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# Characterization and propensity of white spot syndrome virus extracted from imported specific pathogen free (SPF) pacific *Litopenaeus vannamei* brooders progeny by performing SF9 cell line culture

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**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. This investigation was done to characterize the cell line culture from specific pathogen free *Litopenaeus vannamei* and its susceptibility to revise the cytopathic effects of white spot syndrome virus. A cell culture was successfully developed from insect cell SF9. Cytopathic changes like enlarged cells, focal lesions, shrunken and clumped cells were observed in white spot syndrome viruses (WSSV) infected SF9 cell cultures from 24 to 120 h duration. In the present study, conditions for the successful primary culture of insect SF9 cells for WSSV infection have been established. The conformation of WSSV infection in SF9 cell line was by polymerase chain reaction using target gene of WSSV419 like proteins and followed by electrophoresis. The WSSV result was positively as 550 bp in SF9 cell line and in the control sample. This is the first report on the development of primary cell culture of WSSV, host species of *L. vannamei* using insect cell line SF9.**

**Key words:** White spot syndrome viruses (WSSV), SPF, *L. vannamei,* SF9 cell line, cytopathic effect, pathogen, economy

## INTRODUCTION

Crustacean cell culture has gained momentum due to viral diseases affecting commercially important species. Hence, cell culture techniques were developed: (a) to assist in understanding the mechanism of host pathogen-nesis interaction (Chen et al., 1989), (b) to produce large amount of viral material for their characterization and (c) to improve tools for diagnosis and cure of diseases. Attempts have been made to establish several cell culture systems of shrimps (Al-Mohanna and Nott 1987; Chen et al., 1986, 1995; Ke et al., 1990; Nadala et al., 1993; Hsu et al., 1995; Toullec et al., 1996; Mulford and Austin, 1998; Mulford et al., 2001; Uma et al., 2002) and other crustaceans (Peponnet and Quiol, 1971). Yet, for reasons that remain obscure, all endeavors to develop cell cultures from marine invertebrates have been ineffective so far (Rinkevich et al., 1994; Bayne, 1998; Rinkevich, 1999; Mothersill and Austin, 2000) despite the acknowledged need for cell cultures from species that are important in aquaculture or in the pharmaceutical industry (Rinkevich et al., 2005).

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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; Roper et al., 2001). Successful attempts on the development of primary cell culture derived from hepatopancreas are few (Toullec et al., 1996). Recently, the successful development of primary shrimp cell cultures has been reported from many laboratories (Chen et al., 1986, Hsu et al., 1995; Luedeman and Lightner, 1992; Nadala et al., 1993; Toulled et al., 1996). Lu et al. (1995b) and Tapay et al. (1997) developed an *in vitro* quantal assay for yellow head virus (YHV) and China Baculo-like virus (CBV) using primary lymphoid cells of the white shrimp *Penaeus vannamei* and *Penaeus stylirostris*.

With the rapid expansion of high density aquaculture of penaeid shrimp, the detection of infectious diseases, especially of viral etiology, has become increasingly important. Several viruses including *Baculovirus penaei* (BP) (Couch, 1974; Lightner, 1983), *Penaeus monodon*-type baculovirus (MBV) (Lightner and Redman, 1981; Lightner, et al., 1983a) baculoviral midgut gland necrosis virus (BMNV) (Sano, et al., 1981) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner, et al., 1983b), white spot disease (Inouye, et al., 1994; Momoyama et al., 1994; Nakano, et al., 1994) and yellow head disease (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993) are known to be the causative agents of disease and mass mortality of cultured penaeid shrimp. Although, several papers have been published on these viral diseases of cultured shrimp, almost all of the studies were limited to histopathology observations using light and electron microscopy.

Presently, an effort has been made to characterize the white spot syndrome viruses (WSSV) post infected tissues of lymphoid organ, gill and mid-gut gland using insect cell line namely SF9 of the offspring's of SPF *L.vannamei* brooders originating from Thailand.

## MATERIALS AND METHODS

### Sample collection and processing

A total of 20 individuals with ABW 5 - 7g SPF *L.vannamei* offspring's of SPF brooders with ABW 30 -60 g were randomly collected from prime division of South Indian grow out ponds. Suspected WSSV infected insect cells enlightening a hypertrophic appearance were obtained from moribund shrimps. WSSV infection in shrimp was confirmed by polymerase chain reaction (PCR) using PCR primers for target genes such as WSSV419 like protein which is similar to VP28 and their amplification size and by specific clinical symptoms like the presence of white spots, lethargy and reddish discoloration of the body.

