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Development and characterization of a new fish cell line from Honeycomb grouper, *Epinephelus merra*

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ABSTRACT

A new cell line HGF was developed from fin tissue of *Epinephelus merra* Bloch, 1793. The cell line was maintained in Leibovitz's L-15 supplemented with 15% FBS and cells have been subcultured 45 times. The HGF cell line consists predominantly of fibroblastic-like cells. The cells were able to grow at temperatures between 25° and 32°C with optimum temperature of 28°C. The growth rate of fin cells increased as the FBS proportion increased from 2 to 20% at 28°C with optimum growth at the concentrations of 15 or 20% FBS. After confluence, the cells were sub-cultured with a split ratio of 1:2. The cells showed fibroblastic-like morphology and reached confluence on the fourth day after subculture. Polymerase chain reaction amplification of mitochondrial 16S rRNA and COI indicated identity of these cell lines with those reported from this animal species, confirming that the cell lines were of honeycomb grouper origin. The cells were successfully cryopreserved and revived at passage numbers 10, 20 and 30. The bacterial extracellular products from *Vibrio cholerae* MTCC 3904 was found toxic to this cell line.

Key words: *Epinephelus merra*, Fish cell line, HGF, Honeycomb grouper

The value of regional total production of groupers – one of the most important commodities in aquaculture and fish export in the Asia-Pacific region – in 2001 was around US$160 million (Rimmer *et al*. 2004). Forty species of groupers are reported from Indian waters and *Epinephelus tauvina* and *E. malabaricus* are potential species for aquaculture (Nammalwar *et al*. 1997). Countries are trying to increase their production by exploitation from the wild and through aquaculture (Sadovy *et al.* 2003). Experimental culture of groupers was initiated in India during 1992 (Hamsa and Kasim 1992) and broodstock development, sex inversion and captive spawning of *E. tauvina* and *E. malabaricus* were standardised in 2002 (Pillai *et al.* 2002). Captive breeding and larval rearing techniques were developed for honeycomb grouper *E. merra* also (Jagadis *et al.* 2006).Infectious disease and of these infections of iridovirus (Qin *et al.* 2003) and nodavirus (Chi *et al.* 1999) were identified as the most important constraints for grouper culture. Establishment of fish cell lines is essential for identification of infectious viruses from fish. About 283 cell lines have been established from finfish around the world (Lakra *et al.* 2010). Cell line from *Epinephelus coioides* (Qin *et al.* 2006), *E. awoara* (Lai *et al.* 2003), *E. akaara* (Zhou *et al.* 2007), *E.s quoyanus* (Ku *et al.* 2009), and *E. akaara* (Huang *et al.* 2009) reflect the economic and aquacultural importance of groupers; but not a single cell line is available from *Epinephelus merra* Bloch, 1793. It is important to establish a continuous cell line for the management of viral diseases of fishes. In this background, we attempted to establish cell lines from fin of honeycomb grouper for isolating and identifying viruses causing diseases in this species.

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MATERIALS AND METHODS

Preparation of tissues for primary cell cultures: Fish samples were collected from the Keelakarai and Mandapam, Tamil Nadu and transported in live condition to the laboratory. They were acclimatized in the laboratory for 2 weeks. The sterilization of surface of the fish was carried out by immersing the fish in 80% (v/v) ethanol. Fin tissues were collected from the fish using sterile instruments and aseptic techniques and each tissue was processed individually. Containers were surface-disinfected with betadine (10% povidone iodine) and all processes carried out in a laminar flow cabinet. Tissues were washed four times in phosphate buffer saline (PBS) containing antibiotics (500 IU/ml penicillin, 500 µg/ml streptomycin and 2.5 µg/ml fungizone) before preparing explants.

