

Establishment, characterization, and viral susceptibility of two cell lines derived from goldfish *Carassius auratus* muscle and swim bladder

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ABSTRACT: Goldfish *Carassius auratus* are common aquarium fish and have a significant economic and research value, having considerable worth to fisheries as a baitfish and the ability to adapt to a range of habitats. Two cell lines were established from goldfish muscle and swim bladder tissue, in order to create a biological monitoring tool for viral diseases. Cell lines were optimally maintained at 30°C in Leibovitz-15 medium supplemented with 20% fetal bovine serum. Propagation of goldfish cells was serum dependent, with a low plating efficiency (>16%). Karyotyping analysis indicated that both cell lines remained diploid, with a mean chromosomal count of 104. Results of viral challenge assays revealed that both cell lines shared similar patterns of viral susceptibility and production to infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, snakehead rhabdovirus, and spring viremia carp virus. Both cell lines demonstrated a higher sensitivity and significantly larger viral production than control brown bullhead cells for channel catfish virus. These newly established cell lines will be used as a diagnostic tool for viral diseases in this fish species and also for the isolation and study of goldfish viruses in the future.

KEY WORDS: Cell lines · *Carassius auratus* · Goldfish · Viral susceptibility · Fish

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INTRODUCTION

The common goldfish *Carassius auratus*, originating from eastern Asia, has spread to inhabit much of the world's freshwater aquatic systems. They are economically, scientifically, and ecologically important, serving as a recreational aquarium fish and, more importantly, as a baitfish for larger carnivorous ornamental fish and invertebrates. Baitfish, including goldfish, account for a US \$4.4 million net worth market annually (USDA 1998, 2006), and, as such, this species has attracted interest for improved aquaculture techniques and research applications (Anderson 1975).

Goldfish are in some ways the aquatic equivalent of the laboratory rat, mainly due to their size and abundance. They have been used in studies of physiological development, toxicological implications of water quality, and pathogenesis (Weigand 1995, He et al. 1997, Talaat et al. 1998, Altinok 2004, Yue & Orban 2004). The introduction of goldfish into stagnant bodies of water has been implemented in some countries as an ecological control for mosquitos (Gerberich 1946). Having such worldwide exposure has increased the possibility of diseases and pathogens in goldfish (Shea 1983, He et al. 1997). Several viruses cause disease in goldfish, including goldfish Virus Type 1 (GFV 1)

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(Berry et al. 1983), goldfish Virus Type 2 (GFV 2) (Shea 1983, Shea & Berry 1984), and herpes-type viruses (Jung & Miyazaki 1995). Viruses are known intracellular pathogens, and the establishment of permissive cell lines from host animals is essential for the isolation, identification, and study of pathogenic viruses. Studies on goldfish viruses, however, have been limited due to a lack of sensitive cell culture systems. Most attempts to establish cell lines from goldfish have been limited to descriptions of primary cultures (Wang et al. 1995, Akimoto et al. 2000, Li & Fukuda 2003, Kondo & Watabe 2004). Currently, there is only 1 cell line commercially available, which was derived from goldfish fin (ATCC No. CCL-71).

The focus of the present study was the establishment and characterization of 2 new cell lines derived from goldfish tissues: muscle (GFM) and swim bladder (GFSB). The subcultured cells were evaluated for optimal growth conditions, as well as their stability in liquid nitrogen, karyologic typing, and piscine viral susceptibility. These newly established goldfish cell lines will enhance current attempts in establishing effective diagnostic methods for detecting and monitoring viral infection in this important aquatic species.

MATERIALS AND METHODS

Primary cultures. Primary cell cultures were initiated by aseptically collecting muscle and swim bladder tissue from 4 juvenile goldfish *Carassius auratus* (body weight: 35 to 45 g, body length: 14.5 to 17 cm). Similar tissues were pooled and transferred to an antibiotic-incubation medium (AIM) containing $1 \times$ Leibovitz-15 medium (L-15) (Sigma-Aldrich), 1000 U streptomycin ml^{-1} , 1000 μg penicillin ml^{-1} , 25 μg amphotericin B ml^{-1} and 250 μg gentamicin ml^{-1} . Tissue samples were minced with sterile dissecting blades and scissors following a 2 h incubation at room temperature and washes with AIM. Approximately 25 tissue fragments (1 to 2 mm^3) were individually explanted into 25 cm^2 Primaria tissue culture flasks (Falcon Primaria, Fisher Scientific) containing AIM. After allowing the tissue to attach for 4 h at room temperature, liquid from the flasks was aspirated and 5 ml of growth medium was added to each flask.

