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Development of cell culture system from the giant freshwater prawn *Macrobrachium rosenbergii* (de Man)

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Abstract A new cell culture system (MRH) was developed for the first time from 2 months old freshwater prawn, *Macrobrachium rosenbergii*. Primary cultures were developed from heart tissues by explant culture technique. Cell outgrowth was obtained from the heart explant after 14 days of explant culture. The culture medium used was Leibovitz-15 supplemented with 20% Fetal Bovine Serum along with 1% prawn hemolymph serum, 0.1% glucose, 0.5% NaCl and antibiotics (Penicillin 10,000 Units ml^{-1}, Streptomycin 10,000 µg ml^{-1}, Amphotericin B 500 mg ml^{-1}) with a final osmomolality of 470–550 mmol kg^{-1}. The pH of the growth medium found suitable for the growth of the cells was 7.20. The viability of cells was found to be 60% when revived after a month of storage in liquid nitrogen.

Keywords *Macrobrachium rosenbergii* · Cell culture · Giant freshwater prawn · MRH

Introduction

It is globally recognized that the occurrence of infectious diseases in aquaculture systems are the inhibiting factors in the development of the sector. The out break of viral diseases under intensive culture system causes heavy losses to the shrimp industry [1]. The recent report of white tail disease (WTD) in freshwater prawn hatcheries and farms has sent shock waves to the prawn farming in India [2, 3]. The new disease has been reported in many hatcheries located at Nellore (Andhra Pradesh) and Chennai (Tamil Nadu), India. The two viruses, *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) have been found to be associated with WTD [4].

Formulating preventive and disease control measures in aquaculture requires information on pathogenesis, host-pathogen interaction and the defense mechanisms. Cell lines from fish and shellfishes have enormous applications in virology, immunology, ecotoxicology, physiology and genetics [5]. The crustacean cell culture has gained potential scope for the development of diagnostic reagents and probes for use in the shrimp, crayfish and lobster industries [6]. Despite numerous attempts, no established cell line from crustaceans including the freshwater prawn has been reported to date [7–12]. The present study reports the development of a cell culture system (MRH) from the heart of *M. rosenbergii* for the first time.

Materials and methods

Specimens

Two months old prawns (*M. rosenbergii*) weighing 10 g were obtained from Central Institute of Fisheries Education, Mumbai, Maharashtra. After acclimatization, the prawns were disinfected with 0.1% potassium permanganate solution and released into clean fibre reinforced plastic (FRP) tanks and hygienically maintained with adequate aeration and frequent water exchange. They were maintained at a temperature range of 28–31°C and were daily fed with flesh of molluscs and crustaceans.

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Media and supplements

The growth medium used in the study was Leibovitz-15 (L-15, Sigma Chemicals, Sigma Aldrich, St. Louis, MO, USA) supplemented with heat treated 20% FBS along with 1% prawn hemolymph serum, 1 g l^{-1} glucose, 5 g l^{-1} NaCl with a final osmomolality at 470–500 mmol kg^{-1} (pH 7.20). After the initiation of primary culture, prawn hemolymph serum was added at 1% final concentration. Prawn hemolymph serum was prepared from the hemolymph of the fresh water prawn, *M. rosenbergii*. The donor freshwater prawn was obtained from a hatchery after PCR screening for the absence of viral and other infections. After extracting the hemolymph, it was kept for overnight at 4°C. This was then centrifuged at 300*g* for 10 min at 4°C. The clear supernatant serum was separated from the clot using a sterile Pasteur pipette, transferred to a fresh micro-centrifuge tube. The serum was pre-filtered using a 0.45 μm membrane and filter sterilized using 0.22 μm membrane filter (Durapore, Millipore, Bangalore, India). The serum was stored at −20°C until further use.

Explant preparation

For setting up the primary cultures, the prawns were sacrificed by plunging in ice for 5–10 min. They were washed thoroughly with tap water. The surface was disinfected using sodium hypochlorite solution (500 ppm available chlorine) for 5 min followed by a thorough rinse with sterile double distilled water. The hepatopancreas, eye stalk and heart tissues were aseptically excised and rinsed individually with phosphate buffer saline (PBS), 70% ethanol and iodine antiseptic (Betadine, 0.5% w/v iodine). The head and unwanted parts of the body were carefully discarded while the remaining tissues to be used for explants preparation were washed as above. Explants of 1 mm^3 size were prepared and washed thrice with PBS containing antibiotics (Penicillin 10,000 Units ml^{-1}, Streptomycin 10,000 μg ml^{-1}, Amphotericin B 500 mg ml^{-1}) for 5–10 min.

