*Asian Fisheries Science* 8 (1995): 151-157.
Asian Fisheries Society, Manila, Philippines
https://doi.org/10.33997/j.afs.1995.8.2.006

# A New Cell Line from *Puntius schwanenfeldi* Sensitive to Snakehead Fish Cell Line C-Type Retrovirus

INDRANI KARUNASAGAR[1], S.D. MILLAR[2] and G.N. FRERICHS[2]

[1] *Department of Fishery Microbiology*
*University of Agricultural Sciences*
*College of Fisheries*
*Mangalore - 575 002, India*

[2] *Institute of Aquaculture*
*University of Stirling*
*Stirling FK94LA, Scotland*

## Abstract

A new cell line was established from the caudal peduncle of a healthy, juvenile warmwater cyprinid, *Puntius schwanenfeldi*. The cell line has been designated PSP. Cultures grew well in Leibovitz L-15 medium supplemented with 10% fetal calf serum at 25-30°C and have been passaged more than 60 times since initiation over 2.5 years ago. The PSP line was refractory to infection with a range of viruses associated with outbreaks of the epizootic ulcerative syndrome (EUS), including infectious pancreatic necrosis virus (IPNV) serotype Sp and Ab, sand goby birnavirus and snakehead rhabdovirus, but was found to be sensitive to C-type retrovirus released from a persistently infected striped snakehead (*Ophicephalus striatus*) cell line.

## Introduction

Although a considerable number of cell lines have been developed from teleost fish in the past 30 years, relatively few of these have been derived from warmwater species. *Puntius* spp. are economically important riverine fishes found throughout India and Southeast Asia, and are one of the groups of freshwater fish most severely affected by annual outbreaks of the epizootic ulcerative syndrome (EUS), which have resulted in heavy losses in wild and cultured fish stocks throughout the region since 1980 (Roberts et al. 1994). A viral agent has always been considered a likely primary cause of EUS, but evidence for this remains equivocal. As it is generally recognized that homologous cell cultures are most appropriate for the isolation of viruses from infected fish, the availability of *Puntius* spp. cell lines would facilitate virological studies on EUS and other warmwater fish disease syndromes. The establishment of a cell line from *Puntius schwanenfeldi* is presently reported.

## Materials and Methods

### *Establishment of* Puntius *Cell Line*

Clinically healthy juvenile *P. schwanenfeldi* obtained from Bangkok, Thailand, were used to initiate primary cell cultures. Caudal peduncle tissue excised from sacrificed fish was decontaminated by immersion in iodophor disinfectant, minced in Dulbecco's phosphate buffered saline (PBS) and the fragments subjected to a series of 0.5-1 h digestions at 20°C in 0.25% trypsin (Flow Laboratories) according to the procedure of Wolf and Quimby (1976a). A pooled suspension of harvested cells in Leibovitz L-15 medium (Flow Laboratories) containing kanamycin (250 $\mu g \cdot ml^{-1}$), penicillin (200 $IU \cdot ml^{-1}$), streptomycin (200 $\mu g \cdot ml^{-1}$) and supplemented with 20% fetal calf serum (FCS) was distributed in 20 $cm^2$ culture flasks and incubated at 28°C.

Confluent primary cell monolayers were observed for 14 d and a cell line then established by sequential trypsin-EDTA (Flow Laboratories) dispersion of cell sheets and sub-culturing at a 1:2 split ratio as described by Wolf and Quimby (1976b). Serum supplementation was reduced to 10% for sub-cultures and antibiotic-free medium used after the sixth passage.

### *Growth Studies*

The effect of temperature on the growth of *Puntius* cells was determined by seeding 25 $cm^2$ culture flasks with $2 \times 10^5$ cells in L-15 medium supplemented with 10% FCS and incubating replicate cultures at 15, 20, 25, 30 and 37°C. Flasks were observed daily for degrees of confluency and monolayers trypsinized at 7 d. Harvests from paired flasks were pooled and cell numbers determined by trypan blue dye exclusion test (Greene et al. 1964).

Optimum serum requirement was similarly determined by seeding 25 $cm^2$ flasks with $2 \times 10^5$ cells and adding 7 ml growth medium to give duplicate cultures with 2, 5, 10 and 20% final serum concentrations. All cultures were incubated at 30°C and examined daily. When the cultures at the most favorable serum concentration were almost confluent, all cell sheets were trypsinized, paired harvests were pooled and cell numbers determined.

The effect of varying split ratios was studied by sub-culturing confluent monolayers at 1:2, 1:4, 1:6 and 1:8 dilutions. Duplicate flasks were incubated at 30°C, observed daily, and cell numbers determined when confluency was reached.