Primary cell cultures were initially maintained in air at 25°C in L-15 medium supplemented with 20% heat-inactivated defined fetal bovine serum (FBS), 1% antibiotics (100 μg penicillin ml^{-1} , 100 U streptomycin ml^{-1}), and 0.05% growth factors (25 ng epidermal growth factor ml^{-1} and 25 ng fibroblast growth factor ml^{-1}) (Sigma). This method was adapted accordingly from previous cell line establishment efforts (Lu et al. 1999, Wang et al. 2003, Zhao et al. 2003, Zhao & Lu

2006). Each cell line was originally seeded in 25 cm^2 tissue culture flasks and later passed into 75 cm^2 tissue culture flasks for cultivation (Corning Life Sciences). Muscle and swim bladder cells were subcultivated after approximately 4 to 5 d, at a 1:3 ratio, using a 0.25% trypsin-versene solution (Sigma). After several generations, the standard FBS was reduced in concentration to 10%, and growth factors were no longer added to the media.

Growth studies. Growth characteristics of the cell lines were assessed at selected temperatures, FBS concentrations, and with different growth media. Growth rates at 5 different incubation temperatures were compared: 15, 20, 25, 30, and 37°C over 12 d. A seeding concentration of 1×10^5 to 2×10^5 cells at Passage 15 or a later passage was used in 25 cm^2 tissue culture flasks. On alternate days, 2 flasks from each temperature were trypsinized, and 8 counts (4 flask $^{-1}$) were performed using a hemocytometer. Analogous procedures were performed for the effects of various concentrations of FBS (0, 5, 10, 20%) and the different cell culture media (L-15, RPMI 1640, Minimum Essential Medium and Medium 199) on cell growth over 10 d.

Cell-plating efficiency. Cell lines in passages >15 were used to determine the plating efficiencies of GFM and GFSB. Cell densities of 200, 500, and 1000 cells flask $^{-1}$ were seeded in duplicate in 25 cm^2 tissue culture flasks at 25°C in L-15 medium with 10% FBS. After 12 d, the medium was discarded and the cells were fixed with 5 ml of crystal violet–formalin stain–fixative for 15 min, rinsed with tap water, and air-dried. The stained colonies were then counted under the microscope, and plating efficiency was calculated as described by Freshney (1994).

Cryopreservation of cells. The ability of goldfish cells to be preserved in liquid nitrogen (N_2) and their stability were assessed in freezing medium using previously described methods (Freshney 1994). Cells growing logarithmically for GFM and GFSB lines were harvested and resuspended at cell densities of 3×10^6 to 4×10^6 cells ml^{-1} . Cell suspensions were carefully mixed with an equal volume of L-15 freezing medium consisting of 20% dimethyl sulfoxide (DMSO) and 20% FBS, resulting in a final concentration of 10% DMSO and FBS. Aliquots (1.0 ml) were dispensed into 1.5 ml sterile plastic vials (Nalge Nunc) and sealed. Cryogenic controlled-rate freezing containers were used to slowly bring the vial temperatures to -80°C for 48 h before being transferred to liquid nitrogen. After a storage period of 380 d, vials containing the cells were thawed at room temperature (22°C) and viable cells were counted by trypan blue exclusion (Wang et al. 2003). After counting, cells were reseeded in 25 cm^2 tissue culture flasks, and subsequently cell growth and morphology were observed.

Karyotyping of cells. Chromosomal counts were performed on Passage 9 for GFM and Passage 11 for GFSB. Cells were seeded in duplicate 75 cm² tissue culture flasks in L-15 medium with 20% FBS. After a 24 h incubation, spent medium was replaced with 10 ml of fresh medium containing 0.1 ml colcemid solution (1 µg ml⁻¹), and the cultures were allowed to incubate for 15 h before being harvested and prepared for fixation (Earley 1975). After fixation to microscope slides, phase-contrast microscopy of >100 random metaphase spreads was used for each chromosomal count.