Following the confirmation of WSSV infection, the non-viral infected SF9 cell cultures were discarded. All the cultures were observed with an Olympus IM inverted microscope for the formation of confluence cell sheet and the presence of cytopathic effect (CPE). The cells with CPE foci were collected and then prepared for analysis as described by Bioserve Biotechnology, India (P) Ltd. Prior to the experiment, all shrimps were sterilized as described by Chen et al. (1986) using 5% sodium hypochlorite. Subsequently, tissues including lymphoid tissue, 'Oka organ', located at the anterioventral surface of hepatopancreas (Oka, 1969), heart and hepatopancreas were removed and rinsed in double strength (2X) Leibovitz's L-15 medium for 3-4 times.

### Growth and maintenance of SF9 cells

*Spodoptera frugiperda* (SF9) insect cells grown in suspension, serum-free medium (SFM) was used in our experiments for the culture of WSSV cells. 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). Flasks: Sterile, disposable 50, 125 and 500 ml flasks were used for culturing the cells. For small culture volumes (10 ml), Nalgene polycarbonate shaker flasks were used. Before use, the flasks were rinsed several times in MQ water and allowed to air dry. Sterilization was done for 15 minutes at 121°C, and air dried for 15 min.

### Media preparation for SF9 cells growth

Grace's insect medium (Gibco) was supplemented with 1x with Pluronic F-68, 10% (100 x), (Invitrogen). 5 ml of PenStrep (antibiotics) was added to 500 ml medium and final PenStrep concentration was maintained as 1% for every 50 ml conical flask and 45 ml of Grace medium containing 1 % PenStrep with 5 ml FBS (fetal bovine serum) 0.5 ml Pluronic F-68, 10% (100 x) (PA) was prepared and maintained in four conical flasks.

### Thawing and adding of cryogenic cells

The cells that have been frozen in DMSO at a density of 10 mill.cells/ml from a cell culture that is only a couple of days old was used. Two tubes, containing 1 ml of cells (10 mill. cells/ml), out of the liquid nitrogen freezer was thawed in a 26°C water bath. For every 1 ml aliquot of cells, resuspention in 5 ml suspension medium (+PA); 20 mill.cells/ml, 10 ml suspension medium (+PA) was used (15 ml/conical approx).

By centrifuging for 7 min at 1000 rpm the supernatant was decanted. The resuspended cells in desired amount of suspension medium was 30 ml of suspension medium for 20 mill cells /ml; expected confluence is 0.667 mill cells/ml, initially, when cells grew at low density (0.5-1 mill. cells/ml). Cell suspensions were transferred into 50 ml spinner flask.

### Determining cell density and viability

200 µl cell suspensions were withdrawn and the cell count was taken. 20 µl cell suspensions were mixed with 20 µl trypan blue which was sucked by capillary forces into the flow chamber. In 10x magnification, the cells were counted in 5 x 4 grids. Dead cells appeared as brown spots, whereas healthy cells had a bright ring. Actual number of cells were counted in grids sized as 25 x 2 (correction for dilution in dye) x factor $10^4$ = 0.5 mill. cells /ml.

### Freezing cells and procedure

Freezed cells were counted under the conditions which are at a density of ≥1 x 107 viable cells/ml. By using a freezing medium composed of 50% fresh growth medium and 50% conditioned growth medium (day 2 to 4 cell conditioned media collected from SF9 cultures during subculture procedure) and DMSO to a final concentration of 7.5%, freezing medium was prepared instantly, filter-sterilized before the experiment and chilled at 4°C until used.

Desired quantity of Sf9 cells in spinner flasks were harvested when the cells are in mid-log exponential growth and have a viability of >90%. The viable and total cell counts were calculated to the volume of freezing medium required to yield a final cell density of ≥1x107 viable cells/ml. The cells from cell suspension were centrifuged at 100 x*g* for 5 to 10 min. Supernatant was decanted and the cell pellets were resuspended in the pre-determined volume of chilled freezing medium. Dispensed aliquots of this suspension (frequently mixing to maintained a homogeneous cell suspension) into cryovials and the freezing rate should be decrease to 1°C per minute. The vials were transferred to liquid nitrogen (vapor phase) storage.