Tissue explants: The fin tissues were minced in 10 ml

PBS using 2 scalpel blades until a suspension of small tissue fragments was obtained. Each tissue suspension was then aspirated into a 10 ml pipette, placed in a 50 ml tube and centrifuged at 100 *g* for 5 min at 4°C. The supernatant of each tissue was removed and the pellet resuspended in 6 ml PBS. The tissue fragments were placed into 25 cm^2 cell culture flasks with fetal bovine serum (FBS) for attachment of explants and kept overnight at 28°C. After ensuring the attachment of the explants 7 ml of complete growth medium (Leibovitz's L-15 supplemented with 20% FBS), antibiotics (penicillin, 100 IU/ml; streptomycin 100 µg/ml) and fungizone (2.5 µg/ml) were added slowly. The flasks were incubated at 28°C in a normal atmosphere incubator and left undisturbed to allow optimum cell attachment, before examination by light microscopy. Half of the medium was changed every week. Nikon equipped with phase optics was used to observe and photograph living cell cultures every third day for primary cell cultures and subcultures.

Subculture, maintenance, storage and revival: When a confluent monolayer was formed in the primary culture, the cells were washed with calcium and magnesium free PBS (CMF-PBS) 3- times and the cells were harvested with 0.25% trypsin-EDTA solution (invitrogen). The cells were subcultured at 1:2 to 1:3 ratios and maintained in the complete L-15 medium with 20% FBS, 200 IU/ml penicillin, 200 µg/ml streptomycin and 0.5 µg/ml amphotericin B. After 10^{th} subculture, the concentration of FBS in medium was reduced to 10% (L-15–10), and antibiotics and antimycotics were reduced to the normal concentrations of 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The subcultures were stored in the liquid nitrogen (–196°C) in the freezing medium, which consisted of L–15 plus 50% FBS and 10% dimethyl sulphoxide (DMSO). Briefly, cells with 80% confluence were harvested as described earlier. These cells were resuspended to 2×10^6 cells/ml and aliquoted to cryovials (1.5 ml cell suspension per vial). The cryovials were kept at –20°C for 2 h, –70°C overnight and then transferred to liquid nitrogen containers (–196°C). For revivals, a cryovial was thawed quickly in water bath at 37°C and centrifuged at 200*g* for 5 min at room temperature. The cells were resuspended with L-15 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, and seeded in a 25cm^2 tissue culture flask. The viability of the revival cells was estimated by trypan blue staining and the cells were counted on a hemocytometer.

Effect of temperature and FBS on cell growth: To examine the effect of temperature and varied concentration of FBS on fin cell growth, the cells at the concentration of 5×10^4 were inoculated into 25 cm^2 cell culture flasks and incubated at 28°C for 2 h. The batches of culture flasks were then incubated at selected temperatures of 20, 26, 28, 30, and 37°C. Temperature experiments were performed using normal growing medium supplemented with 20% FBS. Every other day, duplicate flasks at each temperature regime were washed with CMF-PBS twice, after which 0.2 ml of 0.25% trypsin solution was added to each flask. When the cells rounded up, the cell density was measured microscopically by using a haemocytometer. The experiment was carried out for 5 days. The experiments were conducted in triplicates. The cell growth in different concentrations of FBS (5, 7.5, 10, 15 and 20%) was carried out using the same procedure as mentioned above at 28°C. In our earlier study, we found no significant difference in the number of viable cells for FBS and equal mixture of FBS and NBCS at 15% serum concentration. To reduce the cost factor, 15% equal mixture of FBS and new born calf serum (NBCS) could be used for the normal maintenance of the fin cells *in vitro* (Swaminathan *et al.* 2010). So 15% equal mixture of FBS and NBCS for the maintenance of the fin cells during this study.

Cytotoxicity test of bacterial extracellular products: The cytotoxicity of bacterial ECP from *Vibrio cholerae* MTCC 3904 to fin cells was tested. The cellophane plate technique of Liu (1957) was used. Briefly, sterilized cellophane sheets were placed on the surface of Brain Heart Infusion Agar plates and inoculated by spreading 0.5 ml of a 24 h old broth culture of *V. cholerae* with a sterile swab and incubated at 37°C. After incubation for 48 h, cells were washed off the cellophane with a minimum volume of PBS. All the cell suspensions were centrifuged at l0000g for 30 min at 4°C. The supernatants were filtered through a 0.22µm pore-size membrane (Millipore), freeze-dried and reconstituted in PBS to a final volume of 10 ml. All ECP samples were stored at –30°C until use. The cells were grown as a monolayer in 24-well plates at 28°C using L-15 medium supplemented with 5% FBS. For the toxicity test, the cell line was inoculated with 0.1 ml serial dilutions of ECP. For negative controls, plates were inoculated with sterile saline. Plates were incubated at 28°C and the effects of ECP on the cells were observed after 12 h for 3 days.