Ribosomal RNA (rRNA) analysis. DNA extractions from 6.0×10^7 cells for GFSB and GFM cells at Passage 22 and 20, respectively, were completed according to the method previously described by Wang et al. (2003). Two oligonucleotide primers were designed to amplify the 16S and 18S goldfish mitochondrial DNA. The 16S fragment (123 bp) was amplified using the following primers: forward (16S-F) 5'-GCGACCACGGAG-GAAAAA-3' and reverse (16S-R) 5'-CGTTGATCG-GCTTGATTAG-3'. The 18S fragment (234 bp) was amplified using the following primers: forward (18S-F) 5'-GCGAGACGAGCCACCACCTATC-3' and reverse (18S-R) 5'-CCCCCGGCCGTCCTCTTA-3'. The PCR reaction mixture (25 µl) contained 2.5 µl PCR buffer (10×), 2.0 µl dNTPs (2.5 mM), 2.0 µl MgCl₂, 0.4 µl forward primer (100 ng), 0.4 µl reverse primer (100 ng), 0.2 µl *Taq*-DNA polymerase, 1.0 µl DNA template (0.5 µg), and 16.5 µl double-distilled water. The cycling conditions involved an initial denaturing phase at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension phase at 72°C for 5 min and a holding temperature of 4°C after completion.

The PCR fragments were cloned into a pCR2.1-TOPO vector using a Topoisomerase cloning kit (Invitrogen, Catalog No. K4575-02). Plasmid DNA containing the PCR fragments was identified by *EcoRI* digestion. Three positive clones containing the inserts were sequenced using capillary-based DNA sequencers of the Center for Genomics, Proteomics and Bioinformatics Research Initiative genome infrastruc-

ture facility of the University of Hawaii at Manoa. The obtained sequences of PCR fragments were compared to known sequences in the National Center for Biotechnology database using the Basic Local Alignment Search Tool (Altschul et al. 1990).

Viral susceptibility. Newly established goldfish cell lines were tested for their susceptibility to 3 fish rhabdoviruses (infectious hematopoietic necrosis virus, IHNV; snakehead rhabdovirus, SHRV; and spring viremia carp virus, SVCV), 1 fish herpesvirus (channel catfish virus, CCV), and 1 birnavirus (infectious pancreatic necrosis virus, IPNV) (Table 1), according to the methods described by Lu et al. (2003). Virus stocks were prepared and viral yields were determined using previously established cell lines. Chinook salmon embryo (CHSE-214) cells (Nims et al. 1970) were used for IHNV and IPNV, while SVCV and SHRV were prepared in epithelioma papulosum cyprinid (EPC) cells (Fijan et al. 1983), and CCV was titered in brown bullhead (BB) cells (Wolf & Quimby 1969). GFM and GFSB cells (5×10^5) were incubated at 25°C in a 25 cm² tissue culture flask until a subconfluent monolayer (80 to 90%) was achieved, after which the culture was inoculated with each viral stock at multiplicities of infection ranging from approximately 0.01 to 0.1. Following viral absorption for 1 h at virus-specific optimal temperatures, the unabsorbed virus was removed by washing cell monolayers twice with L-15 medium devoid of FBS. Then, 5 ml of L-15 medium containing 5% FBS was added to each flask, and infected cultures were transferred to incubation temperatures optimal for viral production (SHRV and CCV at 25°C; IHNV, IPNV, and SVCV at 19°C). Infected cultures were subsequently monitored daily for virus-specific cytopathic effects (CPE). Cultures were frozen during the experiment at -80°C when >80% of the cells demonstrated CPE or at Day 10 of the study. Viral susceptibility of goldfish cells was evaluated by the presence of virus-specific CPE in comparison with cultures in control flasks. In conjunction, the amount of viral production was determined by TCID₅₀ (50% tissue culture infectious dose) assay using the method described by Reed & Muench (1938).

Table 1. Summary of 5 viruses used for this study

Virus	Abbreviation	Family	Source
Channel catfish virus	CCV	<i>Herpesviridae</i>	Wolf & Darlington (1971)
Infectious hematopoietic necrosis virus	IHNV	<i>Rhabdoviridae</i>	Amend et al. (1969)
Infectious pancreatic necrosis virus	IPNV	<i>Birnaviridae</i>	Wolf et al. (1960)
Snakehead rhabdovirus	SHRV	<i>Rhabdoviridae</i>	Johnson et al. (1999)
Spring viremia carp virus	SVCV	<i>Rhabdoviridae</i>	Fijan et al. (1971)

RESULTS

Cell morphology

The morphology of GFSB cells of *Carassius auratus* remained consistently fibroblastic from their initial inception to the current passage (Fig. 1A,C). In comparison, the GFM cells were principally epithelial in nature in the primary culture, with very few cells having spider-like projections (Fig. 1B); however, the cells became uniformly epithelial in later passages (Fig. 1D).

