

Establishment of Cell Lines from Catfish (*Clarias batrachus*) and Snakeheads (*Ophicephalus striatus*)

G.D. LIO-PO¹, G.S. TRAXLER² and L.J. ALBRIGHT³

¹
Aquaculture Department
Southeast Asian Fisheries Development Center
Tigbauan, Iloilo 5021
Philippines

²Pacific Biological Station
Nanaimo, B. C. V9R 5K6
Canada

³
Department of Biological Sciences
Simon Fraser University
Burnaby, B.C. V5A 1S6
Canada

Abstract

Primary cell cultures from catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus*) were prepared from whole fry and fingerling organ tissues of the brain, fins, gonad, heart, kidney, liver, skin and spleen. Four methods were tried: method A wherein explants were placed onto the surface of 25-cm² Primaria flasks (Falcon), allowed to attach for an hour before addition of Leibovitz medium (L-15) supplemented with 15% fetal bovine serum (FBS)(L15-15); Method B wherein explants were inoculated into 25-cm² Primaria flasks (Falcon) already containing L15-15; Method C which required forcing minced organ sections through a stainless steel sieve with the aid of a syringe plunger into a petri dish containing L15-15 medium; and Method D wherein immersed sections of minced tissues to 0.5% trypsin-EDTA slowly agitated using a magnetic stirrer for one hour at 25 °C. Method B was most effective in the establishment of cell cultures from both fish species. Passage numbers of the cells are to date catfish gonad (CFG) P-56, catfish heart (CFH) P-51, catfish kidney (CFK) P-7, catfish liver (CFL) P-8, catfish spleen (CFS) P-54, snakehead gonad (SHG) P-26, snakehead heart (SHH) P-22, snakehead kidney (SHK) P-19, snakehead liver (SHL) P-49 and snakehead spleen (SHS) P-76. Attempts to derive primary cell cultures from organ tissues of the brain, fins, skin and whole fry were unsuccessful. Established cells were fibroblastic. The cells grew rapidly and became confluent 24 h after seeding at 20 and 25 °C. Both SHS and CFS were susceptible to a virus iso-lated from EUS-affected fish in the Philippines. The cells were best maintained at 20 °C and stored in liquid nitrogen or -70 °C.

Introduction

The isolation of viral pathogens from infected fish is highly dependent on the availability of susceptible cell lines. Indications of the presence of a viral etiology in the pathogenesis of the epizootic ulcerative syndrome (EUS) was reported by Roberts et. al., 1986. Subsequently, Frerichs et. al., (1986) reported the isolation of a rhabdovirus from EUS outbreaks among snakeheads and freshwater eels (*Fluta alba*) in Thailand and Burma. Although, Noga and Hartmann (1981) and Frerichs et. al., (1986) reported the establishment of cell lines from adult catfish gill, gonad and kidney tissue and from snakehead fry fins, respectively, these cell lines are not readily available. It was therefore necessary to establish cell cultures from EUS-susceptible fishes to investigate the role of viruses in the development of EUS in these fish.

The objectives of this study were to establish cell lines from indigenous catfish and snakeheads as well as to determine if these cell lines were susceptible to the virus isolated from an EUS-lesioned fish.

