

Evaluation of tissues of Indian major carps for development of cell lines by explant method

M. A. Joseph, R. K. Sushmitha, C. V. Mohan, and K. M. Shankar

Fish Pathology Laboratory, Department of Aquaculture, College of Fisheries, Mangalore 575 002, India

Tissues from various developmental stages of Indian major carps such as embryo, spawn, fingerling and adult were evaluated for their potential to develop cell lines by explant method. Among the various organs/tissues such as gonad, embryo, skin, scale, fin, gill, caudal peduncle, heart, liver, kidney and swim bladder that were screened, heart and caudal peduncle were found to be suitable in terms of attachment, growth and ability to undergo subcultivation. Cells from the caudal peduncle of mrigal could be passaged 13 times while those from the heart of mrigal and catla could be passaged 10 times.

FISH cell culture has widespread applications in virology, toxicology and as *in vitro* models in cytogenetics, biomedical research and physiology¹. As of now, more than 100 fish cell lines have been established for a variety of applications. The list of potential aquaculture species and their intensive culture is growing in Asia and concomitantly there is a constant threat of diseases, particularly of viral etiology. In the last 10 years, commercial culture of Indian major carps has been established in several parts of the country. Mortalities of cultured carps due to unexplained etiologies, have been on the rise in India². In epizootic ulcerative syndrome (EUS), which has taken a heavy toll of fresh and brackishwater fish, the role of virus is still suspected³. Therefore, studies using specific fish cell lines would help in understanding the role of viral involvement, if any, in many of these complex disease syndromes of uncertain etiologies.

A cell line has been established from the gill of Indian major carp, mrigal, *Cirrhinus mrigala*². Caudal fin and scales have been used to develop primary cell cultures of catla, *Catla catla*, mrigal, *Cirrhinus mrigala* and rohu, *Labeo rohita*, success reported only with rohu caudal fin⁴. Recently, successful primary culture of the heart tissue of Indian major carps, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* has been developed by explant method⁵. Keeping in view the need for developing more cell lines from Indian major carps, the present study evaluated the potential of several organs from different developmental stages for development of cell cultures by explant method.

Tissues from various stages of Indian major carps (*C. catla*, *L. rohita* and *C. mrigala*) such as embryo, spawn, fingerling and adult were collected under standard aseptic

conditions. Developing gonads from 2-year-old male and female carp, were collected during late April and early May. Embryos were collected from fertilized eggs of induced bred rohu and mrigal, after washing with 1% iodophore solution for 2 min. Two-day-old rohu and mrigal spawn were used for collecting caudal peduncle. Skin, fin, gill, caudal peduncle, heart, kidney, liver and swim bladder were collected from fingerlings of catla, rohu and mrigal, maintained in aquaria, changing the water several times so that it was clean. In all the cases the tissues were pooled in cold PBS-antibiotic-antimycotic solution (Sigma Chemicals, USA). The tissues were evaluated for attachment, growth and ability to undergo subcultivation.

Primary cultures were initiated from the above tissues according to our earlier procedures⁵, with certain modifications in the subcultivation procedure. Briefly, tissues were cut into 1 mm³ size fragments which were seeded into 25 cm² tissue culture flasks (Tarsons, India). After appropriate semidrying and addition of minimum essential medium (MEM) (Sigma, USA) supplemented with 15% fetal bovine serum (FBS) (Sigma, USA), cell growth was monitored. Confluent monolayers obtained were harvested by dispersion using sterile rubber policeman for subculturing. In addition, monolayers were also harvested by trypsinization. A seeding density of 1.5×10^5 cells as determined by a haemocytometer was maintained for subculturing.

Table 1 documents the potential of various tissues in terms of attachment, growth and potential for development of cell lines.