Growth characteristics

Sizable colonies were established after 2 to 3 d in 25 cm² tissue culture flasks for GFM, and a partially confluent monolayer was reached in 4 to 5 d. In comparison, GFSB colonies were established after 4 to 5 d, and a confluent monolayer was reached only after 7 d. After Passage 13 for GFSB, the 2 cell lines shared similar growth patterns.

Optimal growth conditions for the cell lines were dependant on culture medium, temperature, and serum concentration. Our investigation found that L-15 was optimal for growth, with a doubling time of 18 h

(data not shown). Both GFM and GFSB grew well at temperatures from 25 to 37°C, with 30°C supporting superior growth (Fig. 2). In the GFSB study, the cells raised at 25°C maintained a similar growth pattern to those cultured at 30°C (Fig. 2A). Notably, the media in the flasks were left for the duration of each study with few complications. On Day 8 of the GFSB temperature study, the flasks in the 30°C incubator experienced an increase in acidity that was countered with the addition of sodium bicarbonate solution. The change in pH explains the resultant decrease in cell numbers on Day 8 (Fig. 2A). Cultures at lower temperatures showed minimal attachment, resulting in slow growth kinetics (Fig. 2). The doubling times were approximately 46 and 56 h for GFM and GFSB, respectively. Experimental data also showed that the cells did not grow in non-supplemented medium (L-15 alone), yet grew considerably better with higher FBS concentrations (Fig. 2C,D).

Plating efficiency

Plating efficiency for each cell line was determined at seeding concentrations of 200, 500, and 1000 cells. Moderately low plating efficiencies were observed, with GFSB cells (6, 9.4, and 15.7%, respectively) grow-

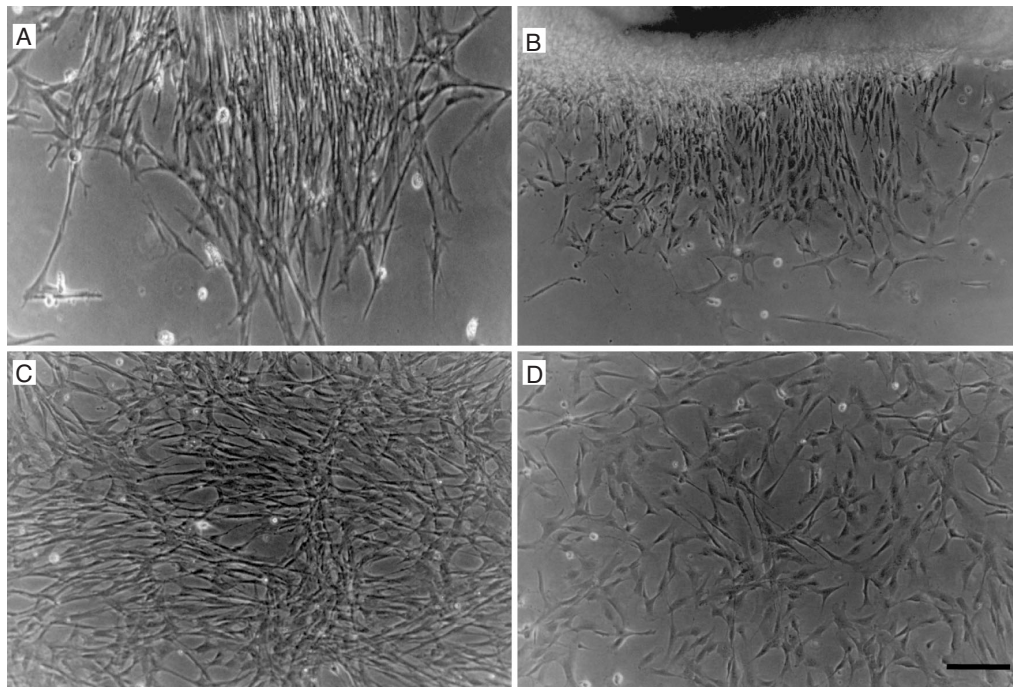


Fig. 1. *Carassius auratus*. Photomicrographs of goldfish cells derived from (A,C) swim bladder and (B,D) muscle tissue. (A,D) primary cultures on Day 3 following seeding tissue explants; (C,D) subcultured cells at Passage 2. Magnification = 100×, scale bar = 100 μm

