang et al. (2011). Six-well tissue culture plates (Nunc; catalogue number: 150339) were used for these tests, and each well was seeded with 7×10^4 cells and incubated at 28°C for 24 h to allow for cell attachment. Batches of plates were incubated at selected temperatures of 20, 25, 28, 35 and 37°C over 12 d for the growth test. Similar procedures were performed for determining the effects of various concentrations of FBS (5, 7.5, 10, 15 and 20%) at 28°C. Daily, cells from the duplicate wells were harvested and counted by six independent haemocytometer counts in both the growth studies.

Cryopreservation and revival. Cells at various passage levels (10, 20, 25 and 35) were used for cryopreservation as per Wang et al. (2003). Cells at log phase of growth were harvested. Two flasks of cultured cells with 80% confluence were trypsinized and centrifuged at 200*g* at 25°C for 10 min. Cell pellets were resuspended in to a density of 4×10^6 cells per ml in complete cryopreservation medium consisting of L-15 with 20% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich; catalogue number: D2650) (9:1). The cell suspensions were then transferred to 2-ml cryo-vials and kept sequentially at −20°C for 4 h, −80°C for 16 h and finally stored in liquid nitrogen −196°C. For viability analysis, frozen cells were thawed in a water bath at 25°C, transferred into 15-ml centrifuge tubes and centrifuged. After centrifugation, the pellet was resuspended in 10 ml L-15 medium and viability was evaluated using a haemocytometer following trypan blue staining. The thawed cells were also seeded into two 25-cm^2 flasks for culture.

Chromosome analysis. Chromosome analysis was conducted for cells at passages 15 and 35 using a method similar to our previous description with minor modifications (Dong et al. 2008). Briefly, the colchicine solution (Sigma-Aldrich; catalogue number: C3915) (0.1 ml of 1 μg/ml) was added into the 80–90% confluence koi carp fin cells. After 3-h incubation at 25°C, the cells were harvested, treated with 5 ml of 0.075 M KCl for 25 min and then prefixed for 5 min in 10 ml fixative (3 parts of methanol and 1 part of acetic acid). After centrifugation at 200*g* at 4°C for 10 min, the cells were resuspended in 2 ml fresh fixative solution. The cell suspension of the fixed cells was dropped onto clean precooled glass slides. After drying, the cells were stained with 10% Giemsa solution for at room temperature. Finally, the slides were observed using a phase contrast microscope and a total of 100 chromosome spreads were counted.

Immunofluorescence microscopy assay. Cells at passages 25 and 40 were grown on cover slips in six-well plates for 24 h, fixed with 4% formaldehyde for 1 h at 4°C, washed with PBS, permeabilized with 0.1% Triton X-100 and blocked in PBS that contained 2% bovine serum albumin (BSA) (HiMedia, Mumbai, India; catalogue number: TC194). After washing with PBS, the cells were incubated overnight at 4°C with primary antibodies, mouse anticytokeratin (pan), clone AE1/AE3 antibodies or mouse anti-vimentin antibodies (Life Technologies;

catalogue number: MA5-13156 and 18-0052). In control cover slips, only PBS with 1% BSA was used in place of primary antibodies. After PBS washing, cells were incubated for 1 h with rabbit anti-mouse IgG FITC conjugate (Life technologies; catalogue number: 81-6711) (diluted 1:50 in PBS containing 1% BSA). The cover slips were washed again in PBS, mounted in VECTASHIELD-mounting medium (Vector Laboratories, Burlingame, CA; catalogue number: H-1200) and images were documented by the fluorescent microscope (Nikon).

Cytotoxicity test of bacterial extracellular products. The cytotoxicity of bacterial extracellular products (ECPs) from *Vibrio cholerae* MTCC 3904 and *Aeromonas hydrophila* was tested with cells at passage levels 28 and 42. The ECPs were prepared according to the protocol described by Balebona et al. (1995). The cells were grown as a monolayer in 24-well plates at 28°C. For the toxicity test, the cell line was inoculated with 0.1 ml serial dilutions of ECPs sample. For negative controls, sterile saline was used in place of ECPs. Plates were incubated at 30°C and the effects of ECP on the cells were observed after 24, 48 and 72 h.