Table 1. Evaluation of tissues of Indian major carps for development of cell line

Species	Tissues	Observation
<i>C. mrigala</i>	Heart and caudal peduncle	Good attachment, growth confluency
	Gill, gonad, kidney, embryo	Good attachment and growth
	Spawn	Good attachment
	Fin and scales	Poor attachment
<i>C. catla</i>	Heart and caudal peduncle	Good attachment, growth and confluency
	Gill, gonad	Good attachment, growth
	Fin and scales	Poor attachment
	skin, swim bladder, liver	
<i>L. rohita</i>	Heart and caudal peduncle	Good attachment, growth and confluency
	Gill, gonad and embryo	Good attachment and growth
	Spawn	Good attachment
	Fin and scales, skin, swim bladder, liver	Poor attachment

Wide variation in attachment of different tissues to the culture flask was noticed after incubation in a semi-dried condition. Tissues from gonads, developing embryo and spawn were found to attach well. Among the external tissues, skin, scale and fin showed poor attachment, while gill and caudal peduncle tissues readily attached to the flask. Explants of internal organs like heart exhibited good attachment, whereas, kidney required longer duration of incubation (24 h) under semi-dried condition. Attachment was very poor with the explants of swim bladder and liver.

Cells emerged around gonad and embryo explants (Figure 1) in 2–3 days. Fibroblast-like cells capable of forming primary culture were seen associated with gonad tissues and could be maintained for 3 days. Although there was good attachment of caudal peduncle explants of spawn, cells did not emerge in 2 days. There was no growth from skin, fin, scale, liver and swim bladder tissues. However, gills, caudal peduncle and heart tissues (Figure 2) from fingerlings showed cell growth within 3 days.

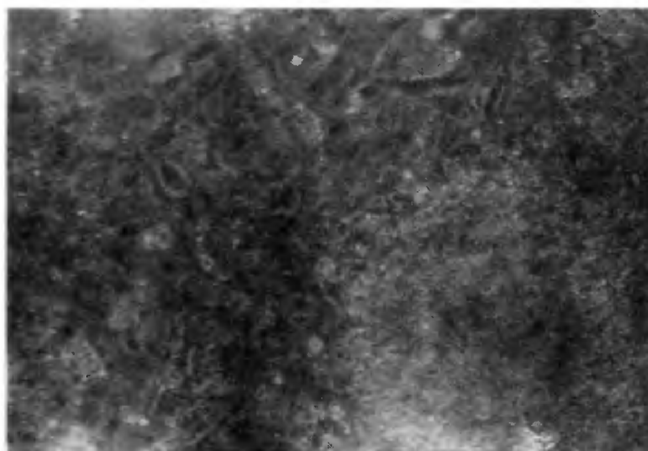


Figure 1. Fibroblast-like cells growing from explant of embryo of mrigal (100 ×).

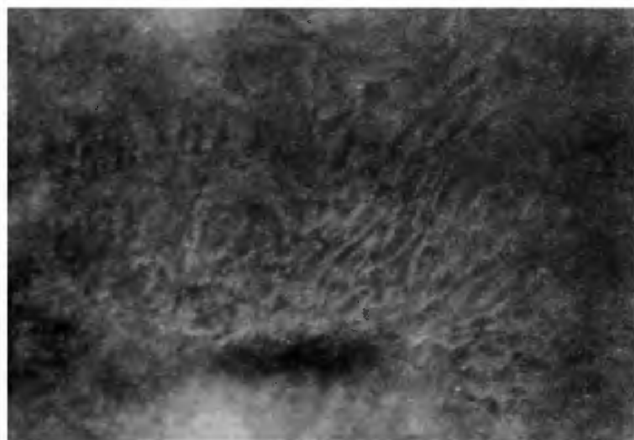


Figure 2. Fibroblast-like cells emerging from heart tissue explant of rohu (100 ×).

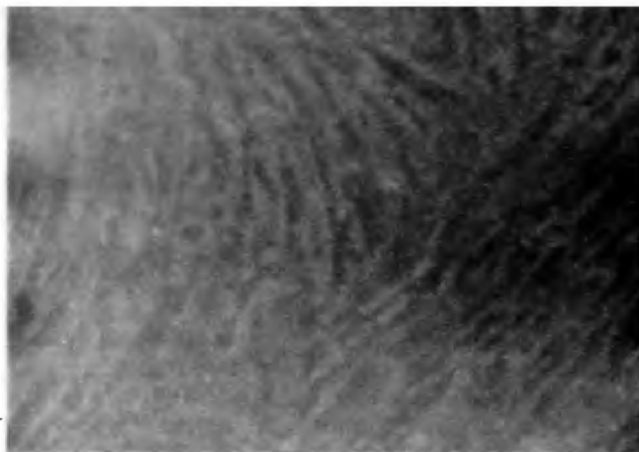


Figure 3. Fibroblast-like cells from caudal peduncle of catla (100 ×).