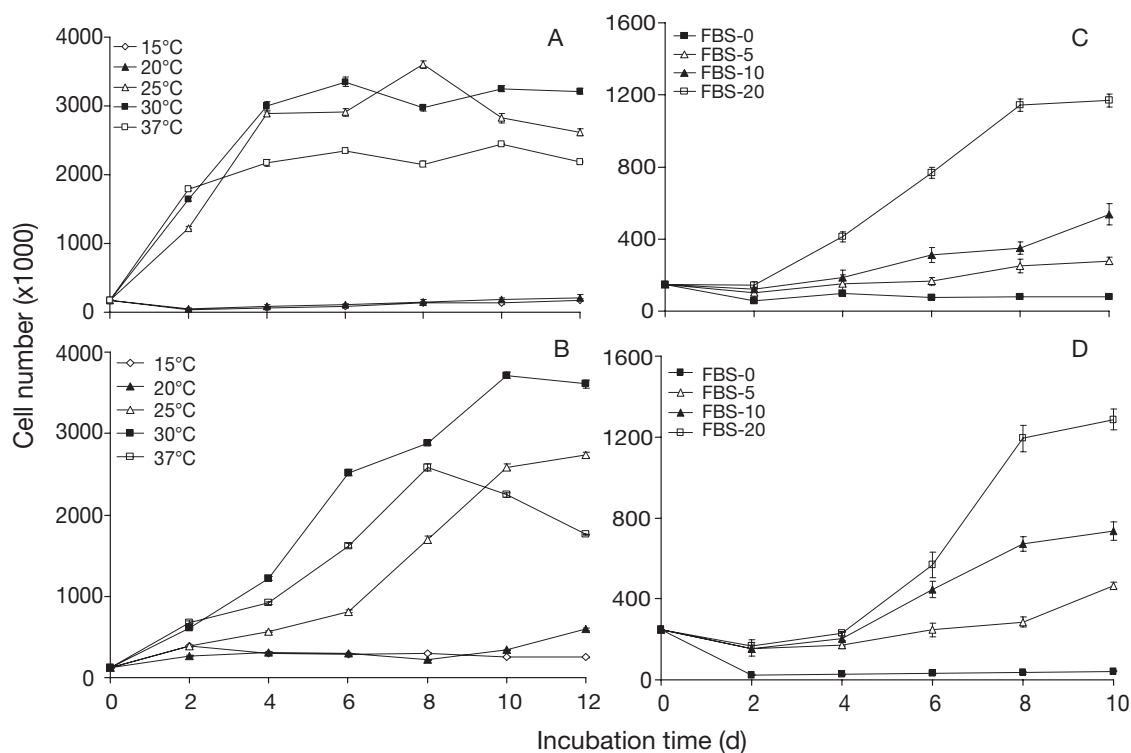


Fig. 2. *Carassius auratus*. *In vitro* growth curves (mean \pm SD) of 2 established goldfish cell lines. (A,C) Goldfish swim bladder and (B,D) goldfish muscle cells were assessed at (A,B) different serum temperatures and (C,D) selected concentrations of fetal bovine serum (FBS)

ing marginally better than GFM cells (4, 7, and 12.1 %, respectively), with no significant differences between replicates (data not shown). These low percentages coincided with observations of low-seeded flasks, in which cells were widely separated, and had difficulties replicating and achieving a subconfluent monolayer within the 7 d after initial seeding.

Cryopreservation

Evaluation of the viability of GFM and GFSB cells stored in liquid nitrogen established the capability of goldfish cells to survive following >1 yr of storage at -196°C . For GFM and GFSB, respectively, over 92 and 93 % of cell populations from each vial remained viable after the storage period and retained the ability to attach and grow at 25°C . Following storage, no obvious alterations in morphology or growth pattern were observed for GFSB or GFM.

Chromosomal analysis

Chromosomal counts from >100 random metaphase spreads were used for each cell line (Fig. 3). Chromo-

some numbers ranged from 88 to 110 in GFM and from 96 to 112 in GFSB, with a distinct peak for both cell lines at 104 diploid chromosomes (Fig. 4). This finding is consistent with previous karyotyping done in goldfish populations by Ohno et al. (1967) and Hong et al. (2005).

