Establishment of Tissue Culture Cell Lines from Reptiles:

a universal protocol for terrestrial species

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Abstract

Tissue culture cell lines are a critical reagent for the isolation and characterization of obligate intracellular pathogens. While a wide variety of cell lines are available from humans, domesticated mammals, and fish, commercially available cell lines from wildlife species, particularly reptiles, are extremely limited. Continuously dividing cell lines were derived from primary tissues collected from reptiles at the time of necropsy, portions of surgical biopsies, or from embryonic tissues. Primary cell growth was routinely observed from a variety of tissues, including heart, lung, spleen, kidney, skeletal muscle, liver, and gonad. Continued selection for stable, dividing cells of homogenous morphology was achieved through passage in growth conditions determined optimal. Aliquots of cell lines were routinely archived in liquid nitrogen to create low passage stocks. After 10-12 serial passages characterized by stable, predictable growth of cells representing a single cellular morphology, the cell line was considered established and aliquots of working stock cells were archived in liquid nitrogen. For lines characterized by mixed morphology cells, continued passage for selection of single morphology populations was continued. Final cell line characterization included confirmation of host origin by sequencing of the cytochrome oxidase 1 gene, and immunohistochemical characterization. Cell lines were successfully established from various tissues from a variety of reptile species, including snakes (n=8), turtles/tortoises (n=5), crocodilians (n=2), and lizards (n=1). Cell lines were cataloged in a publicly accessible virtual database to permit and promote the dissemination of these reagents to improve disease diagnostics and characterization of captive and free-ranging wildlife species.

Keywords: primary cell culture, cell line, intracellular pathogen, cryopreservation, reptile

Introduction

Tissue culture cell lines are routinely used in human and animal biomedical and virology research. They can assist in understanding the effects of infectious pathogens [1]. Cell lines can also help identify novel infectious agents [2]. Lastly, generating cell lines from tissues can preserve genetic information of endangered animals. This has been demonstrated in a study that established primary fibroblast cultures from tissue of a hawksbill sea turtle (*Eretmochelys imbricata*), which may ultimately contribute to the conservation of this endangered species [3].

Specific cell lines are chosen for their availability, rapid growth rate, and suitability for virus isolation [4]. Human, mammal, and fish cell lines are available to purchase from American Type Culture Collection (ATCC), the largest commercial source of cell lines. There are currently over 11,000 known reptile species, and only four reptile cell lines available to purchase [5]. The only reptile cell lines available from ATCC are heart and spleen cells from the Russell's viper (*Daboia russelii),* heart cells from the iguana (*Iguana iguana),* and heart cells from the common box turtle (*Terrapene carolina)*.

Lack of reagents has been an obstacle to the use of cell lines in reptile research. Without cell lines, reptile viral studies cannot be done with the speed and controlled conditions of cell line research. Using live animals is more time-consuming, more expensive, involves more permits, and removes an individual from already declining populations. Of the 8,493 species monitored by IUCN, 1458 (17%) of these are listed as Vulnerable, Endangered, or Critically Endangered [6]. For this reason, some scientists have created cell lines for use in their own research and are found in the primary literature. Snake cell lines have been created from Western Plains garter snakes (*Thamnophis radix haydenii*), jararaca (*Bothrops jararaca*), and the South American

rattlesnake (*Crotalus durissus terrificus*) [7-9]. Lizard cells lines have been created from five species of Australian dragon lizards (*Tympanocryptis punguicolla, Tympanocryptis* sp., *Ctenophorus fordi, Amphibolurus norrisi,* and *Pogona vitticeps*) and rough-tailed geckos (*Cyrtopodion scabrum*)[10, 11]. The order with the most cell lines generated is the chelonians, but most are aquatic species. Cell lines include the Chinese soft-shell turtle (*Pelodiscus sinensis*), green sea turtle (*Chelonia mydas*), loggerhead sea turtle (*Caretta caretta*), hawksbill sea turtle (*Eretmochelys imbricate*), and the commercially available common box turtle (*Terrapene carolina*) [3, 12-20]. Three cell lines have been created from the Chinese alligator (*Alligator sinensis*), including liver, heart, and muscle cell lines [21].

