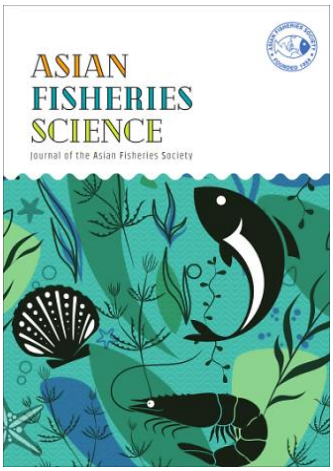

ISSN: 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2019.32.02.006

SHORT COMMUNICATION

Primary Cell Culture from Gills of Striped Bass *Morone saxatilis* (Walbaum, 1792)

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Abstract

Despite the relevance of primary cell culture from the gill tissue, there are no reports on the branchial cell lines of the striped bass *Morone saxatilis* (Walbaum, 1792). The importance of establishing cell lines from *M. saxatilis* branchial tissue represents an alternative to studying infective physiological and biochemical processes, which may facilitate research without compromising valuable live animals. Branchial tissue explants were cultured in 2 × Leibovitz L-15 (pH 7.2 ± 2) medium supplemented with 20 % foetal bovine serum (FBS) and 2 × antibiotic-antimycotic adjusted to 578 mOsm.kg^{-1}. Primary cell cultures obtained showed excellent cell adhesion and proliferation. The cell monolayer consisted of epithelial cells, and the culture was maintained for approximately 30 days. This is the first report describing primary branchial cell line culture from striped bass.

Keywords: isolated cells of fish, marine fish, lobina/lubina, L15 medium, epithelial cells

Introduction

Striped bass *Morone saxatilis* (Walbaum, 1792) production has been increasing at 3-5 million tonnes annually (FAO 2016). Nonetheless, high culture densities represent high risks in intensive aquaculture because suboptimal environmental conditions produce stress and susceptibility to infectious and non-infectious diseases (Assefa and Abunna 2018). The branchial epithelium is easily affected by pathogens and adverse environmental factors that could interfere with oxygen or CO_2 transport and alter ionic, osmotic and acid-base balance when it is in direct contact with the external environment (Speare et al. 1989; Machado et al. 2003; Mitchell et al. 2011). The possibility of having primary cell culture facilitates performing studies focused on knowledge in depth of the mechanisms of these functions with greater speed and simplicity (Avella et al. 1994).

In Mexico, bass farming is a recent development in the northwest zone of the country off the coasts of Ensenada, Baja California (B.C.), Mexico with an annual average production of 10,000 tons. A local company has an inland hatchery that provides fry. Nonetheless, *M. saxatilis* is a susceptible species to the *Pagrus major* red sea bream iridovirus (RSIVD), and the viral haemorrhagic septicaemia virus (VHSV) discovered first in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Olesen and Jorgensen 1992; Lorenzen et al. 1999). *In vitro* cell culture would allow isolating and characterising pathogens for research on the infective process at molecular levels, the development of diagnostic procedures and vaccines.

Alternatively, cell culture can be useful for studies on environmental toxicological effects and understanding physiological responses when facing natural variables, such as temperature, salinity, pH, among others, which could help to reveal various processes of membrane transport to acid-base balance regulation and metabolite excretion (Sobhana et al. 2009; Ma et al. 2017). Hence, it would be possible to study the animal response to the pathogen using cell lines without having to sacrifice expensive breeders. Therefore, the objective of this study was to develop branchial cell line of *M. saxatilis* in L-15 medium supplemented with FBS at 20 %.

Materials and Methods

Biological material

Samples of adult striped bass *M. saxatilis* (56 ± 20 g) were provided by the Marine Fish Culture Unit of

Facultad de Ciencias Marinas (FCM, School of Marine Sciences) of Universidad Autónoma de Baja California (UABC); fry were obtained from the Pacifico Aquaculture (Ensenada, B.C., Mexico). The fish were maintained in 5000 L tank with seawater with constant aeration and fed to satiety two times a day (EWOS, Cargill, CAN).

Primary cell culture of gills

Fish (n = 3), were transferred in 10-L tanks with seawater to the Molecular Ecology Laboratory of FCM-UABC and were anaesthetised with 2 phenoxy-ethanol (2 $mg.L^{-1}$) for 5 min, placed in an ice-bed and rapidly decapitated. The operculum was cut, and gill arches were dissected completely with lamellae. Animals were handled according to UABC bioethical guidelines. Branchial arches were disinfected by immersion in ethanol at 70 % prepared with seawater (35 $g.L^{-1}$) for 2 min. The tissue was rinsed for 2 mins with sterile seawater and for about 3 h at 4 °C in seawater with antibiotic-antimycotic 100 × (Biowest) (10,000 $units.mL^{-1}$ of penicillin G, 10 $mg.mL^{-1}$ of streptomycin sulphate and 25 µg-mL of amphotericin B).