Viral susceptibility and cytopathic effect (*CPE*)*:* Viral nervous necrosis virus (VNNV)-infected RTPCR- positive tissue samples (the only virus reported from India) of Asian sea bass, *Lates calcarifer* were collected from Nagapattinam, Tamil Nadu. The virus was isolated from the infected tissues by homogenizing them in L-15 medium with antibiotics and without FBS. The homogenate was frozen and thawed three times before centrifuging at 13000 g for 1h at 4°C and the supernatant was filtered by a 0.22 µm membrane and inoculated into HGF cells grown to 60–80% confluence (3 d after last subculture). After 1 h of adsorption at room temperature, 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 30°C and the cells were examined daily for the occurrence of CPE for 10 days.

PCR for confirmation of origin of cell lines: The origin of the fin cell line was authenticated by partial amplification

and sequencing of 16S rRNA and COI regions of the *E. merra*. Briefly, the samples were homogenized separately in NTE buffer (0.2 m NaCl, 0.02 m Tris–HCl, 0.02 m EDTA, pH 7.4) and centrifuged at 3000 g at 4°C, after which the supernatant fluids were placed in fresh centrifuge tubes together with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, 0.1 mg/ml, proteinase K). After incubation at 65°C for 2 h, the digests were deproteinized by successive phenol/chloroform/isoamyl alcohol extraction and DNA was recovered by ethanol precipitation, drying and resuspension in TE buffer. The universal primer pair sequences of the 16S rRNA (Palumbi *et al.* 1991) and COI (Ward *et al.* 2005) were used in the PCR reactions. PCR was carried out in a VetriTM 96 well thermal cycler. Each PCR reaction was in a 25 µl volume containing both forward and reverse primers (10 µm, 0.5 µl each), $MgCl_2$ (25 mM, 1.5 µl), dNTPs (2 mM, 2.0 µl), PCR buffer (10X, 2.5 µl), Taq DNA polymerase (1U, 0.5 µl), template DNA (0.3–0.4 µg) and nucleic acid free water. PCR cycling conditions included an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, annealing temperature of 50°C for 30 s, 72°C for 45 s and a final extension of 5 min at 72°C. The PCR products were visualized on 1.2% agarose gels and the amplified products were selected for sequencing. The cleaned up PCR products were sequenced in capillary sequencer following manufacturer's instructions at the sequencing facility. The raw DNA sequences were aligned against known sequences from the National Center for Biotechnology Information (NCBI) database and edited using BIOEDIT sequence alignment editor version 7.0.5.2.

RESULT AND DISCUSSION

The present work describes the development and characterization of cell lines derived from fin of honeycomb grouper. In our earlier studies we found that the fin tissue was best compared to the other tissues to develop cell line (Swaminathan *et al.* 2010). So, cell cultures of *Epinephelus merra* were initiated from explants (n=72) of caudal fin tissues. The cells migrated from the different tissue fragments and grew well and formed a monolayer during the first month. Emergence of growing cells from fin occurred 25 days after the explanation of minced tissue (Fig. 1). In primary culture, fin cells adhered well and achieved confluence in 6 days at 28°C. Cells were subcultured in L-15 medium with 20% FBS every 4–5 days for the initial 10 passages. For the first 15 passages, 50% culture medium was replaced with fresh medium at 4 day intervals. After 15th subculture, cells were subcultured at a ratio of 1:3 at 3–4 days interval, and FBS was reduced to 15% in the L-15 culture medium. This cell line was well adapted to grow in L-15 supplemented with FBS and well adopt for growth in medium without any special requirement, such as NaCl unlike some other marine fish