The explants were seeded in 25 cm^2 tissue culture flask (Nunc, Denmark) and kept semidry for few minutes. Adherence of explants was accomplished by incubation with 50 μl of fetal bovine serum (FBS) at 28°C. After 8–10 h, the growth medium was added gently. Fifty per cent of the medium was exchanged once in every 3 days. Daily observations were made using an inverted microscope.

Subculture

For the first subculture, the cells were carefully harvested from the flask surface using TPVG solution (0.1% Trypsin, 0.25% EDTA and 0.2% glucose in PBS 1×) without dislodging the explants. Detached cells were harvested in 5 ml of growth medium and transferred to fresh flasks. The explants were maintained to collect fresh migrating cells. When the confluent monolayer had formed in the primary culture, the old medium was removed and cells were dislodged by treatment with the above TPVG solution twice for 30 s each. Detached cells were resuspended in 5 ml of fresh growth medium (L-15 with 15% FBS and 1% prawn hemolymph serum) and seeded in 25 cm^2 plastic culture flasks.

Growth studies

To determine the effect of different temperatures on cell proliferation, cells at a rate of 1×10^5 were cultured in 20, 24, 28, 32 and 36°C. Duplicate flasks of cells at each temperature were trypsinized and then counted with a haemocytometer every day for a total of 5 days. Similarly, for studying the effect of different concentrations of FBS, the cells cultured at FBS concentrations of 5, 10, 15, 20% with 1% prawn hemolymph serum and 25% were counted at an interval of 5 days.

Cryopreservation

The viability of the cells following cryostorage in liquid nitrogen was evaluated. A 5-day-old cell mono-layer was harvested by trypsinization and concentrated by centrifugation for 10 min at 206*g*. The cell count was adjusted to 1×10^6 cells ml^{-1}/vial of chilled freezing medium consisting of 10% dimethyl sulphoxide (DMSO), 20% FBS and 70% L-15. The cryovials were held at −20°C for 4 h followed by overnight incubation at −75°C. The vials were then transferred to liquid nitrogen containers (−196°C). After an interval of 1 month, the vial was thawed quickly in a water bath at 26°C and the freezing medium was removed by centrifugation at 367*g* for 5 min. The cells were washed once with 5 ml of fresh growth medium with FBS, resuspended in 4 ml of growth medium (L-15 with 10% FBS) and seeded in 25 cm^2 tissue culture flasks. The viability of cells was measured by trypan blue staining and the number of cells was counted using a haemocytometer.

Molecular characterization

DNA isolation

DNA extractions from the MRH cells were carried out following Ruzzante et al. [13] with minor modifications. The concentration of isolated DNA was estimated using a UV spectrophotometer. The DNA was diluted to get a final concentration of 100 ng/μl.

Amplification and sequencing

The 608 bp fragments of cytochrome oxidase subunit I (COI) was also amplified in a final concentration of 50 µl volume with a final concentration of 5 µl of 10× Taq polymerase buffer, 2 µl of MgCl2 (50 mM), 0.25 µl of each dNTP (0.05 mM), 0.5 µl of each primer (0.01 mM), 0.6 U of Taq polymerase and 5 µl of genomic DNA. The primers used for the amplification of the COI gene were COI-a (5′-AGTATAAGCGTCTGGGTAGTC-3′) and COI-f (5′-CCTGCAGGAGGA GGAGACCC-3′) [14]. The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 40 s at 94°C, 40 s at 54°C and 1 min 10 s at 72°C followed in turn by final extension of 10 min at 72°C.

The PCR products were visualized on 1.2% agarose gels and the most intense products were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. The obtained sequences of PCR fragments were compared to known sequences in the NCBI.

Results

The results were obtained from five successive trials with different tissues. Cells from heart, hepatopancreas and eye stalk of *M. rosenbergii* were cultured in 1× L-15 medium supplemented with 5 g l^{-1} NaCl, 1 g l^{-1} glucose and 20% FBS along with 1% prawn hemolymph serum. Most of the explants adhered on to the substrate with in 6 h of seeding. The percentage of attachment was the highest in case of heart explants followed by hepatopancreas and eyestalk explants. The cell culture system was developed from the heart explant. Cell outgrowth from the heart explants was initiated on the 15th day of culture (Fig. 1). The emerging cells showed mostly fibroblast like morphology and were named as MRH. The cells which migrated from the explants were harvested and seeded in fresh culture flask. Slow colony expansion continued with full or partial replacement of the medium at 3 days interval. When we achieved 80–90% confluency over a period of a month, cells were subcultured. There was no cell growth in the hepatopancreas and eye stalk explants.