### *In vitro* multiplication of WSSV from *L. vannamei* using SF9 cell lines

SF9 cell lines were examined for their ability to propagate WSSV. Viral suspension was prepared as described by Boonyaratpalin et al. (1993) with slight modifications. Briefly, 10% (w/v) gill tissue of WSSV infected *L.vannamei was* homogenized in 2X L- 15 medium and filtered through a 0.2 µm sterile membrane. The virus suspension was then diluted 100 times in 2X L-15 medium and 10 µl of the diluted suspension was inoculated into freshly grown SF9 cell cultures. Control wells were inoculated with an extract of normal gill tissue prepared in the same manner. The inoculated plates were incubated at 28°C and observed daily for cytopathic effect (CPE).

### Concentrating the virus

To produce viral multiplicity of infection (MOI) (>10.0), this protocol is used to concentrate the virus from growth medium. The supernatant must be harvested from a non-lytic, serum-free culture. About 33 ml of virus stock were loaded into each of the six 38-ml polyallomer ultracentrifuge tubes. The virus stock was added with 3 ml of sucrose solution per tube and centrifuged at 80,000g for 75 min at 4°C. By decanting the supernatant, relatively pure viral pellet can be visualized which is translucence white, with faint blue colour near the edges. Less pure pellets displayed increased opaqueness and size; their colour ranges from pale yellow to light brown as contamination increases. The pellets were resuspend in 0.5 to 5 ml D-PBS and the cells were further allowed to disrupt completely. The cells were then filtered through a 0.2-µm filter and stored at 4°C.

### Viral susceptibility and storage

White spot syndrome viruses (WSSV) were used to test viral susceptibility of the SF9 cell line. Eighty percent confluent monolayers of SF9 cells were infected with WSSV at a MOI of approximately 0.1. Cytopathic effects (CPE) caused by the viruses were observed daily using an inverted light microscope. For virus titration, 0.1 ml of 10-fold serial dilutions of virus was inoculated into four wells of sub confluent cells in a 24-well plate (NUNC™ Brand Products). The cultures were incubated for seven days at 27°C, and CPE was recorded daily. The virus titres were expressed as the 50% tissue culture infective dose ($TCID_{50}$) according to the method of Reed and Muench (1938).

Virions are quite stable in standard serum-supplemented growth media. They maintained their integrity and infectious competency for days at elevated temperatures, weeks at room temperature, and months to years at 4°C. The virions were stored for longer than 3 months under serum-free conditions by adding 0.1 to 1% BSA to stabilize the virus. The virus stocks were stored in polypropylene containers to prevent nonspecific binding of virus. They were retitered periodically if used as inoculates. Loss in virus titter were minimal (<10%) with this protocol. Virus-containing supernatant was transferred to a sterile, capped centrifuge tube and centrifuged for 5 min at 500 g and decanted. The supernatant was then filtered using a 0.2-µm, low-protein binding filter. The sterile-filtered supernatant was stored in cryotubes and the virus stocks were stored at 4°C and stored in dark conditions avoiding the exposure of light.

### Viral DNA extraction and PCR analysis from infected and control cell lines

Genomic DNA extraction was done using DNA isolation kit from Bioserve Biotechnologies India Private Limited for the infected samples and control ones. Briefly, the procedure is outlined as viral replicative DNA intermediates were isolated from whole cell lysates. Cells recovered after trypsinization and one wash were lysed for 16 h at 37°C in lysis buffer (10 mM Tris▪HCl, pH 7.4/0.5% SDS/10 mM EDTA, pH 7.4/10 mM NaCl) supplemented with proteinase K (200 µg/ml). The covalently closed circular DNA form was selectively extracted from cells recovered by trypsinization. Cells were lysed at room temperature in a lysis buffer not supplemented with proteinase K. In both cases, cellular DNA was precipitated overnight at 4°C with 1 M NaCl. Nucleic acids were extracted after an overnight lysis at 37°C in lysis buffer supplemented with tRNA (40 µg/ml) and proteinase K (200 µg/ml). DNA was extracted by phenol chloroform and precipitated by isopropanol. Nucleic acids were analyzed by gel electrophoresis procedure on a 1.5% agarose gel containing ethidium bromide (1 µg $ml^{-1}$). The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-Rad Laboratories,USA).