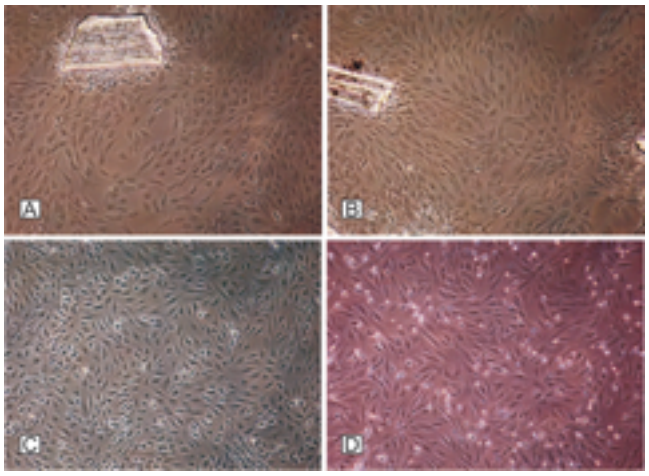


Fig.1. Photomicrographs of Honeycomb grouper fin cells. A and B – Fin explant (200X); C - Monolayer of HGF cells at 20th passage; D – Monolayer of HGF cells at 30th passage (200X).

cells (Fernandez *et al.* 1993). The fin cells were being subcultured more than 45 times since initiation and are designated as Honeycomb grouper fin (HGF) cell line.

In the initial passages, fin cells were composed of a heterogeneous mixture of fibroblastic-like and epithelial-like cells. After 15 subcultures, they were predominantly fibroblastic-like cells. A similar morphological change has also been observed in orange spotted grouper, *Epinephelus coicoides* fin (GF-1) cells (Chi *et al.*1999), and yellow grouper, *Epinephelus awoara* fin (GF), and heart (GH) cells (Lai *et al.* 2003), Asian seabass fry, *Lates calcarifer* (Bloch), (SF) cells (Chang *et al.* 2001), *L. calcarifer* (Parameswaran *et al.* 2006), and *Catla catla* and *Labeo rohita* (Ahmed *et al.* 2009). The change could be due to a common serum-derived factor present in all sera, which has strong mitogenic effect on fibroblast cells thereby inhibiting the epithelial cell proliferation as reported in other animal cells (Freshney 2005).

Optimal growth temperature for HGF cells ranged 26–30°C (Fig. 2A) which was identical with other fish cell lines reported previously (Ahmed *et al.* 2009, Swaminathan *et al.* 2010). However, maximum growth was obtained at 28°C. HGF cells were also able to spread and grow well at 20°C, although it took 48 h for the cells to become well attached When incubated at 30°C, the cells proliferated fast during the first 48 h, but the growth and proliferation slowed dramatically, and the cells appeared to age rapidly. Although cells remained viable during the test period when incubated at 37°C, cell growth was minimal and individual cells looked abnormal. The growth rate of HGF cells increased as the FBS proportion increased from 2 to 20% at 28°C (Fig. 2B). Cells exhibited poor growth at 5% concentrations of FBS, relatively good growth at 10% but maximum growth occurred with the concentrations of 15 and 20% FBS. However, a 15% concentration of FBS also provided relatively good growth, and this is an advantage to maintain this cell line at low cost.

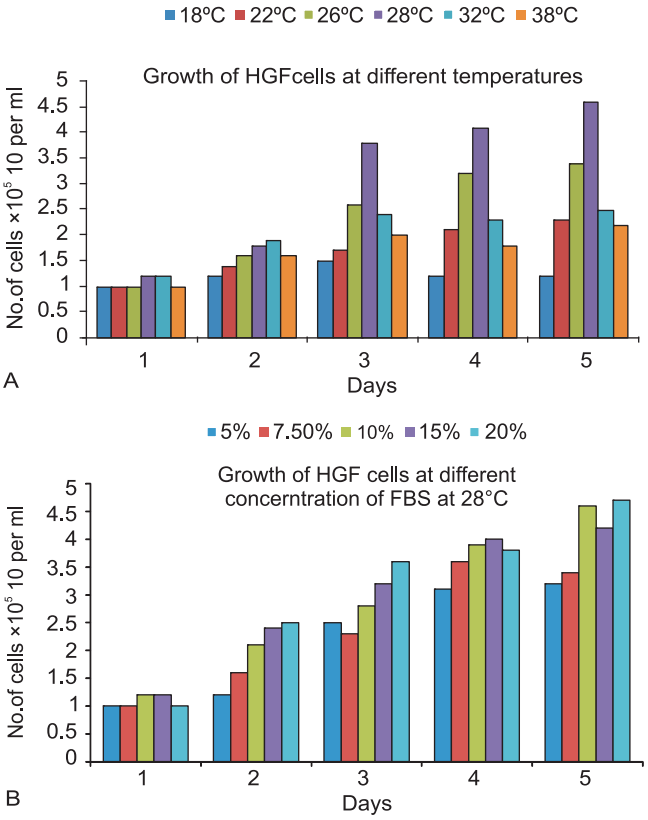
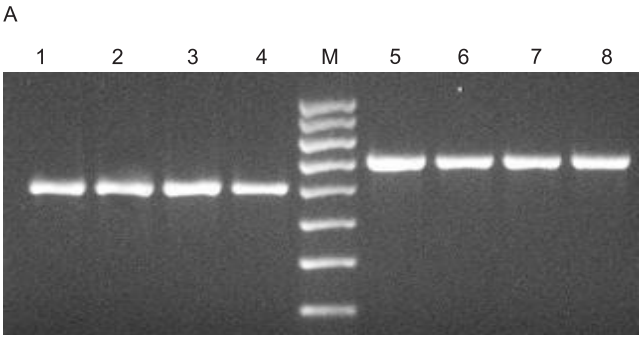