Unlike mammal cell culture, there is no globally accepted protocol for reptile cell culture, contributing to their lack of production. Wolf published an introduction to poikilothermic cell culture that included the known temperature ranges and media requirements by animal type, but many were, and are still unknown [22]. This paper describes a novel protocol that enabled generation of cell lines from the major families of snakes present in the United States including colubrids, boids, pythons, and viperids. The same protocol was utilized to establish cell lines from chelonians, lizards, and crocodilians. Cell lines were cryopreserved and catalogued in a biobank, which is an emerging conservation tool in a time when 20% of all assessed species are threatened. [23, 24]

This novel protocol has been effective in establishing cell lines from a wide range of reptiles. Host specific reagents are not available commercially for research on most wildlife species, especially herpetofauna. Utilizing this protocol will enable researchers to develop their own reagents as needed. This study has increased the diversity of reptile cell lines, and will

contribute to research of infectious diseases and conservation of genetic information from threatened and endangered species including *G. Polyphemus* and *A. sinensis.*

Methods

Tissue Sample Collection

Tissues were opportunistically collected from sixteen reptile species at University of Florida College of Veterinary Medicine department of Zoological Medicine. The samples were from corn snake (*Pantherophis guttatus),* smooth green snake (*Opheodrys vernalis*), boa constrictor (*Boa constrictor*), Burmese python (*Python bivittatus*). amethystine python (*Morelia amethistina*), diamond python (*Morelia spilota spilota),* pygmy rattlesnake (*Sistrurus miliarius*), Venezuelan rattlesnake (*Crotalus durissus*), brown anole (*Anolis sagrei*), Florida softshell turtle (*Apalone ferox*), yellow-bellied slider (*Trachemys scripta scripta*), diamondback terrapin (*Malaclemys terrapin*), Indian star tortoise (*Geochelone elegans*), gopher tortoise (*Gopherus polyphemus*), saltwater crocodile (*Crocodylus porosus*), and Chinese alligator (*Alligator sinensis*). Tissue samples were obtained at the time of necropsy from animals recently euthanized or from surgical biopsy samples. Tissues were taken from organs that showed no signs of disease or decay, including heart, lungs, spleen, kidney, liver, brain, urinary bladder, skeletal muscle, thymus, ovaries and testes. Samples were collected within one hour of euthanasia and within 2 hours from biopsy collection. Tissues from the Indian star tortoise (*G. elegans)* were collected from an individual whose time of death was unknown. Samples were collected in hopes that the tissue was still viable. All tissues were unfixed. All animals that were euthanized were due to quality of life concerns. Collection of samples from all animals were performed in accordance with the University of Florida Institutional Animal Care and Use Committee.

Target tissue types included heart, lung, kidney, liver, spleen, skeletal muscle, gonadal tissue, and thymus. One sample was taken from each organ. Sterilely collected tissue samples of approximately 2 mm^3 were placed into vials containing 3 mL of cold (4˚C) sterile phosphate buffered saline (sPBS; Corning). Primary cell culture was performed within 60 minutes of initial tissue collection. Vials were placed on ice during the transition period.

Primary Tissue Cell Culture

Tissue samples were transferred to individual wells of a six-well tissue culture plate (Corning). Each sample was washed three times with ice cold sPBS and suspended in 1 mL of completed medium (cMEM) (i.e., Earle's Minimal Essential Medium (EMEM; Corning) with 10% fetal bovine serum (FBS; Gibco), 1% non-essential amino acids (NEAA; Corning), 1% penicillin-streptomycin (Corning), 0.1% gentamicin (Gibco), and 0.2% amphotericin B (Gibco)). Tissues were mechanically minced with sterile scalpel blades until pieces were no larger than 0.5 mm in size. Minced tissues were equally divided into six wells of a new tissue culture plate containing 1 mL cMEM in each well or transferred directly into $T_{12.5}$ flasks containing 2.5 mL of cMEM. The plates and flasks were maintained in a 32˚C incubator with 5% CO_2. Samples were checked for contamination, tissue adhesion, and cell growth twice per week. If tissues didn't adhere, medium was aspirated, leaving only enough to just cover the tissue. Medium was replaced after adhesion. After two weeks, medium was changed weekly until first passage.

Subculture of Primary Cells

The first passage was completed when 70-80% confluency was reached, or when cell growth around adhered tissue was overly dense and causing apoptosis of cells. If wells of the same tissue type contained different morphologies, they were kept in separate flasks to achieve

different types of cell lines. The first passage was completed enzymatically using accutase (Gibco). Cells were washed twice with 2 mL sPBS. 0.4 mL accutase was added to each well and incubated at 32˚C until the cells released. Incubation time ranged from three to five minutes. Once cells released, 1 mL cMEM was added to each well. The well contents were mixed by pipetting up and down three times using a 1000 µL micropipette. Once mixed, cells were passed up to a T_{25} tissue culture flask for wells that were ≥ 70% confluent, or they remained in their existing well for samples that were growing too densely and needed cells redistributed.