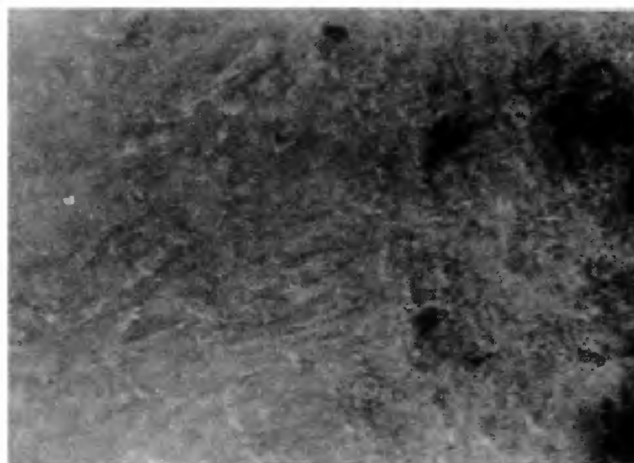


Figure 4. Fibroblast-like cells growing from mrigal kidney explant (100 ×).

The cells from explants exhibited both epitheloid and fibroblast-like shape but later, spindle-shaped cells were seen to dominate the culture. Confluency, with uniform, elongated cells forming a complete monolayer was noticed in caudal peduncle (Figure 3) and heart tissues only. Initial growth was faster in heart explant compared to that of caudal peduncle. Fibroblast-like cells were seen around kidney (Figure 4), gills, gonad and embryo explants but they did not spread out.

Confluent monolayer of cells was best seen with caudal peduncle tissue of fingerlings. The time required to attain confluency at room temperature (28–30°C) varied with the species; 15 days in rohu and approximately a month in catla and mrigal. The monolayer was dominated by both fibroblast-like (spindle shaped) and epitheloid-like (bead like) cells. Cells emerging from heart explant did not spread out uniformly and formed a matrix-like network due to overlap of cell sheets around the tissue (Figure 5). However, as the number of passages

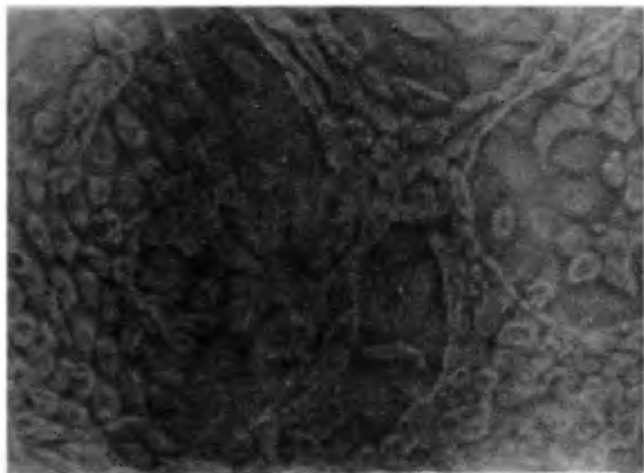


Figure 5. Mrigal heart cell line forming a matrix-like net-work (100 \times).

increased, a monolayer mainly comprising of fibroblast-like cells with a sharp and clear outline developed. These cells were both dendritic and spindle-shape with visible cytoplasmic granules. Epithelial-like cells (bead shaped) were also seen forming a neatly-packed layer.

As confluent monolayer was obtained only with heart and caudal peduncle explants, these were subcultured. Cells harvested by trypsinization for subcultivation yielded individual cells along with clumps. These cells attached well to the flask and regained the original fibroblast-like shape. However, these cells died after a week. On the other hand, monolayers harvested by scraping with a sterile rubber policeman were in clumps and attached well in the new flask. Substantial growth was seen along the edges of these clumps.

Growth performance and monolayer formation were relatively better in the original flask compared to that in the new one. Cells derived from caudal peduncle and heart were observed to have the potential to regenerate and perform well after subcultivation.