The growth pattern/characteristics of cells at different temperatures and FBS concentrations were similar. All cells were able to survive at all temperatures tested, but a higher growth rate was observed at 28°C (Fig. 2). Incubation at 24 and 32°C yielded significant growth whereas the cell number was very less at 20 and 36°C. The cells exhibited low growth at low FBS concentration of 5%, though a relatively better growth occurred at concentrations of above 15% (Fig. 3). After the first passage, prawn hemolymph serum was included in the medium at 1% final concentration as an additional supplement. The proliferation of cells was maximum when the medium was supplemented with 20% FBS and 1% prawn hemolymph serum. The cells after 9th passages were cryo-preserved and revived successfully after a month with 60% viability.

Analysis of mitochondrial COI was performed to verify the origin of MRH cell line. The sequences were submitted for GenBank Accession No (FJ717703- FJ717704). The comparative analysis of the identified sequences demonstrated a 99% similarity for COI region to known *M. rosenbergii* mitochondrial DNA sequences (GenBank Accession No. AB235295). The result indicated that these MRH cells are of *M. rosenbergii* origin.

Discussion

Crustacean cell culture has gained attention as a potent model to assist in the development of diagnostic reagents for the shrimp industry. Various studies on initiating primary cell cultures from the heptopancreas of penaeid shrimp have been reported with limited success [15–18]. Advancement to established cell line status through serial sub-culturing has not yet been reported. Chen et al. [19] reported the formation of confluent monolayers of cells from cultured ovarian tissue fragments from *Penaeus monodon*, with successful subculture for three passages before the cells finally degenerated. We developed MRH cell culture system from cultured heart tissue fragments from *M. rosenbergii* using explant technique. Unlike enzymatic digestion, the explant technique involves spontaneous migration of cells with no damage to the cell surfaces.

The developments of cell culture from freshwater prawns mainly depend on pH and temperature. The pH ranging from 6.8–7.2 has been used by most of the earlier workers [20, 21] as it is the haemolymph pH of the animal. In the present study, we found a pH of 7.20 and temperature 28°C was found suitable for growth of cells from *M. rosenbergii* with supplement of 5 gm $^{-1}$ NaCl, 1 gm^{-1} glucose and 20% FBS in L-15 medium along with 1% prawn hemolymph serum.

The pH range of this basic culture medium is very similar to that of mammalian fibroblasts [22] and to the primary cell culture from shrimp [19]. The addition of salt to raise osmomolality to 470–500 mmol kg^{-1} proved beneficial in maintaining the integrity of the cells. We found that osmomolality at 472 mmol kg^{-1} was ideal for growth of the cells. Previous studies have shown that glucose supplements are very important for crustacean cell culture [20]. Our studies showed that the basic culture

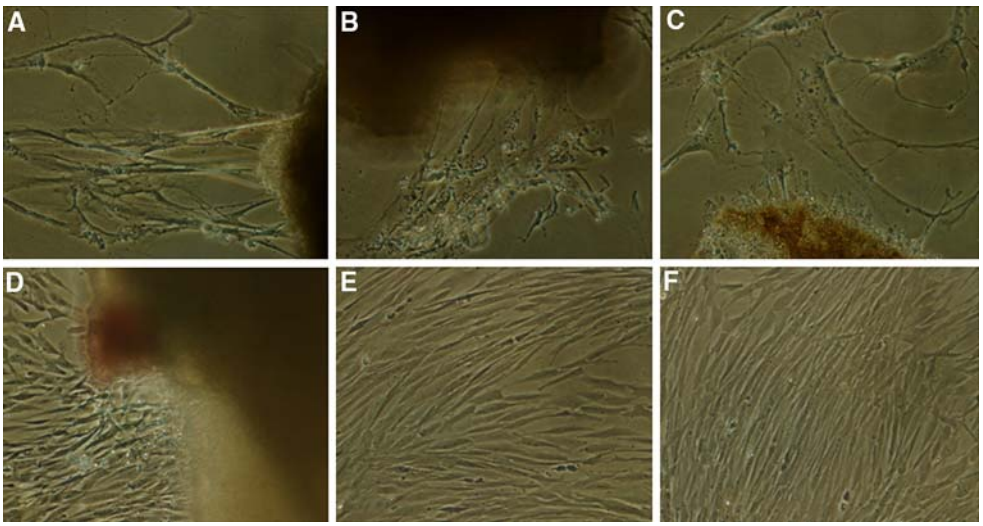

Fig. 1 Phase contrast micrograph of fibroblast like cells derived from the heart explants of *M. rosenbergii* (**a**–**d**). Different heart explants showing radiation of cells (×200; **e**, **f**). Cells showing confluent monolayer (×200)