Subsequent passages were completed using 0.25% trypsin (Gibco) once flasks achieved ≥ 85% confluency. The total volume applied was dependent on vessel size. (0.4 mL per well of a six-well plate or $T_{12.5}$, 0.5 mL per T_{25}, 1.0 mL per T_{75}, and 1.5 mL per T_{150}.) Cells were routinely split to keep the passage rate at once per week. The total volume (cells, Trypsin, and cMEM) of each flask was 2.5 mL in a well or $T_{12.5}$, 5 mL per T_{25}, 12 mL per T_{75}, and 18 mL per T_{150}. All flasks were labelled with species, tissue type, passage number, and the date of every passage or media change.

Flasks were examined twice per week. Morphology, confluency, and presence or absence of dead cells were noted, and photographs were taken to document cell appearance over time. Flasks that had dense growth but were not forming a confluent monolayer were treated with 0.25% trypsin to redistribute the cells. Once cells released, fresh medium was added and the flask was replaced in the incubator without removing any cells. Flasks that had not reached ≥ 85% confluency had their medium changed once per week. Cells were maintained in a humidified incubator at 32˚C with 5% CO_2.

Freezing

Aliquots were frozen once cells were passaged up to a T_{75} size flask (P3 or P4). Cells were released as previously described and transferred to a 15 mL conical tube. Cells were gently pelleted (1700 RPM for 7.5 minutes at 4˚C) and resuspended in a 1 mL freezing solution that consisted of cMEM with an additional 10% FBS and 10% dimethyl sulfoxide (DMSO; BioPlus). Total contents were transferred to a 2 mL cryogenic vial and placed in an isopropanol chamber at -80°C for approximately 24 hours. Aliquots were then transferred to liquid nitrogen (-196˚ C) for long term storage. Additional aliquots were frozen every four to five passages.

Confirmation of Species

For each sample, cells were enzymatically released from the flask, and 1 mL of cells suspended in cMEM was transferred to a microcentrifuge tube. The tube was spun for 5 minutes to create a pellet. DNA was extracted from the pellet using a DNA extraction kit (Zymo). The DNA samples were then submitted to the Zoological Medicine lab for sequencing.

Characterization via Immunohistochemistry

A confluent T_{75} was needed for each sample. Each flask was rinsed twice with 4 mL sPBS. Five mL sPBS was added and cells were released using a cell scraper. The total volume was transferred to a 15 mL conical tube and centrifuged at 1700 RPM for 7.5 minutes. The sPBS was aspirated and the cell pellet was resuspended in 2 mL 10% formalin and kept at 4˚C for 24 hours. The tube was spun again and formalin was aspirated. The cell pellet was resuspended in 200 µL of warmed Histogel (Thermo Scientific) and pipetted onto parafilm to set. Once set, the Histogel and cells were set in a 6-well cartridge and submitted to the Histology lab for characterization.

Results

Twenty-eight novel cell lines were established from sixteen species of reptile and six different tissues of origin. Fourteen cell lines were created eight snake species that included colubrids (*P. guttatus, O. vernalis),* constrictors (*B. constrictor, P. bivittatus, M. amethistina, M. spilota spilota),* and vipers (*S. miliarius, C. durissus).* Two lizard cell lines were established from the brown anole (*A. sagrei.)* Eight cell lines were established from five chelonian species (*A, ferox, T. scripta scripta, M. terrapin, G. elegans, G. Polyphemus*). Four cell lines were created from two crocodilian species (*C. porosus, A. sinensis).* All cell lines were created using the same materials, methods, and growth conditions.

Initial cell growth was most often seen associated and growing out from adhered tissue clumps. Growth in this manner would not result in confluence without cell death, therefore cells were passed for the first time before they reached 70% confluency. In rarer cases, cells initially adhered throughout the growth surface independent of tissue. These cells were able to become more confluent before their first passage.

Cell lines were characterized by consistent growth through at least twelve passages. Early growth was often a mixed population of cells, including cells that had a fibroblast or epithelial appearance (Figure 1). Selective trypsinization was attempted on flasks that had a large population of epithelial-like cells. Repeated passages with short trypsinization times of 60 – 120 seconds and discarding the cells that dissociated resulted in a higher number of epithelial-like cells. This method successfully produced two distinct cell lines from the kidney tissue of the corn snake (*P. guttatus*) (Figure 2). Later passages of the trypsin resistant (TR) kidney cell line changed morphology and appeared similar to the original kidney cell line. Whenever possible, attempts were made to select for epithelial-like and myocardial-like cells. However, most cell lines produced have a fibroblast appearance (Figure 3).