Attachment of explants to the culture flask is the primary requirement for successful cell culture. In this study, attachment of heart tissue was the best, followed by tissues of caudal peduncle, gonad, gill, kidney, embryo and spawn. Successful attachment of heart^{5,6}, kidney⁷ and gill⁴ tissue explants have been documented. Attachment of skin, scales, swim bladder, liver and fin explants was poor, though skin^{6,8}, scales⁹, swim bladder¹⁰, liver¹¹ and fin^{4,12} explants of several fish species have yielded cell lines. As such there was no difference between the three species of Indian major carps with respect to the degree of attachment of different tissues.

Generally, good growth of cells was associated with good tissue attachment to the flask. Emergence of cells was relatively faster with heart explant when compared to other tissues, but monolayer formation was rather

slow, perhaps due to the nature of the tissue. Aggregation of cells with poor spreading resulting in multilayers around heart tissue explants as observed here, have been reported earlier^{5,6}. Caudal peduncle was found to be an ideal tissue for cell culture in this study. In nature, wound healing by regeneration of cells with intense fibroblastic activity of the dermal and hypodermal region is extremely well marked in connective tissue¹³.

Interstitial tissue of developing gonads was seen to have the potential for *in vitro* growth. However, the yolk from developing eggs interfered with the culture, and hence ideally gonads should be collected from either early maturing or spent fish. Ever since the first fish cell line was initiated from the gonad¹⁴, many cell lines have been developed from this tissue⁸. Poor growth with gill and kidney explants of rohu and mrigal observed here agree with the observations made in *Apogon imberbis*¹². Limited growth in kidney tissue was probably due to death of viable cells because of microbial contamination as the melanomacrophage centre in the kidney is a metabolic dumping site for dead cells and systemic pathogens.

Embryonic tissues of rohu and mrigal were found to be ideal for culture though the growth was slow. Embryonic cells have the capacity for growth⁸ due to active mitosis. Though spawn tissue explants attached well, contamination prevented further progress with this tissue. Poor attachment of skin, scale, fin, swim bladder and liver tissues could be the reason for the absence of growth in these tissues.

Contamination is the main obstacle for successful development of carp cell line². Among various tissues evaluated, heart and caudal peduncle tissues of fingerlings were less prone to contamination. Bacterial contamination could not be contained even with 50 X, the internationally suggested dosage of antibiotics (Sigma Chemicals, USA). This may indicate increased resistance in Indian aquatic microflora to antibiotics.

Among the three species of major carps, tissues of mrigal were found to have a better potential for growth and continued subcultivation, followed by catla and rohu. Heart and caudal peduncle were found to be better in terms of attachment, growth and ability to undergo subcultivation with low level contamination. Among the tissues evaluated, cells from the caudal peduncle of mrigal could be passaged 13 times while those from the heart of mrigal and catla could be passaged 10 times, indicating the potential of these two tissues to form cell lines. Fingerlings were found to be the ideal size for removal of necessary tissues.

1. Bols, N. C. and Lee, L. E. J., *Frontiers in Molecular Biology* (eds Hochachka, P. W. and Mommsen, T. P.), Elsevier, Netherlands, 1994, vol. III, pp. 145-159.
2. Sathe, P. S., Mourya, D. T., Busu, A., Gogate, S. S. and Banerjee K., *Indian J. Exp. Biol.*, 1995, 33, 589-594.
3. Lilley, J. H. and Roberts, R. J., *J. Fish Dis.*, 1997, 20, 135-144.