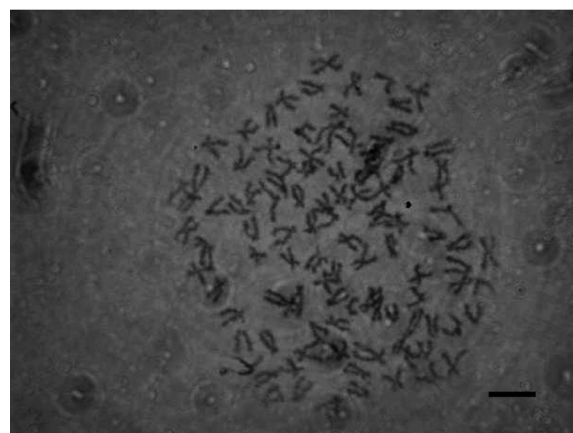


Fig. 3. *Carassius auratus*. Phase-contrast photomicrograph of goldfish cellular chromosomes in a swim bladder cell arrested in metaphase at Passage 11. Magnification = $1000\times$, scale bar = $10\ \mu\text{m}$

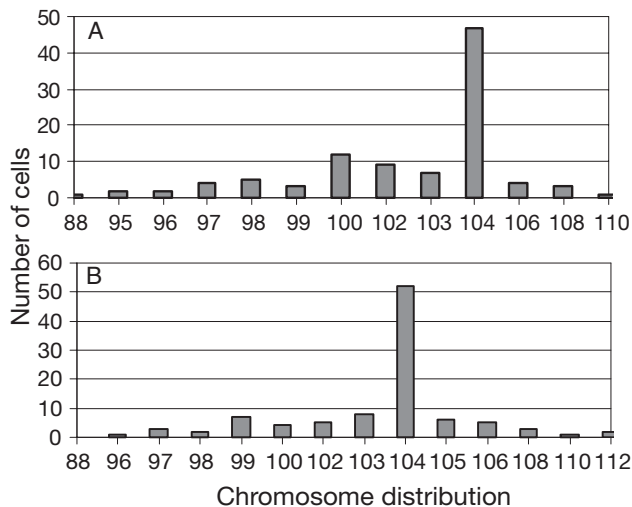


Fig. 4. *Carassius auratus*. Frequency distribution of chromosomes in 100 cells from (A) goldfish muscle and (B) goldfish swim bladder cell lines at Passages 9 and 11, respectively

Sequence analysis of 16S and 18S rRNA

Heteroduplex analysis of mitochondrial 16S and 18S rRNA was performed in order to verify the origin of the cell lines (Kshirsagar et al. 1997, Krieger & Fuerst

2002). Amplification from the 16S and 18S mitochondrial DNA for both cell lines revealed the expected PCR products of 123 and 234 bp, respectively (Fig. 5). Subsequent comparative analysis of the identified sequences demonstrated a 97% match for 16S and a 96% match for 18S to known goldfish mitochondrial DNA sequences (GenBank Accession No. AB111951 and AF047349, respectively). Our data demonstrate that GFM and GFSB cell lines are indeed derived from *Carassius auratus*.

Viral susceptibility

Viral infectivity assays were performed on both cell lines for 5 fish viruses. The assays demonstrated that both GFM and GFSB were susceptible to infection with all viruses tested in the present study: CCV, IHNV, IPNV, SHR, and SVCV (Table 1). Viral infection resulted in extensive CPE in both cell lines, which was observed on various days post-infection, depending on the virus. In general, CPE was observed between 2 and 3 d post-infection for CCV, IHNV, SHR, and SVCV. However, IPNV-infected cells showed a slow progression of viral replication, and initial CPEs did not appear until after 3 d post-infection. These 2 goldfish cell lines