Viral susceptibility and cytopathic effect (CPE). Cytopathic effects were carried out on cells at passage level 30. The cell line was tested for the susceptibility to a virus, viral nervous necrosis virus (VNNV) (the only virus reported from India) isolated from Asian sea bass, *Lates calcarifer*. The virus was isolated from infected tissue (sea bass juveniles tested positive by PCR) by homogenizing the tissues in L-15 medium with antibiotics and without FBS. The homogenate was frozen and thawed three times before centrifuging at 13,000*g* for 1 h at 4°C and the supernatant was filtered through a 0.22-μm membrane and inoculated into monolayer of 80% confluency and allowed to adsorb. Then the supernatant was discarded and the cells were washed with phosphate buffer three times. Following this, L-15 medium with 5% FBS was added to the cells and incubated at 28°C and the cells were examined daily for the occurrence of CPE for 10 d.

*Detection of **Mycoplasma** contamination in CCKF cell line.* In order to detect *Mycoplasma* contamination, cells were grown without antibiotics in the medium for 5 d. Harvested cells (at passages 35 and 55) were transferred into micro-centrifuge tubes and centrifuged at 200*g* for 10 min and supernatants were transferred into micro-centrifuge tubes and centrifuged further at 250*g* to remove debris. Finally, the supernatant was centrifuged at 15,000*g* for 10 min and the pellet was resuspended using 50 μl of buffer solution followed by heating at 95°C for 3 min. The solution was stored at −20°C until use. *Mycoplasma* detection was done by the amplification of a fragment of 715 bp of the 16S rDNA of *Mycoplasma* DNA using the primers MSGO (5′ TGC ACC ATC TGC CAC TCT GTT AAC CTC 3′) and GPO1 (5′ ACT CCT ACG GGA GGC AGC AGT 3′) (van Kuppeveld et al. (1992). Briefly, PCR amplification was performed with 10 μl of nucleic acid in a 100-μl reaction mixture containing 50 mM KCI, 10 mM Tris-HCI (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 50 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 0.2 U of Super Taq polymerase. The thermal profile consisted of 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 2 min. The *Mycoplasma* contamination was also tested using EZdetect^{TM} PCR Kit (HiMedia; catalogue number: CCK009) based on amplification of spacer region between 16S and 23S ribosomal RNA (rRNA) genomic DNA sequence. The amplification products were analysed in 1% agarose gel.

Identification of cell origin. The origin of the cell line was validated by amplification and sequencing of fragments of mitochondrial genes, 16S rRNA and COI from cells following Swaminathan et al. (2010). Briefly, DNA was extracted from CCKF cells (at passage levels 24 and 40) and muscle tissue from ornamental common carp, and fragments of the two genes were amplified using the universal primers. The amplified PCR products were sequenced and the DNA sequences were aligned against known sequences from the National Centre for Biotechnology Information (NCBI) database. DNA from muscle of *C. carpio* koi was used as positive control for PCR amplification and sequencing of the above two mitochondrial genes.

Results

Primary cell culture and subculture. Cell culture was initiated from caudal fin tissues of an ornamental common carp by explant method. The cells migrated from the different tissue fragments and grew well, forming a monolayer during the first month. *C. carpio* koi fin (CCKF) cells were continuously subcultured over 52 times. In primary culture, CCKF cells adhered well and achieved confluence within 3 d at 28°C. Cells were subcultured in L-15 medium with 20% FBS at a ratio of 1:2 every 5 d for the initial ten passages. After ten subcultures, cells were subcultured at a ratio of 1:2 at 5- to 6-day intervals, with FBS being reduced to 15% in the L-15 culture medium. CCKF cells consisted of both fibroblast-like and epithelial-like cells in the early cell passages; however, they became predominantly epithelial cells after 25th passage (Fig. 1*A–D*).