### *Sterility*

Following the withdrawal of antibiotics from growth medium at passage 6, cells were tested at passage 12 for freedom from bacterial, fungal and mycoplasma contamination. Suspensions obtained by scraping off monolayer cultures were inoculated into duplicate tubes of tryptone soya broth (TSB) and fluid thioglycollate medium (FTM). One set of tubes was incubated at 22°C and the other set at 37°C for 7 d. Screening for mycoplasma was carried out by the inoculation of Mycoplasma Test Agar (Flow Laboratories) with cellular material and incubation at 37°C, and also by fluorescence microscopy of fixed coverslip cultures of *Puntius* cells stained with Hoechst 33258 compound (Flow Laboratories).

### *Freedom From Adventitious Viruses*

Supernatant fluids from *Puntius* cell cultures were added to actively growing cultures of bluegill fry (BF-2), chinook salmon embryo (CHSE-214), Atlantic salmon (AS) and striped snakehead (SSN-1) cell lines and incubated for 3 weeks at 25°C. Additionally, ultrathin stained sections of resin-embedded pellets of fixed *Puntius* cells were examined for the presence of virus-like particles by transmission electron microscopy.

### *Virus Susceptibility*

The *Puntius* cells were tested for susceptibility to infection with infectious pancreatic necrosis virus (IPNV) serotypes Sp and Ab, sand goby birnavirus (SGV) (Hedrick et al. 1986), six ulcerative disease rhabdovirus strains isolated from Thailand, Burma and Sri Lanka (Frerichs et al. 1989) and a C-type retrovirus derived from a persistently infected snakehead cell line (SSN-1) (Frerichs et al. 1991). Each test virus stock was assayed on recognized permissive fish cell lines and duplicate 25 $cm^2$ flasks of *Puntius* cells then inoculated at a multiplicity of infection (MOI) of 0.01-0.001 with each preparation. Cultures were incubated at 25°C and observed for the development of cytopathic effects (CPE) for up to 3 weeks.

### *Liquid Nitrogen Storage*

Trypsinized suspensions of actively growing passage 32 cells, adjusted to a concentration of $10^6$ cells·$ml^{-1}$ in L-15 growth medium (10% FCS) + 10% dimethyl sulphoxide (DMSO) freezing mixture, were distributed in 1 ml aliquots in cryovials and stored in liquid nitrogen.

## Results

### *Establishment of PSP Cell Line*

A master seed stock of the *Puntius* cells in liquid $N_2$ was established at passage 32 and the line designated PSP (*P. schwanenfeldi* peduncle). At confluency, a 25 $cm^2$ flask yielded approximately 1.4 x $10^6$ epithelial-like cells.

### *Growth Studies*

The optimum growth temperature for PSP cells was 30°C (Fig. 1.) Cells grew at all temperatures evaluated, although at 15°C multiplication was so slow that confluency was not attained within 7 d. At 20 and 37°C, confluency was reached by 5 d. By contrast, at 25 and 30°C, confluent monolayers were evident by 48 and 36 h, respectively.

PSP cells grew almost equally well at serum concentrations of 5-20%. A somewhat poorer yield per unit time was obtained at the 2% level (Fig. 2).

At optimum serum concentration (10%) and incubation temperature (30°C), a cell split ratio of 1:2 reached confluency at 24 h, a 1:4 split required 48 h and a 1:6 split became confluent at 72 h. Confluent cell sheets could also be obtained following a 1:8 split, although this required up to 6 d incubation.

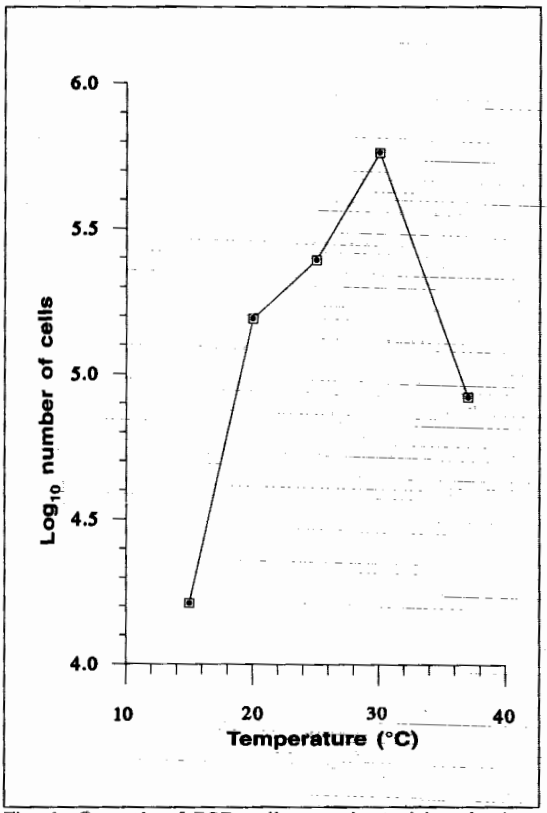


Fig. 1. Growth of PSP cells at selected incubation temperatures (cell number at day 7).