The disinfected explant (1 cm^3 pieces of branchial lamellae), was transferred into Petri dish of cell culture with a growth surface of 22.1 cm^2 TPP® (Merck KGaA, Darmstadt) tissue culture dish, adding 1 mL of medium (2 ×) Leibovitz L-15 (Sigma-Aldrich) (pH 7.2 ± 2) supplemented with 20 % of FBS and (2 ×) antibiotic-antimycotic with osmolarity of 578 $mOsm.kg^{-1}$. Cultures were maintained at 32 °C in an incubator (IC400, Yamato Scientific, America) and examined with an inverted microscope Axio Observer A1 (Zeiss, Darmstadt) every other day, the period in which one-third of the medium was changed for a new one under sterile conditions.

Subculture

When confluency was reached, the cellular monolayer was dissociated from the plate with a cell scraper, and 1 mL of fresh growth medium (2 ×) L-15, 20 % of FBS and (2 ×) antibiotic-antimycotic were added. One-third of the medium was changed every other day. The subculture was performed in a 24-well TPP tissue culture test plate with a growth surface of 1.8 cm^2 and incubated at 32 °C.

Results and Discussion

The branchial tissue cells adhered to the Petri plate surface in 48 to 72 h; round-shaped cells (Fig. 1A) and epithelioid flat-shaped (Fig. 1B) cell migration was detected. A cellular monolayer was formed in the periphery, which lasted surrounding the explant with a high cellular density for one week (Fig. 2). Two weeks later the cells initiated migration in colonies on the plate surface (Fig. 2). Average cell length was from 8.5 ± 1.5 µm and nucleus size from 5.5 ± 0.5 µm. Cell growth from the explant lasted 30 days to reach monolayer confluency of 90 % mainly consisting polygonal shape epithelial cells (Fig. 3). The subculture should be performed immediately starting from this stage because cells become necrotic and start damaging the plasmatic membrane, causing the monolayer to detach.

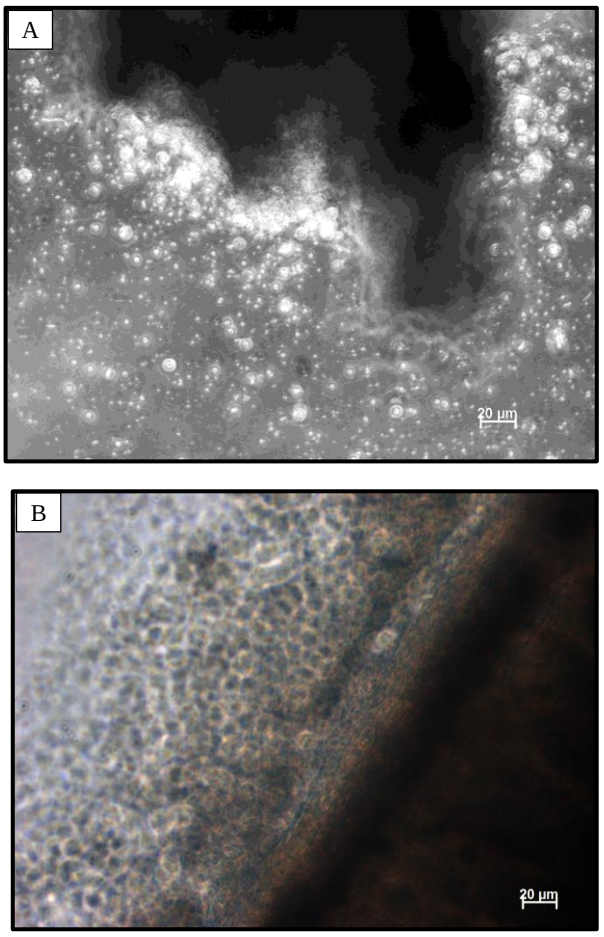

Fig. 1. Round and epithelial cells spreading from *Morone saxatilis* gill explants (48–72 h culture). A) round cells; B) epithelial cells.

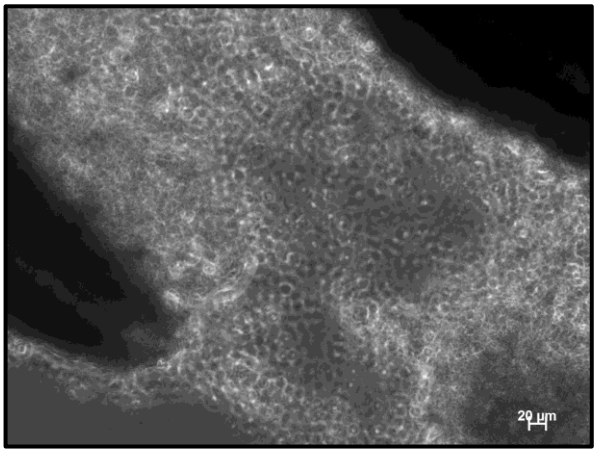

Fig. 2. A branchial monolayer of *Morone saxatilis* forming and surrounding the explants (2-week culture).