Optimal growth of the newly established cells was dependent on temperature and serum concentration. The cells were able to grow at temperatures between 20 and 37°C. But no significant growth was observed at 20 and 35°C. However,

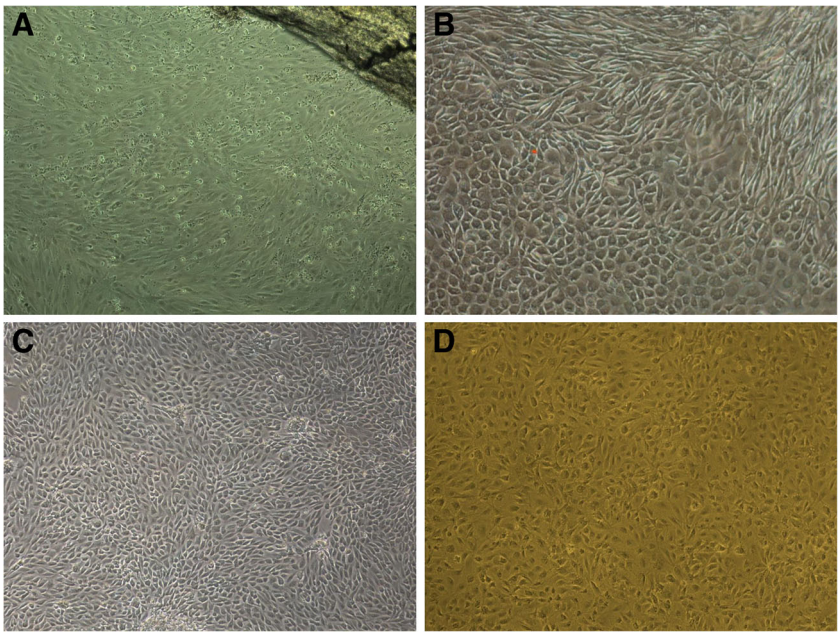

Fig. 1. *(A)* Explant of caudal fin of *Cyprinus carpio* koi showing radiation of cells (100×). *(B)* CCKF cell line at 10th passage showing heterogeneous population of fibroblastic-like and epithelial-like cells (100×). *(C)* CCKF cell at 25th passage (100×). *(D)* CCKF cell at 45th passage (100×).

maximum growth occurred at 28°C. The growth rate of CCKF cells increased as the FBS proportion increased from 0 to 20% at 28°C. Cells exhibited poor growth when 5% FBS was added to L-15 medium, with relatively slow growth at 7.5% FBS and growth rate increased parallel with the increased FBS concentration (Fig. 2*A*, *B*). The CCKF cells grown at 28°C and 15% FBS (Fig. 3*A*, *C*) showed healthy with sharp edges. Even though cells stayed alive at 37°C, cell growth was minimal and individual cells looked abnormal (Fig. 3*B*) and likewise, morphology of the cells when grown in 5% FBS-supplemented medium was without sharp edge and they did not appear in good health (Fig. 3*D*).

Cryopreservation. The CCKF cell line was cryopreserved at different passages. Cell population remained over 90% viable when stored at LN_2 for a period of 4 mo. All viable cells attached and grew well following seeding at 28°C. No apparent morphological changes of these cells were observed following storage.

Chromosomal analysis. Chromosomal counts from 100 random metaphase spreads were made for cell line (Fig. 4*A*, *B*). Chromosome numbers ranged from 88 to 110 in CCKF, with a distinct peak for CCKF cell line at 100 diploid chromosomes. The majority of the cells (66%) had a diploid chromosome number ($2n=100$).

Immunofluorescence microscopy assay. To identify the phenotype of CCKF cells, mouse anticytokeratin (pan), clone AE1/AE3 antibodies (Invitrogen) or mouse anti-vimentin antibodies (Invitrogen) were used in an immunoflousresence microscopy assay as in (Chaudhary et al. 2013). All the CCKF cells were strongly positive for cytokeratin, an epithelial cell marker (Fig. 4*C*). No reactivity was observed in control and in cells incubated with anti-vimentin antibodies. These results confirmed that the CCKF cells are epithelial cells.

Cytotoxicity tests. The ECPs from *V. cholerae* MTCC 3904 and *A. hydrophila* were cytotoxic for the CCKF cell line. Cytotoxic effects were observed after 24 h of inoculation of ECPs. CCKF cells became rounded, having serrated edges (Fig. 4*D*) and detached from the surface leading to the destruction of the monolayer by 72 h. The susceptibility of the CCKF cell line to VNNV revealed no CPE on these cells up to 10 d, and even after ten blind passages and the supernatant of the cell line and CCKF cells were negative by RT-PCR. This indicates that CCKF cells were not susceptible to VNNV.