For PCR analysis, a 1.0 µl of sample DNA (approximately 100 ng/µl) was added to PCR mixture containing 100 mM Tris HCl (pH 8.3), 500 mM KCl (pH 8.3),2.0 µl $MgCl_2$ (25 mM), 2.0 µl dNTP's (2.5 mM), 1.0 µl primer forward and reverse (each of 10 pm/µl) and 1 u/µl of Taq Polymerase. The PCR was conducted in Gene Amp PCR System 9700 (Applied Biosystem, USA). The amplification condition for WSSV was one cycle of 94°C for 5 min then 38 cycles of 94°C for 30 s , 55°C for 30 s, 68°C for 45 s and 68°C for 7 min. The PCR products (6 - 10 µl) were separated by electrophoresis in 2% agarose gels containing ethidium bromide (1 µg $ml^{-1}$). The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-Rad Laboratories,USA).

## RESULTS

The cell culture was successfully developed from insect cell SF9 in serum-free medium (SFM). After seeding the SF9 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. The cells were observed to be spherical in shape. The primary cell culture could be maintained for 12 weeks with seven passages without any undesired effect on the cells. The cytopathic effects was observed in the primary cell culture infected by WSSV. Clumping of cells in a circular fashion and focal lesions of CPE could be observed in 24 h as shown in Figure 1. At 48 h, the cells appeared shrunken in places that showed clumping in 24 h (Figure 2). At 72 h, detachment of the cell clumps and appearance of circular clear areas without cells due to cell lysis occurred (Figure 3). At 96 h, clear circular areas increased in number (Figure 4). Complete "peeling off" cell layer from the culture vessel and scattered abnormal enlarged cells

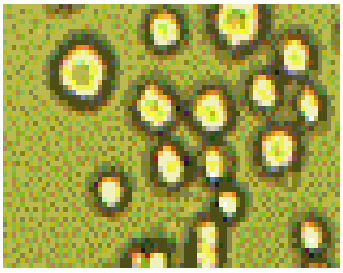

**Figure 1.** SF9 insect cells showing CPE 24 h infection with WSSV extract from infected shrimp gill tissue.

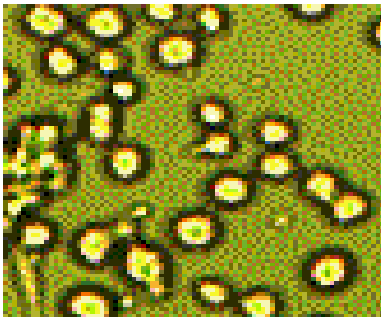

**Figure 2.** SF9 insect cells showing CPE 48 h post infection with WSSV extract from infected shrimp gill tissue.

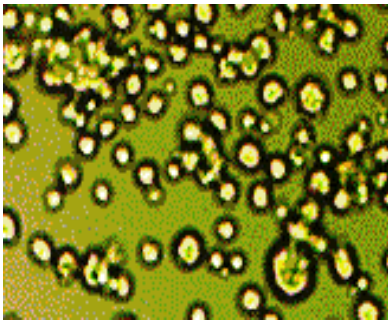

**Figure 3.** SF9 insect cells showing CPE 72 h post infection with WSSV extract from infected shrimp gill tissue.

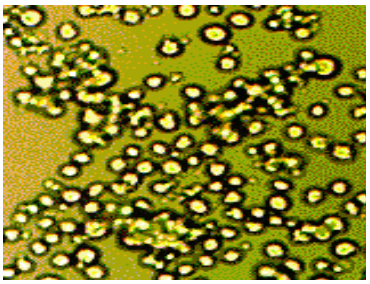

**Figure 4:** SF9 insect cells showing CPE 96 h post infection with WSSV extract from infected shrimp gill tissue.

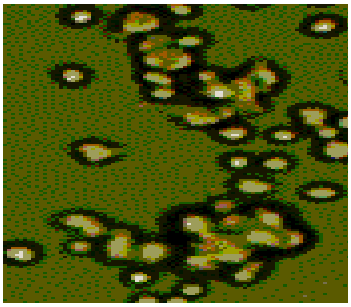

**Figure 5.** SF9 insect cells showing CPE 120 h post infection with WSSV extract from infected shrimp gill tissue.

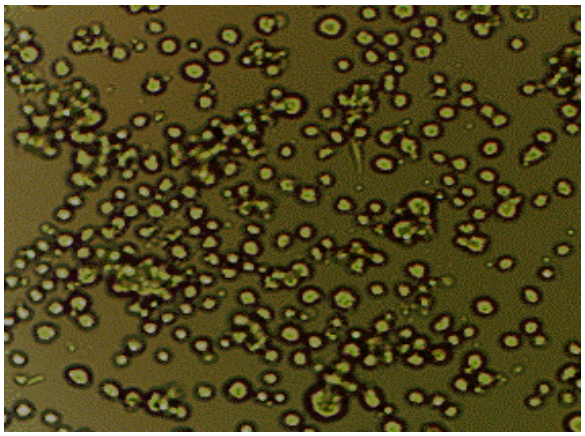

**Figure 6.** Normal SF9 cells after 4 days.

could be observed in 120 h (Figure 5). Normal SF9 Cells and WSSV Infected SF9 Cells after four days is shown in (Figures 6 and 7).