Fig. 2. Growth response of the HGF cell line at the 20th passage to (A) selected temperature and (B) selected foetal bovine serum (FBS) concentrations.

The HGF cells were cryopreserved at 10th, 20th and 30th passages. The cells were recovered after 6 months from storage and grew to confluence within 5 days. The average viability of the cells after cryopreservation was estimated to be 83% with the same morphology. Cryopreservation of cell lines is necessary for long-term storage. The viability of different cell lines was demonstrated that all the cells have the ability to survive following storage at –196°C by various workers (WSF, WSBM and WSHST cells, Wang *et al*. 2003; SISK, Hameed *et al.* 2006; LCF and LCE, Lakra *et al.* 2006; RE and CB cells, Ahmed *et al.* 2009).

Partial sequence information of 16S rRNA (590 bp) and COI (655 bp) were used to further confirm the origin of the newly established HGF by PCR. The amplified PCR fragment was sequenced and this DNA fragment matched perfectly with the genomic sequence of 16S rRNA and COI previously reported for *E. merra* (e values 0.0 and 0.0 respectively; NCBI genebank accession numbers AY947629.1 and DQ107899.1). The mitochondrial 16S rRNA, 12S rRNA and COI gene sequence alignment was used as reliable molecular methods to accurately identify the origin of cell lines of many fish species such as rohu (Ahmed *et al*. 2009) and grouper (Ku *et al*. 2009). The sequence data (Fig. 3A and 3B) confirmed that the newly established HGF cell lines were derived from *E. merra*.



16S ribosomal RNA gene 590bp

ATCAAAAACATCGCCTCTTGCTAAACCGAAAGTATAAGAGTCCCGCCTGCCCTGTGACT
ATATGTTTAACGGCCGCGGTATTTTGACCGTGCGAAGGTAGCGCAATCACTTGTCTTTT
AAATGGGGACCTGTATGAATGGCACAACGAGGGCTTGACTGTCTCCTCTTTCAAGTCA
ATGAAATTGATCTCCCCGTGCAGAAGCGGAGATAAACTCATAAGACGAGAAGACCCTA
TGGAGCTTTAGACACTAAAGCAGGCCAGTATTAATACCCCAAATATGAGGCACGAATA
AACTGAATTTCTGCCCTAATGTCTTAGGTTGGGGCGACCGCGGAGCAACAAAAAACCC
CCGCAAGGACCGAATGTACTACATTCACAACCAAGAGCGACAGCTCTAATTAACAGAC
ACTTCTGACCAATAAGATCCGGCAACGCCGATCAATGGACCAAGTTACCCTAGGGATA
ACAGCGCAATCTCCTCTTAGAGCCCATATCAACGAGGAGGTTTACGACCTCGATGTTGG
ATCAGGACATCCTAATGGTGCAGCCGCTATTAAGGGTCGTTTGTTCAACGATTAAAGTC
CTACGT