Detection of ***Mycoplasma*** *contamination.* The target band (715 bp) was not found on agarose gel when PCR product from cell line were analysed for *Mycoplasma* contamination. This confirmed that the CCKF cell line was free of *Mycoplasma* contamination. Likewise, no target band (350–400 bp) of spacer region between 16S and 23S rRNA *Mycoplasma* genomic DNA sequence could be detected in CCKF cells by the EZdetect™ PCR Kit (Fig. 5*A*).

Identification of cell origin. Sequence analysis of mitochondrial 16S rRNA and COI was performed in order to verify the origin of the cell lines. Amplification from the 16S rRNA and COI genes for both cell lines and muscle tissue of koi carp revealed the expected PCR products of 562 and 642 bp, respectively (Fig. 5*B*). Subsequent comparative analysis of the identified sequences demonstrated a 98% match for 16S and a

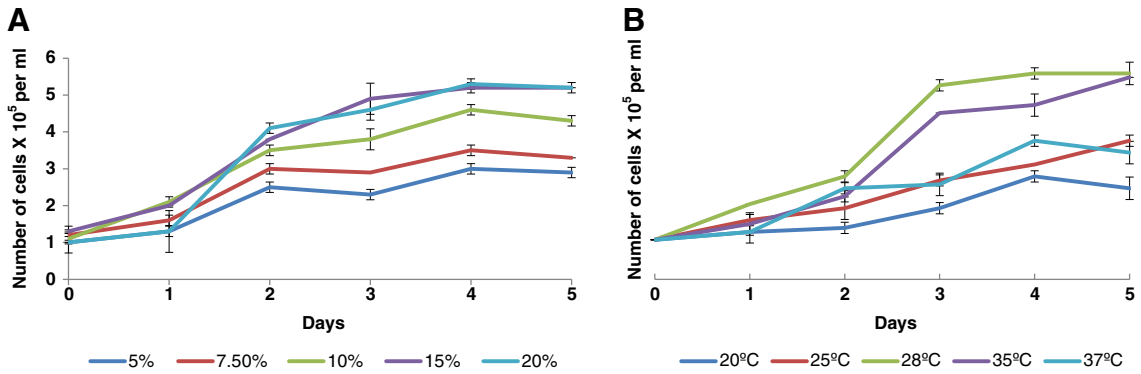

Fig. 2. *(A)* Growth response of the CCKF cell line to selected foetal bovine serum (FBS) concentrations (data are means±SD of three measurements). *(B)* Selected temperature (data are means±SD of three measurements).

97% match for COI to known koi carp mitochondrial DNA sequences. DNA sequencing and comparative analysis demonstrated that mitochondrial genes sequences amplified from fresh koi carp muscle tissue and from CCKF cell line were identical. The phylogenetic tree of 16S rRNA and COI sequences of CCKF cell line showed that it had high sequence homology with known 16S rRNA and COI sequences of *C. carpio* koi (Fig. 5*C*, *D*).

Discussion

The aim of the present study was to establish a cell line, CCKF from fin tissue of koi carp which has very high demand in the international ornamental fish market. The CCKF cells were evaluated for optimal growth conditions, their stability in liquid nitrogen, karyotyping and susceptibility to bacterial extracellular products (ECPs). Several cell lines have been developed for the isolation of cyprinid herpesvirus 3 elsewhere, viz., five koi caudal fin-derived cell lines (Hedrick et al. 2000; Pikarsky et al. 2004; Miwa et al. 2007; Sunarto et al. 2011), a common carp brain-derived cell line (CCB) (Neukirch et al. 1999), a common carp fin-derived cell line (Neukirch et al. 1999), etc. In this study, a CCKF cell line from caudal fin of ornamental koi carp was developed and characterized. The cells showed good growth in L-15 culture medium containing 15% FBS at 28°C. L-15 has been used as a common cell culture medium for growth of cells derived from various fish (Qin et al. 2006; Lakra et al. 2010; Swaminathan et al. 2013). The growth of CCKF cells was influenced by the concentration of FBS, and we chose 15% FBS as optimum for CCKF culture. The changes of growth rate of CCKF at five various temperatures viz., 20, 25, 28, 35 and 37°C were investigated. The CCKF cells were quite susceptible to