### *Sterility*

No bacterial or fungal contamination of PSP cells was detected at any stage of sub-culturing. No mycoplasm contamination was identified when cells were first examined, but *Acholeplasma laidlawii* was isolated at passage 30. The addition of Mycoplasma Removal Agent (MRA) (ICN Biomedicals, Ltd.) to cell culture media at 0.5 $\mu \cdot ml^{-1}$ and incubation for a week appeared effective in eliminating contamination prior to establishment of the master seed stock.

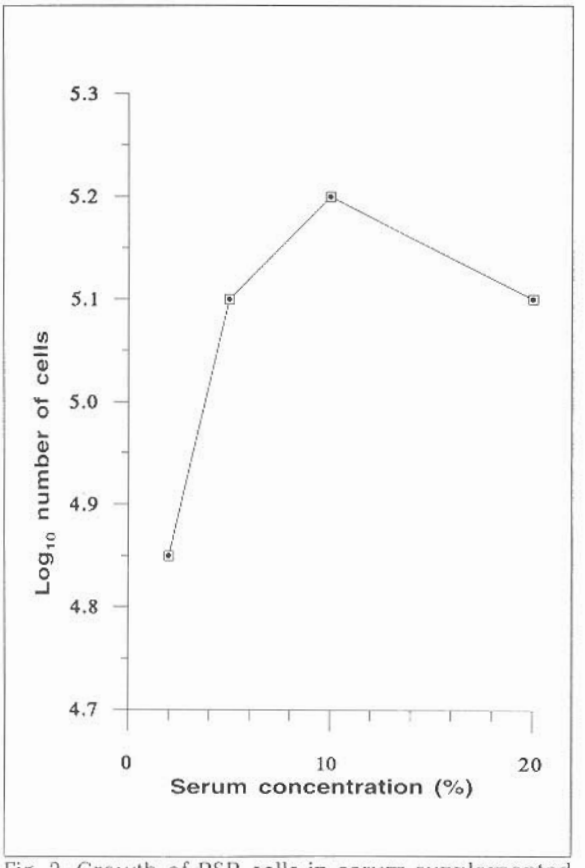


Fig. 2. Growth of PSP cells in serum-supplemented culture medium (cell number at day 4).

### *Adventitious Viruses*

None of the cell lines inoculated with supernatant fluid from PSP cultures developed any specific CPE during a 3-week observation period. No virus-like particles were observed by electron microscopy indicating freedom from virus contamination.

### *Virus Susceptibility*

PSP cells were refractory to infection with IPNV, SGV and the six EUS-associated rhabdovirus strains tested. Cells were susceptible, however, to the snakehead cell line C-type retrovirus. Plaques of cells with a degenerative, mesh-like appearance developed within 7-14 d (Fig. 3) and gradually extended over a further 7-10 d to involve the whole monolayer. The infection was transmissible to fresh PSP cell cultures, and electron microscope examination of cells confirmed the active production of C-type retrovirus particles (Fig. 4).

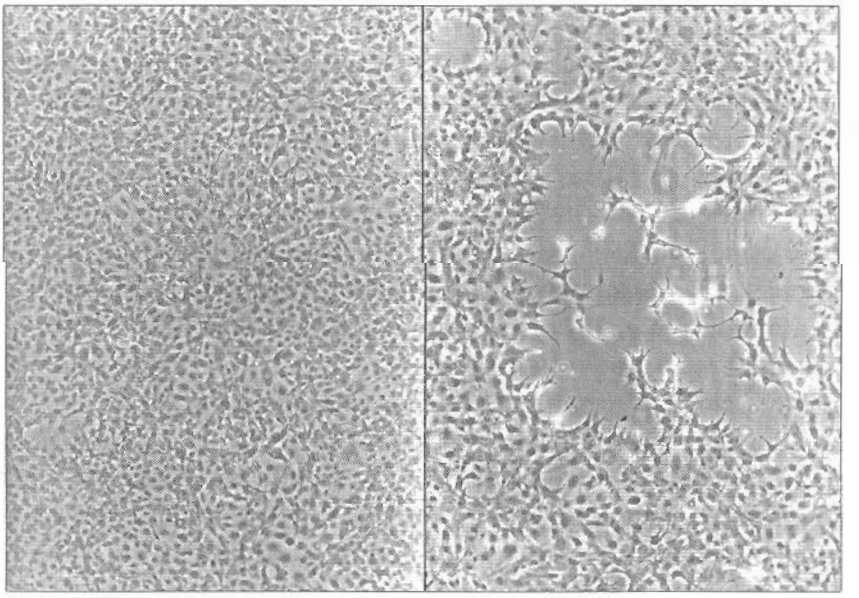

Fig. 3. PSP cell cytopathology (a) normal monolayer (b) plaque formation following infection with snakehead cell line retrovirus (14 d post infection)

