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Primary cell culture from fish gills and kidney using fish serum

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A novel, cost effective and time saving technique for primary cell culture from gills and kidney using fish serum has been developed. Single cell suspension of gills and kidney of *Clarias gariepinus* was obtained by trypsinization. Minimum essential medium supplemented with 10% fish serum in place of 10% foetal calf serum and 20% fish muscle extract, yielded confluent monolayer on 6th and 8th day in gill and kidney tissue respectively at 28°C. Fish serum can be successfully used as media supplement for cultivation and maintenance of primary cell culture from fishes.

Cell lines are commonly used for isolation and propagation of viruses. A number of viral diseases are known to cause widespread losses to aquaculture industry. The International organization of epizootics (OIE) has listed five notifiable diseases, which are all viral in nature. In addition, seven viral diseases have been identified as other significant diseases by OIE. OIE has recommended cell lines to screen the presence of viruses from suspected shipments of aquatic animals and their products. Fish cell lines namely Bluegill fry (BF-2), Epithelioma papulosum cyprini (EPC), Chinook salmon embryo (CHSE-214) and Channel catfish ovary (CCO) are used for this purpose. However these cell lines are derived from cold-water fishes of European region. Efforts are on to develop cell lines from tissues of tropical fish species. In India, cell lines from gill tissue of mrigal & rohu have been reported[4,5]. Successful primary cultures from heart, fin, and kidney have been attempted[1-3,6].

Foetal calf serum (FCS) along with fish muscle extract (FME) is generally used for primary cell culture from fishes[1,2,6]. The objective of the present study was to substitute FME and FCS with fish serum (FS) for the primary cell culture.

Clarias gariepinus is one of illegally introduced exotic fishes in India. This introduction has increased the risk of exposing native fishes to exotic pathogens. Several incidences of ulcerative disease in this fish have been reported which might be of viral etiology. This study was undertaken as a part of our institute mandate to screen the presence of viral pathogens of exotic fishes. This paper describes development of primary cell culture from gill and kidney tissue of *C. gariepinus* for the above purpose.

Media—Minimum essential medium (MEM) with L-glutamine and without sodium bicarbonate (Himedia) was used in the present experiment. Medium was prepared in sterile triple glass distilled water and it's *p*H was adjusted to 7.2 by adding $NaHCO_3$. Penicillin G sodium and streptomycin SO_4 (Hindustan Antibiotics) were added to the media @100 I.U, and 100 g/ ml respectively. Fish serum was mixed to the medium @10%. The reconstituted medium was first passed through a coarse filter paper and refiltered through 0.22 μ filter. The sterility of the medium was checked by putting a drop of medium in nutrient broth. The sterile medium was stored at 4°C for further usage.

Fish serum—Adult *Clarias gariepinus* fishes were procured and stocked in 1-ton FRP tanks. The fishes were fed daily with dried prawns and goat liver. Water was exchanged @ 25% daily. Blood was collected from caudal vein with sterile syringe and transferred to a sterile conical flask and was kept at 4°C for 2 hr. Pooled serum was collected and heat inactivated at 56°C for 30 min. It was stored at –20°C.

Fish—Fingerlings of *Clarias gariepinus* were procured and maintained in a glass aquarium at temperature (28° ± 28°C) for 15 days. The fishes were fed daily 3% of body weight with artificial fish feed. Water exchange was given @ 25% daily. One week before the experiment, the water was treated with $KMnO_4$ @ 2ppm. There after the fishes were starved for 2days prior to the culture to reduce the bacterial gut load and maintained in sterile water. The fishes were sacrificed by covering the operculum with cotton soaked in chloroform and dipped in 70% alcohol for 2 min.

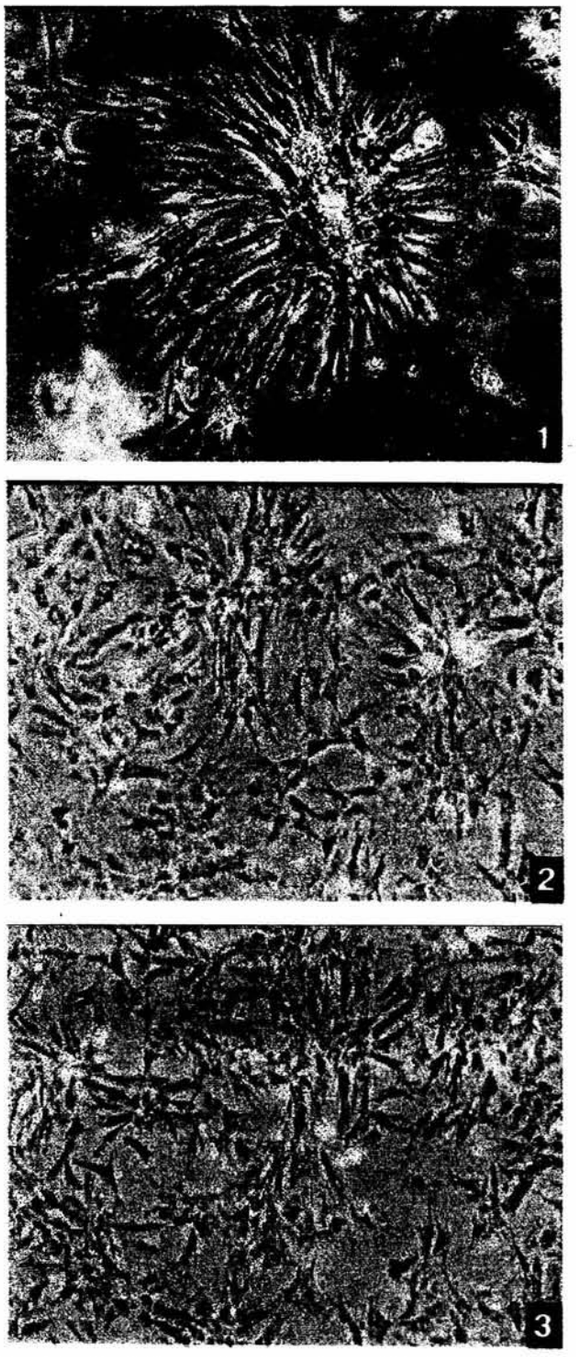
Gill culture—The gill lamellae were cut with a sterile scissors. The gills were thoroughly washed with chilled PBS and transferred to a petridish containing 20% betadine for 5 min. to remove normal microflora. After this treatment, the gills were washed thrice to remove traces of betadine. The tissue was cut into small fragments of approximately 1 cubic mm with sterile scissors and washed twice with chilled PBS. The pellet was suspended in 5 ml of PBS and trypsinized with 1ml of 0.2% chilled trypsin (Sigma) for 2 min. on magnetic stirrer. One ml of fish serum (*Clarias gariepinus*) was added to the cell suspension to inhibit the activity of trypsin. Cells were harvested by filtering cell suspension through a sterile muslin cloth. The filtrate was centrifuged at 2000 rpm for 5 min. and pellet was washed twice with tissue culture medium without fish serum. Finally the pellet was resuspended in tissue culture medium containing fish serum so as to adjust the cell concentration to 1.0×10^5 per ml and 8 ml was seeded in 25 cm^2tissue culture flask (Greiner).