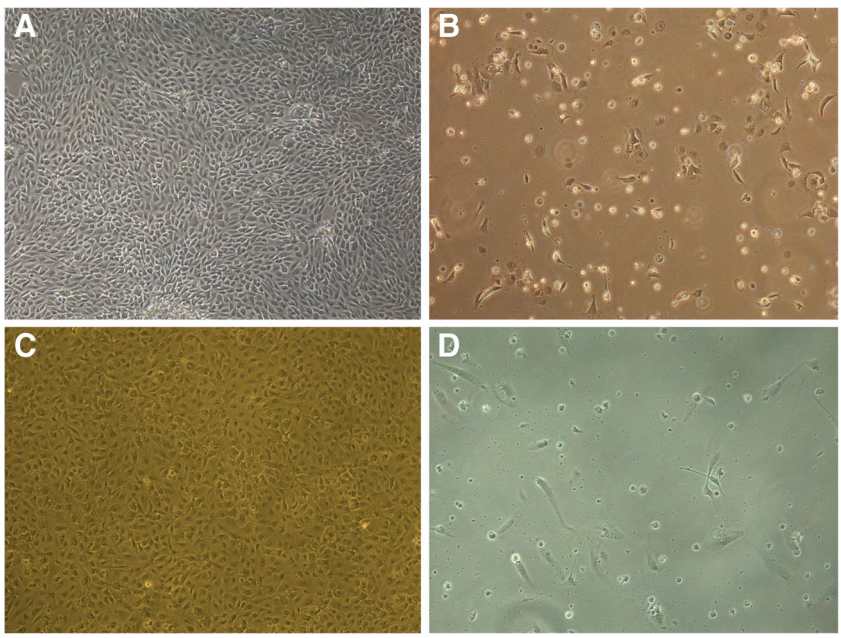

Fig. 3. *(A)* CCKF cells (5th day culture) grown in L-15 medium at 28°C (100×). *(B)* CCKF cells (5th day culture) grown in L-15 medium with 15% FBS at 37°C (100×). *(C)* CCKF cells (5th day culture) grown in L-15 medium with 15% FBS (100×). *(D)* CCKF cells (5th day culture) grown in L-15 medium with 5% FBS at 28°C (100×).