Materials and Methods

Tissue processing and primary cell culture

Catfish and snakeheads, whole fry and fingerlings (2-3 g) were externally disinfected using 70% ethanol, followed by 3 mg per l iodophore solution. Whole fry were then rinsed with sterile NSS (normal saline solution) before being aseptically cut into fragments (about 1 mm³). Brain, fins, gonad, heart, kidney, liver, skin and spleen tissues were removed from each fingerling. Each organ was individually rinsed aseptically three times in petri dishes containing Earles balanced salt solution (EBSS) plus an antibiotic-antimycotic mixture (200 I.U. penicillin G sodium, 200 ug streptomycin sulfate, 50 ug amphotericin B per ml medium). The organs were then minced thoroughly using sterile scissors. These were subjected to one of four treatments: Method A: explants were placed onto the surface of 25-cm² Primaria flasks (Falcon) and allowed to attach for 1 h before the addition of Leibovitz medium (L-15) supplemented with 15% fetal bovine serum (FBS) (L15-15); Method B: explants were inoculated into 25-cm² Primaria flasks (Falcon) already containing L15-15 (R. Hedrick, Univ. Calif.-Davis, pers. comm.); Method C: tissue fragments were passed through a stainless steel sieve with the aid of a syringe plunger into a petri dish containing L15-15 medium; and Method D: tissue fragments were digested with 0.5% trypsin-EDTA slowly agitated using a magnetic stirrer for 1 h at 25 °C (Chen et. al., 1983). For Methods C and D, aliquots of the organ tissue were transferred to 25-cm² Primaria tissue culture flasks (Falcon) containing about 12 ml of L15-15.

At least three flasks were inoculated for each organ tissue. All flasks were incubated at 25 °C. Every two days, half of the medium in each flask was replaced with fresh culture medium. The flasks were also examined micro-

scopically daily for monolayer cell outgrowth around the tissue fragments for four weeks. All media were supplemented with an antibiotic-antimycotic mixture (100 I.U. penicillin G sodium, 100 ug streptomycin sulfate, and 25 ug amphotericin B per ml medium).

Cell passages

Cells in flasks showing moderate cell outgrowths were subcultured using trypsin-EDTA (0.5% Trypsin-5.3 mM EDTA, Gibco). The cell sheets were rinsed once with 1 ml trypsin-EDTA and then flooded with 2 ml trypsin-EDTA for 1-2 min each. After the cells detached, the flasks were shaken vigorously and then fresh L-15 plus 10% FBS (L15-10) with the antibiotic-antimycotic mixture was added. When the cell monolayer became fully confluent, single flask monolayers were subcultured with trypsin-EDTA as described earlier but subcultured onto more flasks at a cell density ratio of 1:2-1:3.

Effect of temperature on snakehead spleen (SHS) cell cultures

SHS cells were seeded in 25-cm² tissue culture flasks and incubated at different temperatures of 5, 15, 25 and 40 °C for one week. In another experiment, 24 h confluent cultures of SHS cells grown in 25-cm² flasks at 25 °C were moved to different temperatures of 15, 20, 25, 30 and 37 °C and incubated for one week. In both experiments, cell proliferation was monitored daily.

Cryopreservation

Confluent one day old cells were trypsinized with trypsin-EDTA, 4 ml L15-10 medium was added and the mixture centrifuged at low speed in a refrigerated centrifuge at 4 °C. The supernatant medium was discarded and the packed cells resuspended gently in a mixture of 90% FBS and 10% dimethylsulfoxide (DMSO). The cells were then subjected to gradual cooling at 4 °C for 1 h, then frozen to -10 °C for 1 h and finally stored at -70 °C (Plumb and Bowser 1983). For liquid nitrogen storage, the cells were mixed with minimum essential medium (MEM) containing 10% FBS (MEM-10) with 10% DMSO and immersed in liquid nitrogen.

Viable cells were recovered from frozen storage by holding the vials in running tap water at approximately 25 °C. Thawed cells were immediately transferred to a culture flask with L15-10 or MEM-10.

Susceptibility to virus

Established cell cultures of SHS, snakehead liver (SHL) and catfish spleen (CFS) in 25-cm² flasks were inoculated with a suspension of 1.5 ml of 10⁶ TCID₅₀ per ml of a virus that was isolated from an EUS fish in 1991 (#91-97). After 1 h, Leibovitz medium with 4% FBS (L15-4) was added and then cells were incubated at 25 °C for one week. The flasks were examined microscopically daily for cytopathic effects (CPE).

