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Establishment of Primary Cell Culture from Hepatopancreas of *Penaeus monodon* for the Study of Whitespot Syndrome Virus (WSSV)

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Abstract

A primary cell culture system was developed from the cells of hepatopancreas of *Penaeus monodon*. Minced tissues of hepatopancreas were seeded (10^6 cells·cm³) and incubated at 28°C in single strength Leibovitz's L-15 medium (pH 7.2) supplemented with 10% fetal bovine serum and antibiotics (ciprofloxacin 100 mg·ml; penicillin 100 IU·ml; streptomycin 100 mg·ml and nystatin 25 mg·ml). The cells showed good attachment to the culture vessels by 24h and formed a confluent monolayer within 72 h. The primary cell culture could be maintained up to a maximum period of 12 weeks by subculturing seven times. When whitespot syndrome virus (WSSV) was inoculated in the hepatopancreas cell culture, characteristic cytopathic effects which include rounding of cells, detachment, lysis of cell sheet with circular clear areas of depletion of cells were observed within 120 h.

Introduction

Shrimp viral diseases have caused severe production and economic losses in the past two decades. A complete understanding of shrimp viruses is dependent upon the development of laboratory techniques for the maintenance and culturing of these viruses and host cells (Hsu et al. 1995). Attempts were made by various researchers in establishing the primary cell culture and continuous cell lines from different organ sources of shrimp (Luedman and Lightner 1992; Purushothaman et al. 1998 and Roper et al. 2001). Successful attempts on the development of primary cell culture derived from hepatopancreas are few (Mike et al. 1990; Toullec et al. 1996). This study was aimed at establishing the primary cell culture from the hepatopancreas of *Penaeus monodon* and to study the cytopathic effects of whitespot syndrome virus (WSSV).

Materials and Methods

Experimental animals

Healthy *P. monodon* shrimps used in this study were collected from the shrimp farms of Tamil Nadu, India. Shrimp samples of 4 to 5 cm size were selected for cell culture.

WSSV

WSSV infected shrimps were collected from shrimp farms located in the Nellore area of Andhrapradesh, India. WSSV infection in shrimp was confirmed by polymerase chain reaction (PCR) using Fast target™ whitespot virus detection kit (Malaysia) and by specific clinical symptoms like the presence of whitespots, lethargy and reddish discoloration of the body.

Media and supplements

Leibovitz L-15 medium at pH 7.2 was used in our experiments for the culture of hepatopancreas cells of *P. monodon* (Owens and Smith 1999). The medium was supplemented with 10% fetal bovine serum, ciprofloxacin (100 mg·ml), penicillin (100 IU·ml), streptomycin (100 mg·ml) and nystatin (25 mg·ml).

Culture procedures

The animals were anaesthetized in cold water at 4°C for 10 min and the body surface was sterilized with 2% tincture and 75% ethanol as described by Ke et al.(1990). The hepatopancreas was aseptically dissected out and quickly transferred to the L-15 medium. The tissue was washed several times and homogenized slowly using glass homogenizer and centrifuged at 1000 rpm for 3 min. The supernatant was aspirated and centrifuged again at 7000 rpm for 10 min. The cell pellet was added to L-15 medium and cell suspension was prepared by repeated aspiration using a sterile pipette. The cells were seeded aseptically in 25 cm² sterile disposable culture flasks and 24 well plates at the rate of 10⁶cells·cm³ and incubated at 28°C. Confluent monolayer of cells was observed by 72 h post seeding. The medium was changed every four days and passaging was done when multiple layers of cells were observed. Passaging was done by mechanical disruption of the layer by repeated pipetting and transfer to culture flasks with medium every 10 to 12 days. To infect the shrimp cell cultures with WSSV, the virus suspension was prepared from the infected shrimp as follows:

The subcuticular epithelial layer and the areas showing white spots were aseptically dissected out from infected shrimp. The tissue was minced by adding L-15 medium in a sterile glass homogenizer and centrifuged at 15,000 rpm for 15 min. The supernatant was filtered through 0.22 m sterile membrane filter (Millex-GV, Millipore) and an aliquot of 100 ml was used as inoculum for

infecting 72 h old confluent hepatopancreas monolayer in 25 cm² culture flasks and 10 ml per well in 24 well plates and incubated at 28°C. The control plates and culture flasks were also maintained under similar conditions.

Results

Primary cell culture was successfully developed from hepatopancreas in L-15 medium. After seeding the hepatopancreas cells, the cells showed attachment to the surface in 24 h. Although more number of unattached floating cells could be observed initially, a monolayer could be observed by 72 h post seeding (Fig.1). The cells were observed to be spherical in shape. The primary cell culture could be maintained for 12 weeks with 7 passages without any undesired effect on the cells. The cytopathic effects observed in the primary cell culture infected by WSSV are presented in table 1. The infected cell cultures were frozen at -70°C and thawed thrice for harvesting the cells. An aliquot of the fluid from the infected culture was used to confirm the presence of WSSV by polymerase chain reaction using Fast target™ whitespot detection kit. Amplification of 356 bp band indicates light infection of WSSV and amplification of 403 bp and 356 bp indicates heavy infection by WSSV. A 232 bp host DNA will also be amplified in all the samples. In uninfected samples only the host DNA fragment (232 bp) is amplified.



Fig. 1. A 72 h old primary hepatopancreas cell layer

Table 1. Cytopathic effects (CPE) observed in WSSV infected hepatopancreas cell culture.

