Primary Shrimp Cell Culture: Applications for Studying White Spot Syndrome Virus (WSSV)

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ABSTRACT: Primary shrimp cell culture from lymphoid organs of *Peneaus monodon* was successfully developed in our laboratory. Minced tissues of lymphiod organs were seeded and cultured in 2x Liebovitz-15 supplemented with 15% fetal bovine serum, 10% shrimp meat extract, and a salt mixture, with an osmolarity of 710-730 mmol/kg. Plates were then incubated at 28°C until 70-80% cell monolayers were formed. Even though these primary lymphoid cell cultures could not be subcultured, they proved to be useful for studying shrimp viruses which could induce unequivocal cytopathology in them. Cytopathic effect (CPE) induced by white spot syndrome virus (WSSV) showed characteristics of cell rounding, detachment and lysis. Quantitative analysis of WSSV infected *P. monodon* was determined using primary lymphoid cell systems. Among those tissues and organs observed, cuticular epidermis gave the highest titer of $10^{8.25}$ $TCID_{50}$/ml. Pathogenesis of shrimp viral infections and the relative virulence of WSSV infecting various species of crustaceans were also examined using this primary shrimp cell culture system.

KEY WORDS: primary shrimp cell culture, white spot syndrome virus (WSSV), *P. monodon*

INTRODUCTION

Disastrous failures have occurred in the shrimp farming industry in Thailand in the past decade mostly due to virus infection. One of the most serious viruses (known in Thailand as systemic ectodermal and mesodermal baculovirus or SEMBV) causes reddish body discoloration and white spots or patches on the inside surface of the carapace and shell of affected shrimp (Kasornchandra et al. 1995; Wongteerasupaya et al. 1995). Similar viruses with similar disease symptoms have now been reported elsewhere throughout Asia (Flegel 1997). This virus has been called white spot syndrome baculovirus (WSSV) by Lightner (1996) and that abbreviation is now widely used but to stand for white spot syndrome virus (i.e., the “baculo” prefix is usually dropped since the relationship of the virus to baculoviruses is now widely questioned). Therefore, in this paper we will use WSSV instead of SEMBV. This virus is a DNA virus of bacilliform to cylindrical morphology with an average size of 120x275±22 nm (Kasornchandra et al. 1995; Wongteerasupaya et al. 1995). Characterization of this virus is based on histological observations, electron microscopy and molecular studies. However, to study its infectivity, one has to rely on *in vivo* bioassay which requires large numbers of animals. Although much new information is available for this virus, many issues remain to be examined, and especially those relating to interaction between this virus and its host. These studies are difficult due to the lack of shrimp cell lines for production of the virus and for *in vitro* studies. Although numerous attempts have been made, no marine shrimp cell line has yet been established. Recently, the successful development of primary shrimp cell cultures has been reported from many laboratories (Chen et al. 1986, Hsu et al. 1995, Luedeman & 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 YHV and China Baculo-like virus (CBV) using primary lymphoid cells of the white shrimp *Penaeus vannamei* and *P. stylirostris*.

In this present study, we prepared primary shrimp cell cultures derived from the lymphoid organ of *P. monodon* and we also demonstrated the use of these primary shrimp cell cultures for propagation of WSSV, for a viral titration assay, for determining the WSSV tissue and organ specificity in *P. monodon*, and for determining the relative virulence of WSSV to various species of crustaceans.

MATERIALS AND METHODS

Primary cell culture preparation

Primary culture of shrimp lymphoid cells are prepared according to the method of Kasornchandra et al. (1998) with slight modifications. Briefly, lymphoid organs were obtained from adult black tiger shrimp *P. monodon* (40-50 g), washed three times in cold antibiotic-buffered mixture consisting of 1,000 IU/ml penicillin, 1,000 µg/ml streptomycin, 250 µg/ml gentamicin, 250 µg/ml Fungizone, and 1M phosphate buffered saline (PBS). After a final wash and further incubation for 10 min in cold antibiotic-buffered solution, the lymphoid organs were then minced to small fragments of approximately 1 mm^3. Four or five tissue fragments were transferred to each well in 24-well plates and 1 ml of the culture medium containing 2x L-15 supplemented with 15% FCS, 10% shrimp meat extract (SME), 1% glucose, 5 g/L NaCl, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml

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Fungizone, osmolarity 720±20 mmol/kg, was added to each well. Plate was then sealed and incubated at 28°C until 70-80% confluent monolayers were formed. These cells were then ready for propagation of the virus. Before propagation of the virus, 0.1 ml of medium in each well was replaced with fresh culture mediim containing 5% FCS. The plate was then ready for use.

Propagation of WSSV

Primary shrimp cell cultures were examined for thier 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 *P. monodon* experimentally infected with WSSV was homogenized in 2xL-15 medium and filtered through a 0.2 µm sterile membrane. The virus suspension was then diluted 100 times in 2xL-15 medium and 10 µl of the diluted suspension was inoculated onto primary shrimp 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). To confirm viral propagation in the shrimp cells, they were fixed with 6.25% glutaraldehyde in cacodylate buffer pH 7.2 and processed for examination with the electron microscopic.