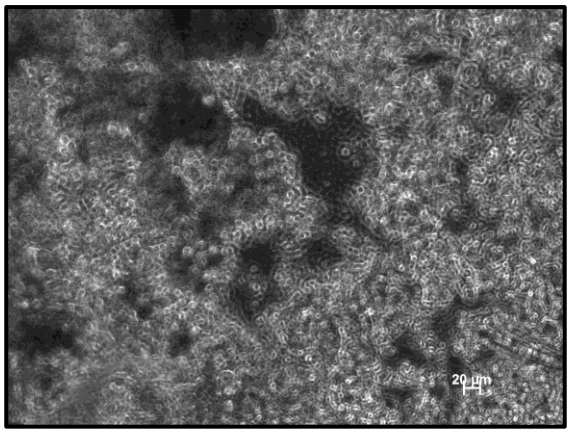

Fig. 3. Confluent monolayer (90 %) of epithelioid cells from *Morone saxatilis* gills (one-month culture).

Subculture

When confluency reached 90 %, the primary culture was sub-cultured (first stage). Few cells survived and recovered rapidly from the scraping. During the first week, these sub-cultured cells maintained a globular shape (Fig. 4A). Subsequently, a monolayer was formed mainly from the epithelial cells that adhered to the plate surface and proliferated (Fig. 4B). The final confluence of the first stage was 90 % (Fig. 5) in approximately 3 weeks and further subcultures were not performed.

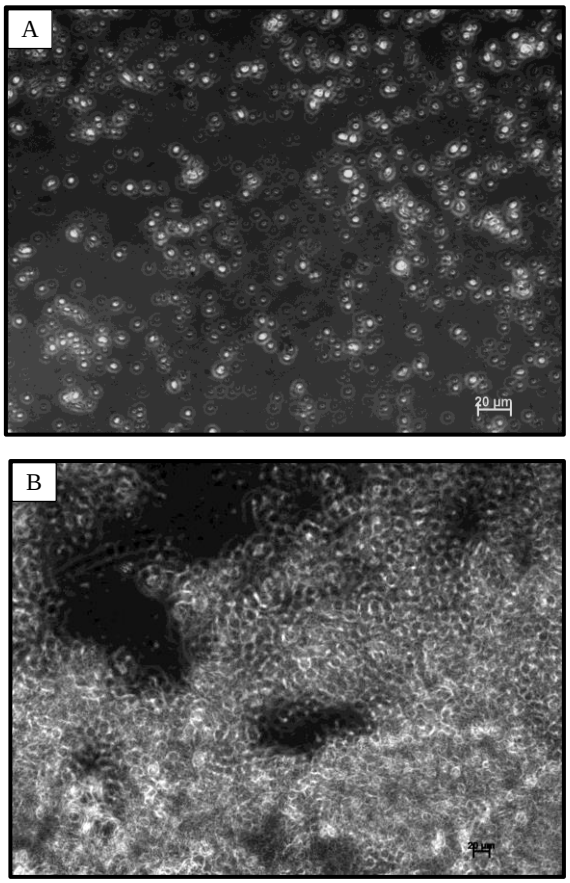

Fig. 4. First (subculture) growth stage, round shape cells and monolayer of epithelioid cells from *Morone saxatilis* gills. A) round cells; B) monolayer of epithelioid cells.

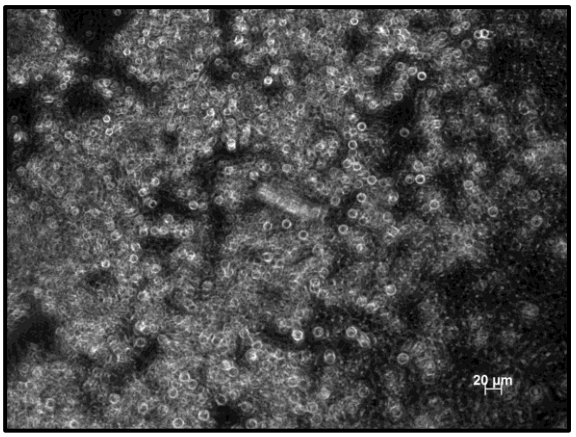

Fig. 5. First (subculture) stage, growth 90 % confluency monolayer of epithelioid cells from *Morone saxatilis*.