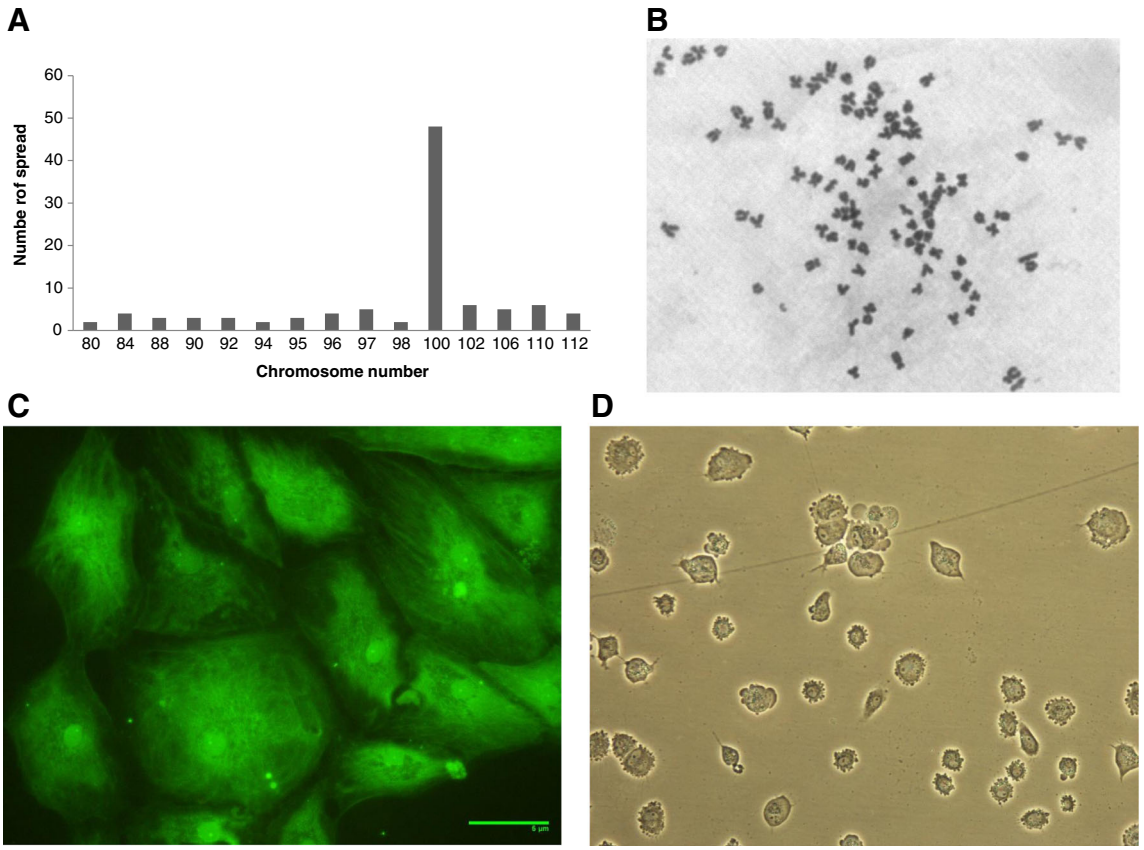

Fig. 4. *(A)* Frequency distribution of chromosomes of CCKF cells in 100 spreads. *(B)* Cellular chromosomes of CCKF cells arrested in metaphase (passage 35). *(C)* CCKF showing presence of cytokeratin marker. *(D)* CCKF cells at 1 d post inoculation to ECP of *Vibrio cholerae* (200×).

the culture temperature, and a dramatic change in the growth rate was observed at 20 and 35°C. Cells died within a few hours when the culture temperature was shifted to 45°C (data not shown). A number of cell lines developed from freshwater fishes are known to grow best at 28–30°C (Tong et al. 1997; Luc Rougee et al. 2007; Ahmed et al. 2009; Ku et al. 2009; Swaminathan et al. 2013). Ossum et al. (2004) also reported that cells from warm water fishes can grow at 15–37°C incubation temperature. Cells from poikilothermic teleosts are not able to grow at their sublethal temperatures (Hightower and Renfro 1988; Bols et al. 1992). For example, the rainbow trout, *Oncorhynchus mykiss*, cell line RTG-2 cannot grow at 26–28°C and cannot survive at 30°C (Mosser et al. 1986). It has been also reported that goldfish, *Carassius auratus*, cells easily change their growth rate in association with temperature shifts (Sato et al. 1990). The population doubling times of goldfish cells at 30 and 35°C were apparently shorter than those at 20 and 25°C (Kondo and Watabe 2004). In our study, we found that the CCKF cells grow temperature between 20 and 35°C and maximum growth was recorded at 28°C. Experimental results showed that FBS is required for the cells to survive and grow and that the higher the concentration of FBS, the faster the growth rate of cells. After 6 d of culture, cells cultured in medium containing 5% FBS grew significantly slower than those cultured in medium containing higher FBS concentrations.

The modal chromosome number (2*n*) in CCKF was found to be 100 at passages 15 and 35, which is identical to the modal chromosome number, reported earlier (Ojima and Takai 1981). In mammals, the immunochemical markers of cytoskeleton has being used to distinguish the two cell types, and these antibodies of fibroblastic or epithelial markers have also being used in fish cell lines to confirm their lineage. In order to confirm the cell type of CCKF cells, immunophenotypic markers such as anti-cytokeratin (pan) AE1/AE3 antibodies or mouse anti-vimentin antibodies were used. The cells were strongly positive to cytokeratin, which are used as specific markers for epithelial; however, the cells were negative to vimentin, a fibroblasts marker. These results indicated that the CCKF cells are epithelial. Similar findings were described by earlier workers viz., a fibroblast cell line positive to fibronectin and desmin in milkfish, *Chanos chanos* (Parmeshwaran et al. 2007); pancytokeratin confirmed their epithelial origin of EAGL cells (Ou-yang et al. 2010); EAGK cells were strongly positive to vimentin, desmin and fibronectin, which are used as specific markers for fibroblasts (Gong