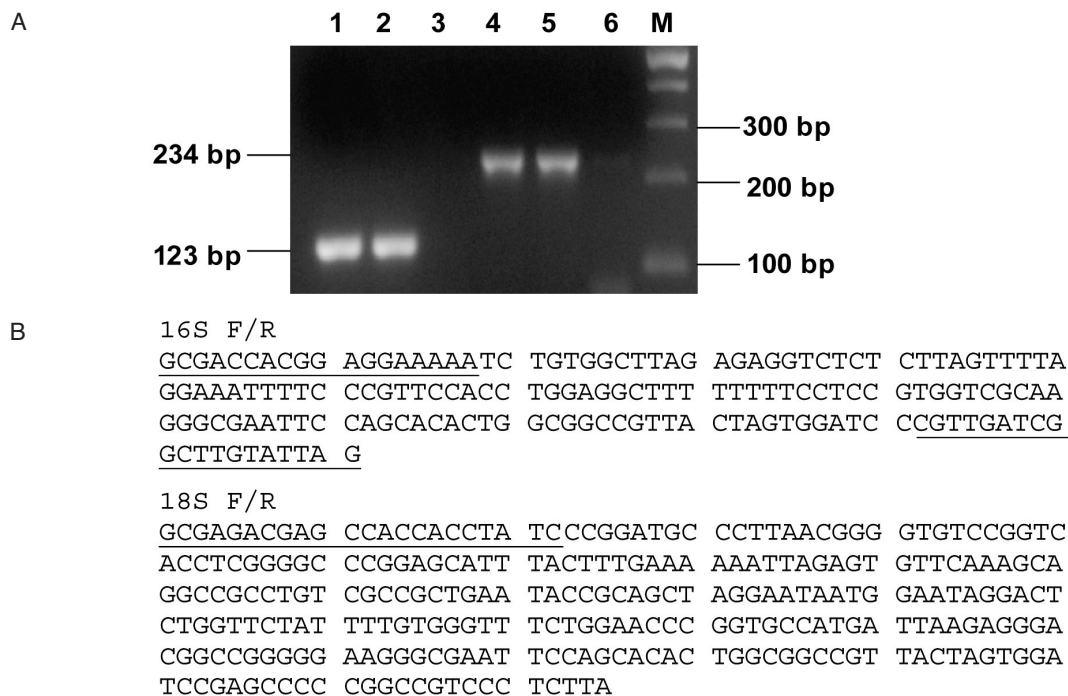


Fig. 5. *Carassius auratus*. (A) PCR amplification of 123 and 234 bp sequences of the goldfish genome using oligonucleotide primers designed from the conserved portions of the 16S and 18S mitochondrial DNA. The 100 ng of DNA isolated from goldfish muscle and swim bladder cells was amplified and then subjected to 2.0% gel electrophoresis. Lane 1: GFM 16S; Lane 2: GFSB 16S; Lane 3: 16S negative control; Lane 4: GFM 18S; Lane 5: GFSB18S; Lane 6: 18S negative control, M (marker): 100 bp DNA ladder (showing range from 100 to 500 bp). (B) Nucleotide sequences of the 123 and 234 bp fragments amplified by PCR using the respective primers. Underlined portions represent positions of the PCR primers

Table 2. *Carassius auratus*. Comparison of the 2 newly established goldfish cell lines (GFM, GFSM) for the propagation of selected fish viruses. CPE app.: cytopathic effects appeared; TCID₅₀: 50% tissue culture infectious dose; GFM: goldfish muscle; GFSB: goldfish swim bladder; BB: brown bullhead; CHSE-214: chinook salmon embryo cells; EPC: epithelioma papulosum cyprinid; ND: not done; virus abbreviations, see Table 1

Virus	Cell line	CPE app. (Day)	TCID ₅₀ U ml ⁻¹ (log titer)			
			Day 0 (seeded)	Day 5	Day 7	Day 10
CCV	GFM	3	3.5	8.0	ND	ND
	GFSB	2	3.5	8.0	ND	ND
	BB	2	3.5	5.7	ND	ND
IHNV	GFM	3	4.5	ND	ND	4.7
	GFSB	4	4.5	ND	ND	4.5
	CHSE-214	2	4.5	ND	ND	6.7
IPNV	GFM	5	3.5	ND	ND	6.3
	GFSB	4	3.5	ND	ND	4.5
	CHSE-214	1	3.5	ND	ND	8.0
SHRV	GFM	2	3.5	ND	7.7	ND
	GFSB	2	3.5	ND	8.3	ND
	EPC	1	3.5	ND	7.7	ND
SVCV	GFM	3	4.5	8.0	ND	ND
	GFSB	2	4.5	8.0	ND	ND
	EPC	2	4.5	8.7	ND	ND

shared similar viral propagation characteristics to the 5 viruses tested: GFSB and GFM are highly permissive to CCV, SVCV, and SHRV, as inoculation led to a high yield of viral production ($10^{7.7}$ to $10^{8.0}$ TCID₅₀ ml⁻¹). Although extensive CPE was observed in IHNV- and IPNV-infected goldfish cells, the viral production in GFSB and GFM was significantly lower than that produced in their respective control host cell lines (Table 2).