In some fish, as the European bass *Dicentrarchus labrax* (Linnaeus, 1758), the primary branchial cell culture showed polygonal epithelioid cells with thin morphology and with a size range of 10 to 50 µm (Avella et al. 1994, 1997). Comparatively, the cells of the striped bass *M. saxatilis* showed similar characteristics. Since 2008, several researchers have reported obtaining approximately 12 branchial cellular fish lines; five of them had epithelial morphology (Lee et al. 2009): common carp *Cyprinus carpio* (Linnaeus, 1758), (Ku and Chen 1992); rainbow trout *Oncorhynchus mykiss* (Bols and Lee 1994); halibut *Paralichthys olivaceus* (Temminck and Schlegel, 1846) (Tong et al. 1997); and Atlantic salmon *Salmo salar* (Linnaeus, 1758), (Butler and Nowak 2004). In the case of the Malabar grouper *Epinephelus malabaricus* (Bloch and Schneider, 1801), both epithelial morphology and fibroblast cells coexisted; nonetheless, while progressing in stages, epithelial cells predominated (Sobhana et al. 2009).

Fish gills usually consist of six cell types; 90 % representing paving or epithelial cells (PVCs) and they are rich in H^{+-}ATPase (proton bomb), and their main function is acid-base transport (Avella and Ehrenfeld 1997; Wilson and Laurent 2002). The tri-dimensional structure of the primary branchial cell culture of *Dicentrarchus labrax* revealed by scanning electron microscopy (SEM) showed the nucleus in a central position surrounded by numerous vesicles that might correspond to the Golgi apparatus and an invagination system (Avella et al. 1994, 1997). These morphological characteristics of cells in the present study were similar to those of the paving cells described in other teleost fish (Avella et al. 1994 and 1997; Wilson and Laurent 2002). To distinguish the cell types, an analysis with cellular morphology techniques, such as indirect/direct immunofluorescence and/or SEM should be undertaken.

Cell culture performed with the explant technique is very useful to understand cell physiological and biochemical processes (Avella et al. 1994; Fernandes et al. 1995); it has the advantage of not involving the use of enzymes or mechanical procedures that harm

cells or eliminate surface proteins that are important for membrane transport. With this method, growth factors and specific tissue hormones of the neighbouring cells are maintained in the culture medium (Avella et al. 1994), favouring good development on the plate. In the primary culture of branchial cells of *D. labrax* and *Oncorhynchus mykiss*, the original branchial epithelium physiology was achieved with the explant method (Avella et al. 1994; Fernandes et al. 1995). While comparing the enzymatic method and that of the primary explants with different organs of the carp *Cirrhinus mrigala* (Hamilton, 1822), the explant was observed to have better adherence and cellular growth (Nanda et al. 2014).

In fish cell culture, different growth media such as Leibovitz L-15, 199 (M-199) medium, Eagle (MEM) minimal essential medium, among others have been assessed, of which L-15 at a pH of 7.4 was reported to be the most appropriate for good growth and cellular adherence (Lakra et al. 2005, 2006; Sobhana et al. 2009; Sahul Hameed et al. 2006; Parameswaran et al. 2006). L-15 provides essential nutrients for seabass cellular development.

Currently, only 31 cell lines of fish have been established compared to more than 3400 mammal cellular lines that have been deposited in the American Type Culture Collection (ATCC) (Lee et al. 2009) and reported in other sources (Parameswaran et al. 2006; Buonocore et al. 2006; Chaudhary et al. 2013; Abdul Majeed et al. 2014; Vo et al. 2015). This situation is likely because primary cell culture is a tedious process as the cells are difficult to isolate, maintain and replicate. Nevertheless, it is important to achieve the development of primary cell cultures of different tissues because their study provides important information on their morphology, physiology and cellular interaction.

This is the first report on the successful culture of primary cell lines from the seabass *Morone saxatilis*, grown from branchial explants in L-15 (pH 7.4) medium supplemented with FBS at 20 % and (2 ×) antibiotic-antimycotic at an osmolarity of 578 mOsm.kg^{-1}. The cellular monolayer seemed to be formed by epithelial cells, but subsequent studies should allow improving cellular proliferation and characterising the explant subtype or subtypes in development.

Acknowledgements

SVH is grateful to CONACYT (National Council for Science and Technology in Mexico) for the post-doctoral scholarship granted for the period 2016-2017 and to RD Cadena-Nava for his disposition in this research. The authors are grateful for the facilities offered by Pacifico Aquaculture S.A. de C.V. and to MA Galaviz-Espinoza from FCM-UABC for providing fish for the study and to O. Alvarado-Muñoz from the Molecular Ecology Laboratory for technical support. We are also thankful for the support provided by the staff of the IIO-UABC Macroalga Laboratory (L. Arual-Rangel, S. Revilla-Lovano, A. Gálvez-Téllez, JM Sandoval-Gil, V. Macias-Carranza) for providing access to the Microscopy Platform; and to D. Fischer for translation and editorial services.

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