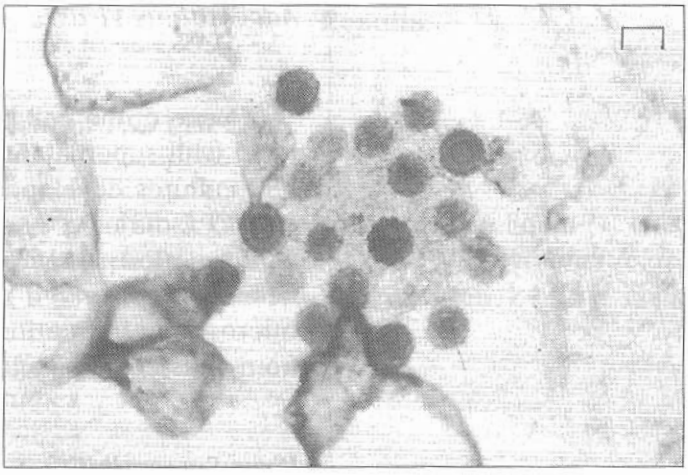

Fig. 4. Ultrathin section showing budding and mature C-type retrovirus particles in PSP cells (uranyl acetate - lead citrate stained). Bar = 100 nm

### *Liquid Nitrogen Storage*

PSP cells at passage 32 were readily recovered by rapid thawing from the frozen state after 2 years storage in liquid nitrogen.

## Discussion

The aetiology of EUS is still unresolved. Field surveys and laboratory investigations over the past decade have indicated the condition to be an infectious disease complex mediated by climatic and possibly other environmental factors. Bacteria and fungi are clearly involved in the pathogenesis of the characteristic skin lesions, but a virus infection is regarded as a likely primary cause (Roberts et al. 1992). A diverse range of viral agents have been associated with EUS-affected fish, but very low rates of recovery from occasional specimens together with the heterogeneity of the isolates do not support the candidature of these organisms (Roberts et al. 1994). The refractory nature of the PSP cell line to infection with selected EUS-associated birnavirus and rhabdovirus isolates, despite the fact that *Puntius* spp. are known to be markedly susceptible to EUS, now lends additional weight to the view expressed above.

It is also not yet known what role, if any, the snakehead cell line C-type retrovirus may play in the pathogenesis of EUS (Frerichs et al. 1991), or what relationship may exist between this virus and the C-type particles hitherto observed in neoplastic lesions of fish. The susceptibility of the PSP cell line to snakehead virus however, particularly with plaque formation, may find application in further qualitative and quantitative studies of piscine retroviruses.

## Acknowledgments

The authors thank the UK Overseas Development Administration and British Council for support of this study.

## References

Frerichs, G.N., S.D. Millar and M. Alexander. 1989. Rhabdovirus infection of ulcerated fish in south-east Asia. In: Viruses of lower vertebrates (eds. W. Ahne and E. Kurstak), pp. 396-410. Springer-Verlag, Berlin.

Frerichs, G.N., D. Morgan, D. Hart, C. Skerrow, R.J. Roberts and D.E. Onions. 1991. Spontaneously productive C-type retrovirus infection of fish cell lines. Journal of General Virology 72: 2537-2539.

Greene, A.E., L.L. Coriell and J. Charney. 1964. A rapid cytotoxic antibody test to determine species of cell cultures. Journal of the National Cancer Institute 32: 779-786.

Hedrick, R.P., W.D. Eaton, J.L. Fryer, W.G. Groberg, Jr. and S. Boonyaratpalin. 1986. Characteristics of a birnavirus isolated from cultured sand goby *Oxyeleotris marmoratus*. Diseases of Aquatic Organisms 1: 219-225.

Roberts, R.J., G.N. Frerichs and S.D. Millar. 1992. Epizootic ulcerative syndrome - the current position. In: Diseases in Asian aquaculture I (eds. M. Shariff, R.P. Subasinghe and J.R. Arthur), pp. 431-439. Fish Health Section, Asian Fisheries Society, Manila.

Roberts, R.J., G.N. Frerichs, K. Tonguthai and S. Chinabut. Epizootic ulcerative syndrome of farmed and wild fishes. Recent advances in aquaculture V. (In press).

Wolf, K. and M.C. Quirnby. 1976a. Primary monolayer culture of fish cells initiated from trypsinized tissues. TCA Manual 2: 453-456.

Wolf, K. and M.C. Quimby. 1976b. Procedures for subculturing fish cells and propagating fish cell lines. TCA Manual 2: 471-474.

*Manuscript received 20 April 1994; accepted 12 July 1995.*