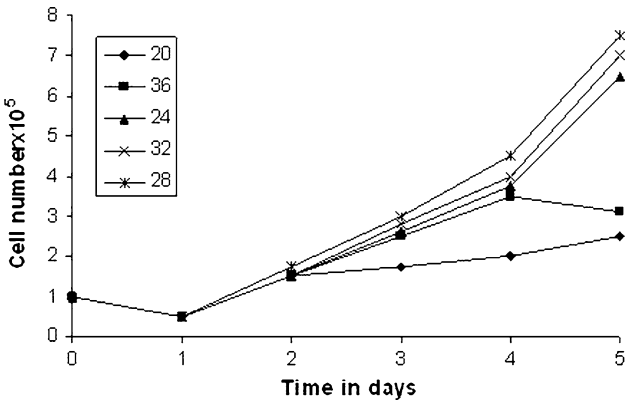

Fig. 2 Effect of temperature on the growth of MRH cells

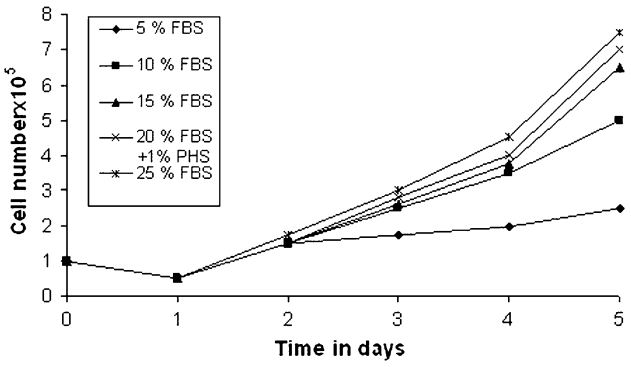

Fig. 3 Effect of different concentration of serum on the cells of MRH

medium with 1 g l^{-1} glucose can enhance the growth of cells. Several growth factors like epidermal growth factor, transforming growth factor β (TFG β), insulin-like growth factor I etc. were listed for developing a subculture system of shrimp cells did not show encouraging results [20].

The highest growth rate of various tropical fish cell lines was observed at 32°C [23, 24], 28°C [25, 26] and 20–25°C [27, 28]. A temperature of 35–37°C was often found lethal to many of fish cells [27]. In the present study, the heart cells exhibited maximum growth rate at 28°C, but were also found growing over a wider range of temperature (20–36°C). This property increases the spectrum of viruses that can be used to isolate.

Bejar et al. [29] and Bradford [30] supplemented the medium with 5% FBS whereas Joseph et al. used 15% FBS and Lakra and Bhonde and Lakra et al. [29–34] used 20% FBS in different fish cell culture experiments. The widely used cell line RTG–2 was successfully adapted to a serum free media [35]. The growth rate of the heart cells of *M. rosenbergii* increased as the FBS proportion increased from 5 to 25%. The best growth was obtained with 25% FBS. However, 20% FBS supplemented with 1% prawn hemolymph serum was considered ideal taking into consideration of the cost factor. Observations on cell culture performance with various types and concentrations of FBS supplementation indicated that untreated serum normally used for tissue culture could be toxic for crustacean cells [21]. Heat treated FBS at 20% level along with 1% prawn hemoloymph serum was used in the present study. In the present study, addition of prawn hemolymph serum enhanced the growth of MRH cells. This strongly validated the effectiveness of serum from same species for successful and improved cell culture system as suggested in previous work of Lakra et al. [33].

Species identification of cell lines is crucial for scientific research accuracy and reproducibility. Hebert et al. [36] have demonstrated the utility of COI gene as a universal barcode, referred as "DNA barcoding" for the genetic

identification of animal life. Cooper et al. [37] used COI region for identification of sixty-seven cell lines used for barcode analysis. Our analysis also proved the utility of COI gene for identification of the newly established cell lines from *M. rosenbergii*. By embracing the use of COI region as an identification system, cell lines could be employed, beyond their traditional application as disease models, as safeguards providing a renewable source of DNA standards for this freshwater prawn species.

During the past decade, a number of workers have succeeded in initiating primary cell culture from hepatopancreas, lymphoid and ovarian tissues of a range of economically important marine penaeid shrimps [8, 18, 38–40]. Advancement to established cell line status through serial sub-culturing, however, has not yet been reported in this group [21]. In the present study, successful confluent monolayer (80–90%) of the heart cells was found to be formed up to 10th passage which is quite encouraging towards the development of continuous cell line from *M. rosenbergii.*

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