The infected cell cultures were frozen at -70°C and thawed thrice for harvesting the cells. Cytopathic effect (CPE) was observed in infected cell lines within 2-5 days post infection (PI) in contrast to healthy SF9 cells which served as control. The infected SF 9 cells were swollen and peeled off from the surface of flask, and their cytoplasm was full of particles inside. Nuclei were peripheral, broken and membrane of dead cells was shrunk. Cell mortality has reached approximately 100% after five to six days period. Infectivity assays for WSSV were conducted

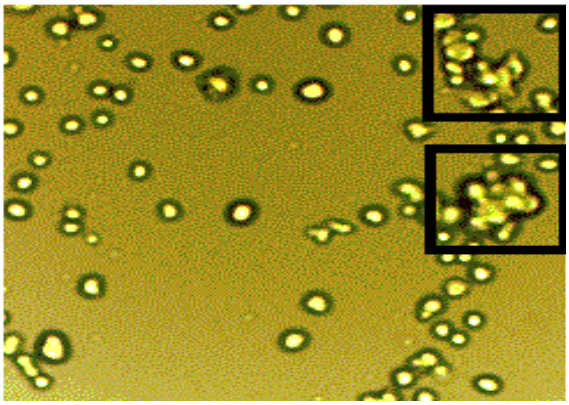

**Figure 7.** WSSV infected SF9 cells after 4 days.

**Table 1.** PCR primers for target genes and their amplification size.

| Species | Target gene | PCR primer's sequences (5'-3') | Amplicon Size | Reference/genebank accession no |
|---|---|---|---|---|
| WSSV | WSSV-419 like protein | *WSSV F1:* TGGCATGACAACGGCAGGAGT<br>*WSSV R1:* CGAGCTGCCTTGCCGGAAATTA | 550 bp | JN165706<br>JN165706 |

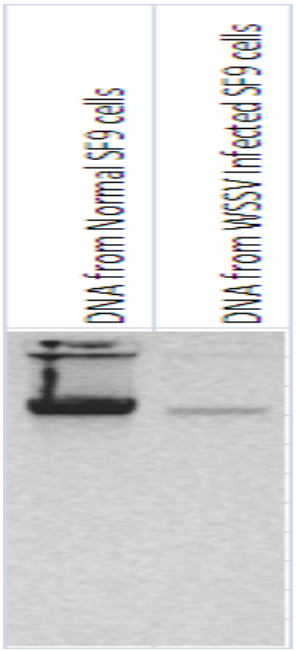


**Figure 8.** The DNA bands were visualized under ultraviolet transilluminator. Lane 1- DNA from uninfected normal SF9 cells; Lane 2- DNA from WSSV infected SF9 cells.

*in vitro* using insect SF9 cells cultures prepared from moth insect *Spodoptera frugiperda.*

Infection with WSSV resulted initially in the appearance of focal areas of CPE as early as 2 day post infection (p.i). As the infection progressed, the affected cells rounded up and the CPE extended. Finally, the cells would detached from the surface of the culture vessel resulting in a plaque-like clearing in affected areas. No CPE was observed in the normal SF9 cell culture controls. However, when the cells of SF9 cells was confirmed to be infected by the WSSV cultured for 96-120 h, so many CPE foci (Figures 4 and 5) were observed.

An aliquot of the fluid from the infected culture was used to confirm the presence of WSSV by polymerase chain reaction using PCR primers for target genes (WSSV-419 like protein) and their amplification size (Gene bank accession No: JN165706 & JN165706) is shown in Table 1. The WSSV positive result was shown as 550 bp in SF9 cell line and control sample (Figure 8). The PCR products (6 - 10 µl) were separated by electrophoresis in 2% Agarose gels containing ethidium bromide (1 µg $ml^{-1}$). The WSSV positive SF9 cell line sample and control showed 550 bp in lanes 2 and 4 (Figure 9). The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-Rad Laboratories,USA).