DISCUSSION

With only 1 permanent cell line available in goldfish *Carassius auratus*, our current research sought to establish more cell lines from this widely distributed species. Specifically, we have established 2 new cell lines derived from goldfish muscle and swim bladder tissue. These 2 cell lines have been continuously passed over 35 times since their establishment in August of 2002. These 2 newly established goldfish cell lines grow optimally in L-15 medium at 25 to 30°C when high concentrations of serum (20% FBS) are used. Although these goldfish cells remain viable following liquid nitrogen storage, their plating efficiency is rather low (<16%). Since general characteristics for transformed cell cultures include serum-independent growth, high contact inhibition, and high plating efficiency (Freshney 1994), our findings suggest that neither GFSB nor GFM cells are transformed in the passages for which they were tested. The non-

transformation status of these 2 goldfish cell lines was further evidenced by their chromosomal typing, showing a diploid chromosomal count of 104, which has been documented in the literature for this aquatic species (Ohno et al. 1967, Hong et al. 2005).

Our viral assays demonstrated patterns in both cell lines showing them to be equally susceptible to 5 known viruses isolated from other fish species. Three of the virus titers were within comparable ranges in GFM and GFSB cell lines contrasted with the respective controls. These data suggest that the newly developed goldfish cell lines are susceptible to infection from a variety of piscine viruses, making them a valuable biological tool for the effective monitoring and detection of marine viral pathogens. Both newly established goldfish cell lines were more susceptible to CCV, and produced significantly larger amounts of

infectious virus (>100-fold) than the control BB cell line. The consistent results from repeated trials in both cell lines suggest that goldfish cells are more permissive to CCV than the BB cells, and would serve as a more sensitive detection tool for this particular virus.

Viruses are the most abundant biological agents in the ocean, and they average 10 billion particles l⁻¹ of seawater (Fuhrman 1999). Viral infections in goldfish have been reported in countries such as Japan, Australia, Taiwan, and the USA (Humphrey & Ashburner 1993, Jung & Miyazaki 1995, Arthington & McKenzie 1997, Goodwin et al. 2006). Due to the size of viral particles and their intracellular replication, the effective identification and study of these microorganisms is largely dependent on the availability of sensitive cell lines. Currently there are only 3 known goldfish viruses (GFV 1, GFV 2, and the goldfish herpes-type virus), though it is likely that more exist. The lack of an established cell culture system for goldfish may have resulted in the limited experience with viral isolation and identification. In addition, some pathogenic viruses are known to be organ- and tissue-specific, which makes the establishment of additional cell lines from different organs and tissues of a host species essential for proper monitoring of viral diseases. In the absence of susceptible cell culture systems, scientists must rely on other methods and techniques, such as electron microscopy and bioassays, which are not as simple and easily reproducible as *in vitro* cell cultures.

With goldfish capable of adapting to a range of habitats throughout the world, expansion to new environ-

ments has the potential to introduce exotic diseases. This increases the likelihood of disease transmission to other species in different regions of the world and threatens the balance of ecosystems. Furthermore, fisheries using goldfish as a feedstock are at risk of spreading diseases and of a general decrease in productivity. Therefore, there is a need to establish goldfish cell lines from various organs and tissues in order to provide an enhanced capability for viral detection and identification in this aquatic animal species. To that end, further research on the newly established GFM and GFSB cell lines regarding their biological properties and functions will facilitate the establishment of these goldfish cells as a permissive biological tool in the study of goldfish viruses. After such establishment and full characterization, these new goldfish cell lines will be available to scientists all over the world for the advancement of research in this field.

Acknowledgements. This research was supported in part by funding from the Center for Tropical and Subtropical Aquaculture, Oceanic Institute, Honolulu, and an Integrated Graduate Education and Research Training grant from the NSF (No. 0549514).

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Geelong, Victoria, Australia*

*Submitted: January 31, 2007; Accepted: March 29, 2007
Proofs received from author(s): August 6, 2007*