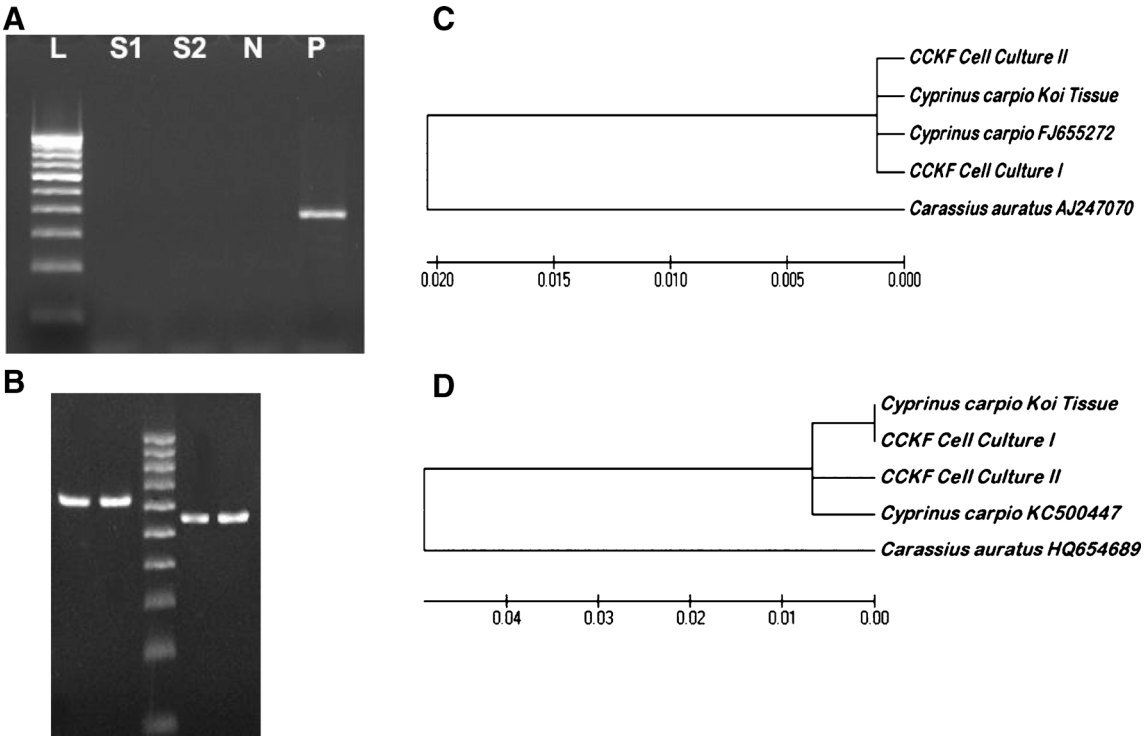

Fig. 5. *(A)* Detection of *Mycoplasma in* CCKF cell line based on amplification of 350–400 bp fragment of spacer region between 16S and 23S rRNA genomic DNA sequence by PCR using EZdetect™ PCR Kit (lane S1 and S2—CCKF cells at passage 55; *lane N*, negative control; *lane P*, positive control provided in the kit; *lane L*,100 bp DNA ladder. *(B)* PCR amplification of 562 and 642 bp sequences of the CCKF cell line and *Cyprinus carpio* koi genome using universal oligonucleotide primers of the 16S rRNA and CO1 genes, respectively. 200–300 ng DNA isolated from CCKF cell line and *C. carpio* koi was amplified and then subjected to 2.0% gel electrophoresis. mtDNA profile with COI primer (*lane 1*, CCKF cells; *lane 2*, *C. carpio* koi tissue); with 16S rRNA primer (*lane 4*, CCKF cells; *lane 5*, *C. carpio* koi tissue); *lane 3*, marker (100 bp DNA ladder). *(C)* Neighbour-joining (NJ) tree of *C. carpio* koi CCKF cell line; wild samples from ornamental fish farms and NCBI GenBank accession FJ655272 based on partial sequence information (562 bp) of 16S rRNA. *Carassius auratus* (AJ247070) was included as out-group species for easy comparison. *(D)* Neighbour-joining (NJ) tree of *C. carpio* koi CCKF cell line; wild samples from ornamental fish farms and NCBI GenBank accession KC500447 based on partial sequence information (642 bp) of COI. *C. auratus* (HQ654689) was included as out-group species for easy comparison.

et al. 2011); CTE cells were strongly positive for cytokeratin, an epithelial cell marker (Chaudhary et al. 2013).