Species of origin for each cell line was confirmed by sequencing the COI gene. An attempt was made to identify cells as epithelial, mesenchymal, or myocardial cells via immunohistochemistry. Slides were stained for cytokeratin to identify epithelial cells, vimentin for mesenchymal cells, and muscle-specific actin for myocardial cells (Figure 4).

Discussion

This study has shown that cells can successfully be propagated from crocodilians, chelonians, and squamates utilizing the same optimized growth conditions. Growth conditions include maintaining cells in a complete growth medium (Earle's Minimal Essential Medium (EMEM) completed with fetal bovine serum (FBS), nonessential amino acids (NEAA), penicillin-streptomycin, gentamicin, and amphotericin B) in an incubator at 32˚C with 5% CO_2.

The number of cell lines produced from each order of reptile largely depended on availability of tissue samples. Snake samples were the most readily available and produced the greatest number of cell lines. Crocodilian and chelonian samples were rarely available, but at least one cell line was successfully propagated from each individual that was necropsied. Lizard samples were infrequently available, and cell lines were only generated from one species (*A. sagrei*).

There were trends noted within cell lines of each order. Tissue samples from snakes, in the absence of bacterial contamination, would readily grow cells from every tissue type attempted. Heart, lung, kidney, and spleen were the most successful for continuous passage and produced most of the snake cell lines. Growth from liver and pancreas tissues would oftentimes look very promising, but rarely survived beyond passage 3, and no liver or pancreas cell lines

were produced. Two attempts were made to grow brain cells but neither attempt survived beyond passage 2. Due to the labor involved to retrieve brain tissue, no further attempts were made. A unique observation was heart tissue from the corn snake (*P. guttatus)* continued to contract 60 days post-mortem and cells growing out from the tissue also contracted. This phenomenon was only witnessed in snake heart samples.

Many crocodilian cells displayed a unique morphology with a bubbly appearance. In non-crocodilian species, this could indicate the cells are close to senescence. Crocodilian cells maintained the same morphology throughout their lifespan, and their appearance did not affect their growth.

Chelonian cells grew readily and were very resilient. The spleen cell line from the Indian star tortoise (*G. elegans)* was the only line to grow from a specimen found deceased and the necropsy may have been performed up to 18 hours post-mortem. The diamondback terrapin (*M. terrapin)* cell lines grew quickly and densely. Early passages were done at a split ratio of 1:1, but that was done several times per week. The ratio was gradually increased to a final split ratio of 1:7 every five days. Even if passed too thin, the cells rapidly rebounded and became confluent within 8 – 10 days.

Lizard cell lines were more difficult to propagate than snakes. Fewer tissues were available than snakes, and initial growth was not as rapid or prevalent. The individual *A. sagrei* that was necropsied to propagate the kidney and lung cell lines was infected with both bacteria and flagellates. Initial cell growth was very promising, and flasks were treated with

both metranidozole (50 µg/mL) to eliminate the flagellates and vancomycin (20µg/mL) for the bacteria. Both treatments were successful after a single dose and the cells were cleared of contamination and continued to proliferate.

Immunohistochemical characterization was attempted to identify the origin of cells. Slides were stained for cytokeratin to identify epithelial cells, vimentin for mesenchymal cells, and muscle-specific actin for myocardial cells. Of the 28 cell lines, only five (18%) had the IHC results that were expected (Figure 3). Nine cell lines (32%) had mixed results, with most stained positive for the expected antibody plus an unexpected antibody. Fourteen cell lines (50%) had negative results for the expected antibody. The antibodies used were chosen for their sensitivity in mammalian species. These unexpected results indicate they are not as efficacious in reptiles. All cell lines stained positive for at least one of the antibodies, but reptiles may express the target antigens in multiple cell types.

A novel protocol was established for propagation of reptile cell lines from a diverse sampling of species. This protocol was successful in creating 28 new cell lines from 16 reptile species. Samples have been cryopreserved in a biobank for long term storage of DNA, potential collaboration, and future research needs.

Work is ongoing and more cell lines are in production to expand the diversity of the collection. Future opportunities exist in establishing protocols for other underrepresented species, attempting new collection methods including tail and toenail clippings from lizards, and

investigation into the proteins expressed in reptiles to improve characterization via immunohistochemistry.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was funded by a grant provided by Morris Animal Foundation.

Acknowledgments

Special thanks to the zoological medicine department and the clinical science research laboratory at University of Florida.

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