Cytochrome oxidise subunit 1 (COI) gene 655bp

CCTTTATCTTGTATTTGGTGCCTGAGCCGGCATAGTAGGAACAGCCCTCAGC
CTGCTTATTCGAGCCGAGCTAAGCCAACCAGGAGCCTTGCTCGGTGACGAT
CAAATCTATAATGTAATTGTGACAGCACATGCTTTCGTAATAATTTTCTTTAT
AGTAATACCAATCATGATTGGAGGCTTCGGAAACTGACTTATCCCGCTTATG
ATCGGCGCCCCAGATATGGCATTCCCTCGAATGAACAACATGAGCTTCTGAC
TTCTCCCCCCATCATTCCTGCTCCTCCTGGCTTCTTCTGGAGTAGAAGCTGGA
GCCGGTACCGGCTGAACAGTTTATCCACCCCTAGCTGGAAACCTGGCCCAC
GCAGGTGCGTCCGTAGATTTAACCATTTTCTCACTTCACCTAGCGGGTGTCT
CATCAATCCTGGGGGCAATTAATTTCATTACAACCATCATCAACATAAAACC
CCCTGCCATCTCTCAGTACCAAACACCCCTATTCGTATGAGCTGTACTAATTA
CAGCAGTACTCCTACTCCTCTCCCTTCCTGTCCTTGCCGCCGGTATTACAATG
CTTCTAACAGATCGTAATCTCAATACTACCTTCTTTGACCCAGCCGGAGGAG
GAGATCCCATTCTCTACCAACACTTACAT

Fig. 3. PCR amplification of 16S rRNA and CO1 of Honeycomb grouper and their partial sequence.

(A) PCR amplification of 590 and 655bp sequences of the honeycomb grouper 16S ribosomal RNA gene and Cytochrome oxidise subunit 1 (COI) gene genome using oligonucleotide primers designed from the conserved portions of the 16S and COI mitochondrial DNA. The 100 ng of DNA isolated from honeycomb grouper tissue and fin cells was amplified and then subjected to 2.0% gel electrophoresis. Lane 1: HGF 16SrRNA; lane 2: HGF 16SrRNA; lane 3: honeycomb grouper 16S positive control; lane 4: honeycomb grouper 16SrRNA positive control; lane 5: HGF COI; lane 6: HGF COI; lane 7: honeycomb grouper COI positive control; lane 8: honeycomb grouper COI positive control M (marker): 100 bp DNA ladder (showing range from 100 to 1000 bp). **(B)** Nucleotide sequences of the 590 and 655bp fragments amplified by PCR using the respective primers.

The ECPs from *Vibrio cholerae* MTCC 3904 proved to be cytotoxic for all HGF cell lines. Cytotoxic effects could be observed within 10 h after inoculation. The morphological changes detected in these cell lines were rounding, detaching and finally monolayer destruction. The ECP from *Vibrio cholerae* MTCC 3904 was cytotoxic to the HGF cell line. Many fish cells have proven suitable for demonstrating the cytotoxic effects of fish pathogenic bacteria, such as members

of the various genuses (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. Nodavirus, a marine fish virus (the only fish virus reported from India) was tested on the HGF cell line but was not susceptible to the virus.

Some pathogenic viruses are known to be organ- and tissue-specific, which makes the establishment of additional cell lines from different organs and tissues of a host species essential for proper monitoring of viral diseases (Luc Rougée *et al*. 2007). In the absence of susceptible cell culture systems, other methods and techniques, such as electron microscopy and bioassays, may be relied upon for the diagnosis of viruses which are expensive and not as easily reproducible as *in vitro* cell cultures. So, the development of HGF cell line would be valuable for isolation of the virus in any disease outbreaks in *E. merra* and also for studying species-specific responses of the viruses at the cellular level.

In conclusion, a fish cell line, HGF, was established from fin of *E. merra* which was subcultured 45 passages and diploid at their present passages. The HGF cells were cryopreserved at this passage level for further use. The cell line can be used for developing cell models for toxicological and genotoxicological studies to replace whole animals and for genetic engineering. The development of HGF cell line would be valuable for isolation of the virus in any disease outbreaks in *E. merra* and also for studying species-specific responses of the viruses at the cellular level.

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