Titration of WSSV

The viral titer was determined in primary lymphoid cell cultures by end-point dilution assay ($TCID_{50}$/ml) according to the method of Rovozzo and Burke (1973). Primary shrimp lymphiod cells were prepared in 96-well plates as described by Lu et al. (1996). Briefly, two to three tissue fragments were seeded in wells of a 96-well plate and a drop of culture medium was added to each well. The plate was then sealed and incubated at 28°C until 70-80% confluent monolayers were formed. The cells were then ready for virus titration. A day before performing the quantitative bioassay, 0.1 ml of medium in each well was exchanged with fresh culture medium containing 10% FCS and the plate was then incubated at 28°C until used. Viral suspension was prepared as previously mentioned. The 10-fold diluted WSSV suspension was diluted serially in 2x L-15 medium, and 0.1 ml of each dilution (10^{-2}-10^{-8}) was then inoculated into 3 wells of a confluent lymphoid cells monolayer. Control wells were inoculated with the same amount of normal gill tissue extract in inoculation medium. The plate was then sealed and incubated at 28°C for 7 days and examined daily for cytopathic effect (CPE). Dilution end-points were calculated by the method of Reed and Muench (1938).

Pathogenesis of WSSV infected shrimp

Specificity of tissues and organs of *P. monodon* for WSSV in experimentally infected shrimp was determined using primary lymphoid cells. WSSV suspension was prepared from gills, lymphoid organs, cuticular epidermis, hepatopancreas, heart, hemolymph, eyestalks, muscle and perieopods of infected animals as previously described. A bacterial-free suspension of these homogenated WSSV infected tissues and organs (0.1 ml) was added separately to sterile test tubes containing 0.9 ml 2xL-15 medium, and then serially diluted 10-fold. A portion (0.1 ml) of each dilution was added to tissue culture wells (3 wells each) followed by incubation for 10 days at 28°C to determine the viral titer.

Virulence of WSSV to various species of crustaceans

The infectivity of WSSV to various species of crustacean was conducted in aquaria at the Marine Shrimp Research and Development Center (MSRDC), Songkhla. Four species of penaeid and metapenaeid shrimp (*P. monodon, P. merguiensis, Metapenaeus ensis* and *M. brevicornis*) and three species of crabs (*Portunus pelagicus, Scylla serrata* and *Sesarma* spp.) collected from uninfected shrimp ponds and reservoirs inside the center and nearby were injected intramuscularly with $10^{6.0}$ $TCID_{50}$/ml at 0.1% body weight (ten shrimp or crabs of each species per dosage). Control shrimp and crabs were injected with L-15 basal medium. Both injected and control animals were kept separately in 30L aquaria equipped with aeration. Chlorinated sea water of 28-30 ppt salinitywas changed daily (75%). Experimental shrimp and crabs were fed 3 times daily. The animals were observed daily for clinical signs for 10 days. Dead and moribund shrimp and crabs were recorded and removed. In order to confirm WSSV infection, portions of the crustaceans were homogenated immediately after death in 2x L-15 medium. The homogenate was then filtered and inoculated onto primary shrimp cell cultures. Moribund shrimp and crabs were also fixed in Davidson's fixative and then subjected to histological preparation. At the termination of the experiment, all remaining crustaceans were subjected to histological analysis and shrimp cell culture assays.

RESULTS

Cells obtained from the lymphoid organ tissue fragments exhibited fibroblastic-like morphology by 18 h post-seeding. After 3-4 days, 70% confluent monolayers were achieved and these cells could be maintained for 8-10 days without changing medium.

Infectivity of WSSV in primary lymphoid cells showed unequivocal cytopathology in which the cells became rounded followed by detachment and lysis. (Fig. 1). The completion of cells lysis occurred within 4-5 days post-inoculation. No CPE was observed in control wells (Fig. 2). To confirm the cause of primary lymphoid cells lysis, infected cells were processed and subjected to electron microscopy. The results revealed the presence of a bacilliform virus having the same size as WSSV in nucleus of the infected cells.

Titration of the WSSV in primary lymphoid cells was $10^{8.0}$ $TCID_{50}$/ml. This titration was repeated twice and similar results were obtained. The recovery of infectious WSSV from selected tissues and organs of infected *P. monodon* is shown in Table 1. Among those tissues and organs examined, cuticular epidermis gave the highest titer of $10^{8.5}$ $TCID_{50}$/ ml., followed by hemolymph and gills. Perieopods, eyestalks, muscle and heart produced moderate titers ranging from $10^{6.0}$-$10^{5.0}$ $TCID_{50}$/ml. Hepatopancreatic tissue gave the lowest titer of $10^{4.0}$ $TCID_{50}$/ml.

The infectivity of WSSV to various types of crustaceans was investigated and compared. *P. monodon* and *P. merguiensis* were the most susceptible to WSSV infection. They produced clinical symptoms after 4 days post-injec-

Figure 1. Primary lymphoid cell culture showing cytopathic effect (CPE) 5 days p.i. with WSSV extract from infected shrimp gill tissue.