4. Lakra, W. S. and Bhonde, R. R., *Asian Fish. Sci.*, 1996, 9, 149–152.
5. Sushmitha, K. R., Joseph, M. A., Shankar, K. M. and Mohan, C. V., *Curr. Sci.*, 1997, 73, 374–376.
6. Webb, C. J., *J. Mar. Biol. Assoc. UK*, 1975, 55, 933–938.
7. Kanchanakhan, S. and Frerichs, G. N., *Diseases in Asian Aquaculture II* (eds Shariff, M., Arthur, J. R. and Subasinghe, R. P.), Fish Health Section, Asian Fisheries Society, Manila, 1995, pp. 369–383.
8. Fernandez, R. D., Yoshimizu, M., Kimura, T. and Ezura Y., *J. Aquat. Anim. Health*, 1993, 5, 137–147.
9. Smith, R. W. and Howlihan, D. F., *J. Comp. Physiol.*, 1995, 165, 93–101.
10. Chen, M. M., Chen, S. N. and Kou, G. H., *Rep. Fish. Dis. Res.*, 1995, 16, 25–35.
11. Ghosh, C., Zhou, Y. L. and Collodi, P., *Cell Biol. Toxicol.*, 1994, 10, 167–176.
12. Alvarez, M. C., Otis, J., Amores, A. and Guise, K., *J. Fish Biol.*, 1991, 39, 817–824.
13. Thomas, L. E. M. and Bucke, D., *J. Fish Biol.*, 1973, 5, 115–119.
14. Wolf, K. and Quimby, M. C., *Science*, 1962, 135, 1065–1066.

ACKNOWLEDGEMENT. we thank Dr P. Keshavanath, Professor and Head, Department of Aquaculture, for his encouragement throughout the period of study and Mr N. Basavaraja for the facilities provided.

Received 8 July 1998; revised accepted 14 September 1998

A test of simple models of population growth using data from very small populations of *Drosophila melanogaster*

V. Sheeba and Amitabh Joshi*

Evolutionary Biology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur P.O., Bangalore 560 064, India

Three simple discrete-time models of density-dependent population growth were fit to data from eight small laboratory populations of *Drosophila melanogaster* subjected to either stabilizing or destabilizing food regimes. The exponential logistic model gave the best fits to data from both types of populations. In contrast, the linear logistic and hyperbolic models did not yield good fits, especially in the case of the destabilized populations. Parameter estimates from the exponential logistic model were in close agreement with those from previous studies using very large *Drosophila* populations subjected to the same type of stabilizing and destabilizing food regimes. Our results suggest that the simple exponential logistic model can reasonably well capture the major features of the dynamics of *Drosophila* populations even for very small population sizes where one may expect demographic stochasticity to play a major role in determining the population size.

ALTHOUGH the importance of demographic stochasticity in population ecology has been appreciated for a long time by theoreticians^{1–8}, most empirical work on population dynamics has been structured around deterministic models^{9–13}. Many of the commonly-used models of population growth are extremely simple and deterministic^{14–16}, yet empirical results have typically shown reasonable agreement with predictions of the deterministic models^{10–13,17}. However, such studies have typically

used populations large enough to render the effects of demographic stochasticity on their dynamics unimportant. In recent years, the dynamics of small populations has been receiving considerable attention in ecology, especially because of conservation concerns^{18–22}. Most attention, however, has been focused on the impact of demographic stochasticity on the likelihood of extinction of small populations^{23–27}. On the other hand, the effect of demographic stochasticity on the dynamics of small populations, has rarely been studied empirically²⁸. It is, therefore, of considerable interest to assess whether deterministic models of population growth and dynamics can adequately capture the essential features of the dynamics of very small populations, or whether we need to explicitly incorporate demographic stochasticity into our models of population dynamics in order to make them applicable to smaller populations. In the study reported here, we fitted three simple discrete-time models of population growth to data from eight small (average size $N = 74.69$, st. dev. = 57.23) laboratory populations of *Drosophila melanogaster* to examine how well these simple deterministic models fit data from populations that may be expected to show relatively large amounts of demographic stochasticity.

We studied the dynamics of eight small populations of *D. melanogaster*, each of which was maintained in a single 8-dram vial (25 mm d × 95 mm h). Two sets of four populations each were derived from a large ($N \sim 2000$ adults) and outbreeding ancestral laboratory population (JB-1), derived from the UU populations of Mueller (UU and JB maintenance described, respectively, in refs 29, 30), that had been maintained in the laboratory at low larval densities for many years. At the beginning of the experiment, sets of 8 males and 8 females were placed into each of 8 vials and allowed to lay eggs for 24 h. The adults were then discarded, and the vials used to initiate the experimental small populations, which were maintained for a further 10 generations in the laboratory, as described below. Four of the populations were subjected to a food regime which combined low levels of food (3 ml food medium per

*For correspondence. (e-mail: ajoshi@jncasr.ac.in)