## DISCUSSION

Research on crustacean viruses is hampered by the lack of continuous cell lines susceptible to them. To overcome this problem, previously challenged immortal insect moth

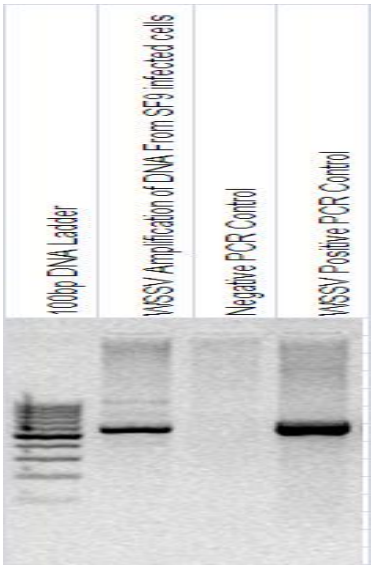


**Figure 9.** PCR amplification results of WSSV infected cell line; Lane 1- 100 bp DNA ladder (100 - 1000 bp); Lane 2- Insect cell line SF9 sample heavily infected with WSSV; Lane 3- Negative PCR control; Lane 4- WSSV positive PCR control.

and *Spodoptera frugiperda* cell lines with shrimp white spot syndrome virus (WSSV), followed by serial, split-passage of whole cells, showed that this produced cells persistently expressed WSSV antigens (Gangnonngiw et al., 2010). The study was done to determine whether such insect cultures positive for WSSV antigens could be used to infect shrimp *L. vannamei* with WSSV. As such the results are comparable with studies on primary culture of hepatopancreas from *Penaeous monodon* (Uma et al., 2002), *P. orientalis* (Ke et al., 1990), *Palaemontes argentinus* (Sousa et al., 2005), *Marscupenaeus japonicus* (Zilli et al. 2003).

Various studies on initiating primary cell cultures from the heap-topancreas 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 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.

The present study showed that susceptibility was observed when monolayer cell sheet derived from normal insect cells SF9 of *L. vannamei* was infected with WSSV. In contrast, other studies reported that *P. stylirostris* and *P. vannamei* Oka cell were susceptible to a non-occluded baculo-like virus (possibly similar to WSDV) (Lu et al., 1995) and YHV (Tapay et al., 1997) respectively, 2-3 days after incubation. However, this experiment showed that CPE foci were only observed in the cell culture derived from WSSV infected SF9 cells 2-4 days after formation of confluent cell sheet. A similar result was obtained when ovary of *P. monodon* was cultured (Chen et al., 1986). There was survival of the lymphoid tissue and ovary tissue duction of shrimp baculoviruses. Cell lines derived from *Culex tritaeniorhynohurs*, *Culex salinarius*, *Spodoptera frugiperda* and *Armigeres subalbatus* have been exposed to *Baculvoirus penaei* (BP), a related shrimp baculovirus (Summers et al., 1977). Unfortunately, no successful viral infection was obtained in these insect cell lines. As compared to the *in vivo* system, an *in vitro* culture system is equally important for the detailed study of animal viruses. Because no cell lines have been established from shrimp, several insect cell lines have been investigated for the replication and production of shrimp viruses.

The lack of continuous cell lines of shrimps makes us not to be able to fully understand the infections of shrimp pathogenic viruses that often cause serious damage to aquaculture industries. Nevertheless, several investigators have demonstrated that the primary cell cultures were susceptible to certain shrimp viruses (Chen and Kou, 1989; Lu et al., 1995; Tapay et al., 1997). Itami et al. (1999) reported that WSSV induced CPE on primary cultures of lymphoid organs from the kuruma shrimp, *M. japonicus,* within 8 days. In our preliminary study, primary cultures of the SF9 insect cells were also permissive for WSSV infection. Thus, the primary culture system developed in the present study will facilitate large-scale *in vitro* experiments, including bioassay of virus infectivity and biochemical characterization of virus infections. 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 at 550 bp. Similarly, WSSV infected cell culture fluid exhibiting moderate infection are 356 and 232 bp host DNA and WSSV infected cell culture fluid exhibiting heavy infection are 403, 356 and 232 bp host DNA (Uma et al., 2002). From the available reports, this study appears to be the first report on the CPE of WSSV in primary cell culture derived from the insect cells of moth. The inadequacy of the methods for cell line establishment tested in the present study may suggest that more growth factors are needed for the establishment of shrimp virus cell lines.

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