Results

Of the techniques used, the direct inoculation of organ explants in culture flasks containing the medium described as Method B, consistently yielded successful primary cell cultures from the gonad, heart, kidney, liver and spleen of both catfish and snakeheads. Method A yielded poor explant outgrowths while Methods C and D were successful in some but not in all of the tissues (Table 1). All attempts to derive primary cell cultures from the brain, fins, skin and whole fry were unsuccessful. Initial outgrowths of cells from explants were detected 5-23 d after initial culture. Extensive cell migration was observed 9-28 d after initial inoculation.

The first passages of the primary cell cultures from catfish and snakeheads were conducted on day 29. The first cell culture passages, P-1 to P-2, took approximately 23 d to achieve at least 80% confluency when incubated at 25 °C. Cell culture passages, P-2 to P-3, took 3 d for cells to develop complete confluency. On the fourth passage complete confluency within 24 h was observed. This rapid cell multiplication was consistently observed in subsequent passages.

Cell cultures were successfully established from the spleen, kidney, heart and gonad of catfish. Passage numbers of the cells are to date: catfish gonad (CFG) P-56, catfish heart (CFH) P-51, catfish kidney (CFK) P-7, catfish liver (CFL) P-8, catfish spleen (CFS) P-54 (Table 2). The CFL cells were lost to contamination at P-8 before these could be cryopreserved. Microscopically, catfish cells were initially a mixture of epithelioid and fibroblastic cells. After several passages, microscopic observations showed fibroblastic shaped cells rapidly propagating and becoming completely confluent within 24 h of passage after P-3 at 25 °C. Cell density was estimated using a hemacytometer. Passage of a cell density of at least 2×10^2 cells per ml or one-third of a monolayer on a flask was required for complete confluency. Cell passage every 7 to 10 d was required to maintain viability of the cells. Consequently, cell culture from representative fish organ cell cultures was subcultured once a week.

Cell cultures from snakehead spleen, kidney, heart, gonad and liver tissues were successfully grown as monolayer cultures in-vitro. Passage numbers of the cells are to date: snakehead gonad (SHG) P-26, snakehead heart (SHH) P-22, snakehead kidney (SHK) P-19, snakehead liver (SHL) P-49, snakehead spleen (SHS) P-76 (Table 2). The snakehead cells were likewise initially a mixture of epithelioid and fibroblastic cells. After several passages these became consistently fibroblastic, multiplied rapidly and became completely confluent within 24 h of passage at 25 °C incubation. One third of flask cell density or 2×10^2 cells per ml, when passed in fresh L15-10 medium, achieved full confluency within 24 h of passage after P-3. Cell passage at 7 to 10 d was required to maintain viability of the cells. Subsequently, cell cultures were passed once each week.

Newly seeded SHS cells grew to complete confluency in 24 h at 25 °C but were killed at temperatures of 5 and 40 °C. At 15 °C, the cells grew slowly and reached only 50% confluency after three to five days incubation. Confluent cells showed tolerance to temperatures of 15, 20, 25, 30 and 37 °C.

Table 1. Primary cell culture development from different organs of catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus*) using four different processing methods, cultured in Leibovitz medium supplemented with 15 % fetal bovine serum and antibiotics and antimycotic incubated at 25 °C.

Fish species	Organs	Methods			
		A	B	C	D
<i>Clarias batrachus</i>	brain	-	-	-	-
	fins	-	-	-	-
	gonad	-	+	+	-
	heart	-	+	+	+
	kidney	-	+	-	-
	liver	-	+	-	+
	skin	-	-	-	-
	spleen	-	+	+	-
<i>Ophicephalus striatus</i>	brain	-	-	-	-
	fins	-	-	-	-
	gonad	-	+	-	+
	heart	-	+	+	+
	kidney	-	+	+	-
	liver	-	+	+	-
	skin	-	-	-	-
	spleen	-	+	+	+

- A explants were placed onto the surface of tissue culture flask to attach for 1 h before adding L15-15 medium with antibiotic/antimycotic
 B explants were inoculated into tissue culture flask already containing L15-15 medium
 C minced organ fragments were pushed through a stainless steel sieve using a syringe plunger into Petri dish with L15-15 medium
 D minced tissue fragments were exposed to 0.5% trypsin-EDTA with agitation for 1 h
 - no cell proliferation detected
 + primary cell proliferation developed

Table 2. Tissue cultures established at 25 °C from various organs of catfish (*Clarias batrachus*) and of snakeheads (*Ophicephalus striatus*).