The nucleotide sequence analysis of mitochondrial gene fragments of a cell line is very useful to verify the origin of the cell line and check the cross-contamination with other cell lines if any in the laboratory. To confirm that the cell line originated from *C. carpio* koi, partial amplification and sequencing of 562 and 642 bp fragments of 16S rRNA and COI for cell line were done. The sequence analysis of both 16S rRNA and COI fragments showed 99 and 100% similarity with respective gene fragment amplified from the tissue of *C. carpio* koi. The results confirmed that the CCKF cell line was of *C. carpio* koi. Similarly, amplification and sequencing of the COI region was used for identification of 67 cell lines Cooper et al. (2007). 16S ribosomal RNA gene sequence has also been used to confirm the origin of fish cell lines of (Luc Rougee et al. 2007; Lakra et al. 2010; Yan et al. 2011; Swaminathan et al. 2013). This suggests the utility of 16S rRNA and COI sequence as a valid and universal marker for species identification of established fish cell lines. The 18S rRNA sequence data (Wang et al. 2003) and 12S rRNA sequence data (Ahmed et al. 2009) have also been used to identify the origin of newly established cell lines in previous studies.

The ECPs from *V. cholerae* were cytotoxic to the CCKF cell line and morphological changes similar to those described by Bejar et al. (1997) in the SAF cell line and by Sahul Hameed et al. (2006) in the SISK cell line due to the toxicity of *Vibrio* ECP. Previously, many fish cells have proven suitable for demonstrating the cytotoxic effects of pathogenic bacteria including *V. cholerae* (Swaminathan et al. 2010; Chaudhary et al. 2013) and other bacteria (Ahmed et al. 2009; Ku et al. 2009). The susceptibility of cell lines to viral infection is the basis for isolating and characterizing fish viruses. The tissue homogenate of Asian sea bass infected with VNNV was inoculated into CCKF cell line and found that the CCKF cell line was not susceptible to this virus. It has been reported that in vitro viral replication generally requires permissive cell lines derived from the same host species (Lu et al. 1999).

Fish cell lines can vary greatly in their sensitivities to different aquatic pathogenic viruses. If a suspected virus-infected sample is inoculated into cell line of different species, the virus may not be able to grow and a negative result can be obtained. In early viral infection (when less viral load is expected), a positive result may be obtained only when the cell line of same species is used. Therefore, successful establishment of cell lines from various fish will improve diagnosis of viral disease and prevention. This cell line had been deposited in the National Repository of Fish Cell Line (NRFC), National Bureau of Fish Genetic Resources, Lucknow, for dissemination to other researchers for scientific research. The CCKF cell line can be used as a diagnostic tool for isolation, propagation and detection of viruses specific to cyprinids.

There are many examples from the past and the disease outbreaks caused by various trans-boundary aquatic animal disease epizootics (e.g. epizootic ulcerative syndrome of fresh and brackishwater fishes, viral nervous necrosis of marine fish, viral diseases of shrimps, haplosporidiosis in oysters, akoya pearl oyster mortalities, etc.) elsewhere in the world. Likewise, transmission of the KHV pathogen via live fish is a major problem worldwide (Hedrick 1996). In India, in a 3-yr disease surveillance programme, KHV has not been detected from koi carp or from Indian major carps (Rathore et al. 2012). These lessons can assist us towards preparing better and improving responses in the event of KHV disease outbreaks in India. This newly established CCKF cell line, together with other cell lines, previously established, will greatly enhance current attempts to develop effective diagnostic methods for detecting and monitoring viral infections in this important aquatic species.

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