Kidney culture—After removing the gills, the same fish were dissected, and kidney was removed aseptically. The tissue was washed with chilled PBS twice and minced aseptically in PBS using sterile scissors. The tissue fragments were again washed in PBS and suspended in 5 ml PBS. One ml of 0.2% chilled trypsin was added to the tissue for 2 min. on a magnetic stirrer. Trypsin activity was arrested by adding 1ml of sterile fish serum. The suspension was filtered through a sterile muslin cloth and centrifuged at 2000 rpm for 10 min. The pellet was washed twice with TCM without fish serum. Kidney cells were reconstituted in TCM containing fish serum and seeded in 25 cm^2tissue culture flask (Greiner) in the same concentration as in the case of gills.

Seven trials were conducted using the above protocol.

The flasks were kept a desiccator wherein CO_2 tension was given by burning out a candle. The desiccator was kept in BOD incubator (28°C). The flasks were left undisturbed for 48 hr and thereafter examined every day. Fifty percent tissue culture medium was changed after 4 days.

The trypsinized cells attached to the flask in clumps and also individually. This finding is in accordance with earlier report[3]. Adherence of gill and kidney cells was observed within 48 hr. Spindle formation in individual cells and clumps was seen on 3rd and 4th day in gill and kidney cells respectively. After 4 days, large elongated fibroblast like cells appeared around clumps (Fig. 1). The cells were found to radiate from clumps and multiply. Individual cells showed elongation and multiplication. Complete



Figs 1-3—(1) Cells radiating from clumps; (2) Monolayer formation from kidney cells; and (3) Monolayer formation from gill cells

Table 1— Stages of primary cell culture using 10% fish serum

Tissue	No. of successful trials	Adherence (days)	Elongation (days)	Multiplication	Monolayer formation (days)	Rounding of cells (days)
Gills	3 /7	2	3	+	6	14
Kidney	4/7	2	4	+	8	16

monolayer was formed in 6 and 8 days from kidney (Fig. 2) and gill tissues (Fig. 3) respectively. Rounding of the cells started to appear by 2nd week in gill and kidney cells. Out of 7 trials made, 3 and 4 successful attempts of gill and kidney tissues respectively were attained (Table 1).

Primary cell cultures from fishes have been established mainly from tissue explants, as it is easier to establish cell culture as explant culture rather than single cell suspension culture. In explant culture tissues are chopped into small pieces and placed in culture dishes in semidried condition to facilitate attachment of explants. The fibroblasts grow out from these explants and form a monolayer. In heart explant culture, monolayer formation occurs in 8 days in catla, 10 days in rohu and 20 days in mrigal[3]. Cells from fin explant of *Tor putitora* are in growing phase from 5-8 days[2]. In the present study, monolayer formation was observed in 6-8 days with single cell suspension. This technique is convenient and time saving as there is no need to keep the explants for 4-6 hr for their adherence to the surface.

Kidney tissue of *Clarias gariepinus* was found ideal for primary culture, as it is comparatively easy to excise the kidney aseptically after removing the visceral organs. The fungal contamination in gill tissue primary culture encountered during initial trials was overcome by treating gill tissue in betadine @ 20% in PBS and adding amphotericin B (Sigma, A-2942) @ 2.5 ml/L. The adherence and multiplication in cells of gill tissue was higher and complete monolayer formed earlier than in kidney tissue culture.

In the present study a successful primary culture from gills & kidney was obtained using minimum essential medium supplemented with 10% FS without using FCS and FME. The growth factors present in FS appear to support growth and multiplication of cells as seen in case of FCS and FME which have been used for successful development of primary cultures[1,2,6]. FME of same fish species has been used in primary cell culture[2,6]. Preparation of FME is time consuming and tedious. Use of FS can obviate the need of FME and costly FCS. Fish serum has not been tried as an alternate source of nutrients for primary cell culture earlier. These results suggest that fish serum can be successfully used in place of fish muscle extract and foetal calf serum. Efforts are on to establish kidney and gill cell lines from the primary culture.

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