Fish species	Organ source	Passage number
<i>Clarias batrachus</i>	gonad	56
	heart	51
	kidney	7
	liver	8
	spleen	54
<i>Ophicephalus striatus</i>	gonad	26
	heart	22
	kidney	19
	liver	49
	spleen	74

However, at 15 °C, the cells started to peel off 24 h post-seeding with the monolayer completely peeling off at day 5. At 37 °C, monolayer detachment from the flask surface occurred on day 6. At 20 °C incubation, the cell passage interval could be extended to two weeks. All established cells suspended in FBS with 10% DMSO and stored at -70 °C remained viable for at least a year. Cells suspended in MEM-10 and stocked in liquid nitrogen have remained viable for the past five years.

The CFS, SHS and SHL cells were susceptible to the # 91-97 virus isolate. CPE developed three to five days after viral inoculation. Cells became granular, rounded and eventually detached from the flask surface and lysed.

Discussion

The report of snakehead cells established from the gonad, kidney, heart, liver and spleen is the first report of such cells established from the organs of this fish species. Frerichs et. al., (1986) used snakehead cells derived from fry of snakeheads to isolate a rhabdovirus.

The CFS, SHS and SHL cells developed CPE within three days of exposure to the virus isolate #91-97. The susceptibility of these established cells makes them excellent potential hosts for the isolation, propagation and *in vitro* study of viruses associated with EUS-affected fish.

The establishment of cell lines from the kidney, gonad and gills of catfish has been previously reported (Noga and Hartmann 1981). Cells established from the heart, liver, and spleen of catfish in this study are being reported for the first time.

All of the cell cultures established from catfish and snakeheads were readily trypsinized within 2 min, and multiplied rapidly when seeded in fresh L15-10 medium, becoming confluent in 24 h. Cells can also be adapted to medium with 4% FBS. Although the cells can be cultivated in both L-15 and MEM, it was more practical to use L-15 because of its phosphate buffer and ability to maintain a stable pH. Optimum temperature for SHS cell growth was at 20-25 °C.

Because of their fast growth rate, it became disadvantageous to maintain the cells in the laboratory as passage of the cells need to be performed every two weeks at 20 °C or every seven to ten days at 25 °C. In addition, the adherence of the cells on the surface of cell culture flasks was weak compared to that of other fish cell lines such as EPC (epithelioma papulosum cyprini) (unpublished data).

The cells established in this study were very sensitive to agitation or movement of the medium over the cell culture flasks, causing cells to detach and float in the medium. Hence, transport of these cells is problematic, as viability of the detached cells cannot be ensured. Moreover, cell attachment can be weakened after prolonged incubation at temperatures as low as 15 °C. Alternatively, cells frozen at -70 °C may be transported in dry ice or in liquid nitrogen.

The potential usefulness of the cell cultures established from catfish and snakeheads in this study is not limited to investigations on EUS-associated virus. Since fish viruses generally have a predilection for organs like the spleen, kidney, liver and gonad, availability of these cell cultures allows more choice of cell lines for screening fish epizootics of uncertain viral etiology. In addition, since research on warmwater viral infections of tropical fishes is increasingly important, these cell cultures from catfish and snakeheads may greatly facilitate initial investigations on viral etiology of infections in other fish species.

Lastly, these cell cultures have potential application as bioindicators of aquatic pollutants in toxicology research (Martin-Alguacil et. al., 1991).

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