Figure 2. Primary lymphoid cell culture 5 days after inoculation with extract from normal shrimp gill tissue.

Table 1. Titer of WSSV recovered from selected tissues and organs of experimentally infected *P. monodon.*

Tissue/Organ	$TCID_{50}$/ml
Cuticular epidermis	$10^{8.25}$
Gills	$10^{7.75}$
Hemolymph	$10^{7.50}$
Lymphoid organ	$10^{7.00}$
Perieopods	$10^{6.00}$
Eye-stalk	$10^{5.50}$
Muscle	$10^{5.25}$
Heart	$10^{5.00}$
Hepatopancreas	$10^{4.00}$

tion and all shrimp died within 9 days post-injection (Table 2). *Metapenaeus ensis* and *M. brevicornis* were also susceptible to WSSV infection. However, some of these shrimp still survived 10 days post-injection (Table 2). *Portunus pelagicus, Scylla serrata* and *Sesarma* spp. were also infected by WSSV. Only a few crabs (*Portunus pelagicus* and *Scylla serrata*) died within 7 and 9 days post-injection. The others still survived 10 days post-injection (Table 2). Only one mangrove crab, *Sesarma* spp., died during this experiment. Histological examination of the moribund and surviving shrimp revealed the presence of hypertrophied nuclei in various tissues of ectodermal and mesodermal origin. The characteristics of CPE induced by their tissue extracts in primary shrimp cells were also similar to those induced by WSSV extracts as previously described. Interestingly, high numbers of hypertrophied nuclei were observed in the infected tissues of surviving crabs.

DISCUSSION

Primary shrimp cell cultures obtained from lymphoid organs of *P. monodon* were successfully developed in our laboratory. These cells can be maintained up to 10 days without changing medium. This is enough time to conduct viral titration assays. The study also demonstrated that our primary lymphoid cells supported growth of WSSV and that CPE induced by this virus exhibited cell rounding followed by detachment and lysis. Similar CPE was reported by Tapay et al. (1997) for Chinese Baculo-like virus or CBV (which produced white spot disease syndrome in China) in primary lymphoid cells derived from lymphoid organs of the white shrimp, *P. stylirostris*. Moreover the viral titer produced in their primary lymphoid cells was as high as that for our primary cells (i.e., giving a $TCID_{60}$ titer of $10^{8.0}TCID_{50}$/ml).

Table 2. Percent survival of seven species of crustaceans after experimental injection with WSSV. Number of animals in each group = 10.

Crustaceans	Days post injection									
	1	2	3	4	5	6	7	8	9	10
Penaeus monodon	100	100	100	100	80	60	20	10	0	0
P. merguiensis	100	100	100	100	100	70	50	20	0	0
Metapenaeus ensis	100	100	100	100	100	80	80	60	40	20
M. brevicornis	100	100	100	100	100	100	80	60	50	30
Portunus pelagicus	100	100	100	100	100	100	80	80	70	60
Scylla serrata	100	100	100	100	100	100	100	90	80	70
Sesarma spp.	100	100	100	100	100	100	100	100	100	90

Susceptibility of tissues and organs of *P. monodon* supporting growth of WSSV was determined in primary shrimp cells. The amount of infectious WSSV recovered was highest from cuticular epidermis followed by hemolymph and gills suggesting that those organs greatly support viral multiplication and would enhance the recovery of WSSV. A similar finding was reported by Tapay et. al. (1997) who demonstrated that cuticular epidermis of experimentally infected *P. stylirostris* was the most susceptible target for CBV multiplication, followed by gills and hemolymph. Thus, those tissues and organs seem to be the best choice for virus isolation.

Many scientists have reported that WSSV is capable of infecting various species of crustaceans including larval insects (Kasornchandra et al. 1997, Lo et al. 1997, Supamattaya et al. 1998). However, the relative virulence of this virus to the various species of crustaceans has not yet determined, due to the lack of cell lines for *in vitro* studies. We described here a method using primary shrimp cell cultures for viral quantitation to determine relative virulence of WSSV for four species of shrimp and three species of crabs. The results suggest that *P. monodon* and *P. merguiensis* are the most susceptible hosts for WSSV infection while the two species of metapenaeid shrimp, *M. brevicornis* and *M. ensis*, were moderately susceptible hosts. The shrimp survivors also showed typical characteristics of hypertrophied nuclei in infected tissues. For the three species of crabs tested (*Portunus pelagicus*, *Scylla serrata* and *Sesarma* spp.), most still survived 10 days post-injection. However, histological examination showed high levels of infection but little tissue damage in these survivors. Therefore, they may be implicated as carriers for white spot disease. Even though no continuous shrimp cell lines are available as yet, primary lymphoid cell cultures established not only in our laboratory but elsewhere have proven to be useful for studying shrimp viruses *in vitro* (Chen and Kou 1989, Lu et al. 1996a,b, Tapay et. al. 1997).

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