Duration	Observations
24 h	Clumping of cells in a circular fashion. Focal lesions of CPE could be observed (Fig. 2).
48 h	The cells appeared shrunken in places that showed clumping in 24 h (Fig. 3).
72 h	Detachment of the cell clumps and appearance of circular clear areas without cells due to cell lysis.
96 h	Increased number of clear circular areas (Fig. 4).
120 h	Complete "peeling off" cell layer from the culture vessel. Scattered abnormal enlarged cells could be observed.

Discussion

Various studies on initiating primary cell cultures from the hepatopancreas of penaeid shrimp have been reported but with limited success (Toullec et al. 1996; Kasornchandra et al. 1998; Owens and Smith 1999). Hepatopancreas are more susceptible to most shrimp viruses than other organs and hepatopancreas cell culture will be helpful in isolation of viruses (Ke et al. 1990). Mechanical disruption of the tissue was adopted in our study as Toullec (1996) has reported that enzymatic dissociation will not be suitable for fragile hepatopancreas tissue thereby leading to poor attachment and survival of the cells.

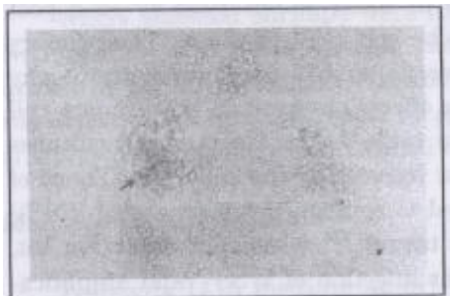


Fig. 2. Primary hepatopancreas cells showing CPE 24 h infection with WSSV



Fig. 3. Primary hepatopancreas cells showing CPE 48 h post infection with WSSV

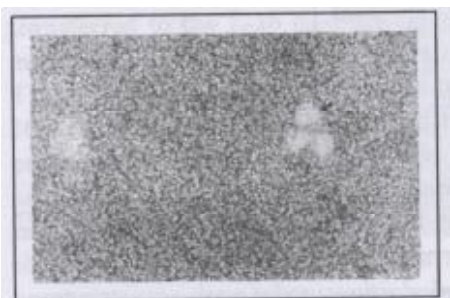


Fig. 4. Primary hepatopancreas cells showing CPE 96 h post infection with WSSV

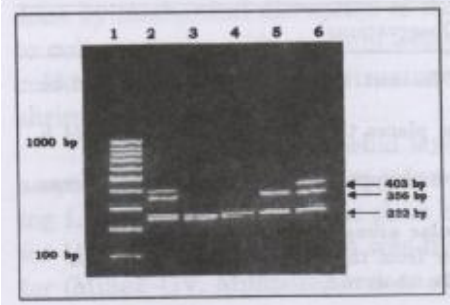


Fig. 5. PCR amplification results
 Lane 1 - 100 bp DNA ladder (100 bp - 1000 bp)
 Lane 2 - Shrimp sample heavily infected with WSSV (Positive Control)
 Lane 3 - Uninfected shrimp sample (Negative control)
 Lane 4 - Uninfected cell culture fluid
 Lane 5 - WSSV infected cell culture fluid exhibiting moderate infection (356 bp and 232 bp host DNA)
 Lane 6 - WSSV infected cell culture fluid exhibiting heavy infection (403 bp, 356 bp and 232 bp host DNA)

Although Toullec (1999) has also reported that mechanical dissociation of hepatopancreas would rupture the cells, this method is found to be simple and gives good results in our study. The cells were observed to be spherical in shape as described by Ke et al.(1996). In recent years, shrimp cell culture attempts are mostly done in lymphoid organ and ovarian tissue of shrimp. But our observations showed that among the shrimp tissues tested such as eyestalk, eyeball, muscle, haemolymph and hepatopancreas, cell culture from hepatopancreas was found to exhibit good growth on seeding and subsequent passages.

Hsu et al (1995) was successful in developing primary cell cultures from lymphoid organ of shrimp using single strength L-15 medium whereas many researchers (Chen et al. 1986; Chen et al. 1989; Ellender et al. 1992; Nadala et al. 1993) have reported that L-15 at double strength concentration is suitable for shrimp cell culture. Observations in this study showed that L-15 in single strength concentration is equally good and suitable for hepatopancreas cell culture from shrimp.

Owens and Smith (1999) have reported that the mean survival of hepatopancreas explant culture is about 48 h in double strength L-15 medium with 20% FBS whereas findings in this study showed that the primary cell cultures could be maintained for a period of twelve weeks by intermittent passages with 10% FBS. The findings also indicate that dissociated cells are more suitable for cell culture than the explants as suggested by Toullec (1999).

It has also been demonstrated in this study that primary cell cultures of shrimp hepatopancreas could be used to study the CPE and isolate WSSV. The CPE included rounding of cells, detachment or lysis followed by the development of circular clear areas with depletion of cells. The CPE produced by WSSV were similar to those described by Tapay et al. (1997); Kasornchandra et al. (1998) and Itami et al.(1999) in the lymphoid cell culture. Review of the available literature on CPE of WSSV has not spelt the type of CPE of WSSV in the cells of hepatopancreas. Hence the recorded observation on the abnormal changes of infected cells in comparison with control cells indicate that the changes are due to CPE produced by WSSV, which was confirmed by PCR of infected cell culture fluid. From the available reports, this study appears to be the first report on the CPE of WSSV in primary cell culture derived from the hepatopancreas of shrimp.

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