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Lymphoid organ cell culture system from *Penaeus monodon* (Fabricius) as a platform for white spot syndrome virus and shrimp immune-related gene expression

S Jose[1], P Jayesh[1], N S Sudheer[1], G Poulose[1], A Mohandas[1], R Philip[2] and I S Bright Singh[1]

1 National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi, India
2 Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, India

Abstract

Shrimp cell lines are yet to be reported and this restricts the prospects of investigating the associated viral pathogens, especially white spot syndrome virus (WSSV). In this context, development of primary cell cultures from lymphoid organs was standardized. Poly-L-lysine-coated culture vessels enhanced growth of lymphoid cells, while the application of vertebrate growth factors did not, except insulin-like growth factor-1 (IGF-1). Susceptibility of the lymphoid cells to WSSV was confirmed by immunofluoresence assay using monoclonal antibody against the 28 kDa envelope protein of WSSV. Expression of viral and immune-related genes in WSSV-infected lymphoid cultures could be demonstrated by RT-PCR. This emphasizes the utility of lymphoid primary cell culture as a platform for research in virus–cell interaction, virus morphogenesis, up and downregulation of shrimp immune-related genes, and also for the discovery of novel drugs to combat WSSV in shrimp culture.

Keywords: BrdU assay, immune-related genes, immunofluorescence, lymphoid cell culture, *Penaeus monodon*, white spot syndrome virus genes.

Introduction

Penaeid shrimp culture is globally in sixth position in terms of value among all taxonomic groups of animals cultivated (FAO 2006). *Penaeus monodon* (Fabricius), one of the most widely cultured penaeid shrimps, is affected by several diseases, among which white spot disease caused by white spot syndrome virus (WSSV) causes the greatest damage. The virus causes total devastation of shrimp culture within 3–7 days of infection (Mamoyama, Hiraoka, Nakona, Koube, Inouye & Oseko 1994; Hao, Thuy, Loan, Phi, Phuoc, Duong, Corsin & Chanratchakool 1999) and almost every species of penaeid shrimp is susceptible. Moreover, the virus can affect other marine, brackish and freshwater crustaceans (Lo, Ho, Peng, Chen, Hsu, Chiu, Chang, Liu, Su, Wang & Kou 1996; Flegel 1997, 2006). WSSV has been recently re-classified as belonging to the family Nimaviridae (genus *Whispovirus*). The virions are ellipsoid to bacilliform-enveloped particles, with a distinctive tail like appendage at one end. The WSSV genome is a large circular dsDNA of approximately 300 kbp (van Hulten, Witteveldt, Peters, Kloosterboer, Tarchini, Fiers, Sandbrink, Lankhorst & Vlak 2001; Yang, He, Lin, Li, Pan, Zhang & Xu 2001). Three WSSV isolates from China (accession no.AF332093), Thailand (accession no. AF369029) and Taiwan (accession no. AF440570) have been completely sequenced with genome sizes of 305, 297 and 307 kbp, respectively. The ICTV whispovirus study group committee recently has chosen the China isolate, WSSV-CN as the type strain (Leu, Tsai & Lo 2008).

Cell cultures are being applied to standardize and validate diagnostic methods and to develop new, safe therapeutants and effective disease control methodologies (Villena 2003). *In vitro* cultures are

Correspondence *I S Bright Singh, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, India (e-mail: bsingh@md3.vsnl.net.in)*

Journal of Fish Diseases

used for the manufacture and characterization of antiviral vaccines (Cox & Hollister 2009; Paillet, Forno, Kratje & Etcheverrigaray 2009), novel drugs (Brandon, Sparidans, Meijerman, Manzanares, Beijnen & Schellens 2004; Deshpande, Nagarkatti, Sombati & DeLorenzo 2008) and bioactive compounds (Brocal, Falco, Mas, Rocha, Perez, Coll & Estepa 2006; Jayaprakasha, Mandadi, Poulose, Jadegoud, Gowda & Patil 2007). In spite of extensive investigations on shrimp viruses, lack of a valid shrimp cell line has hampered research in elucidating pathogenesis and morphogenesis, as well as the development of prophylactic and therapeutic measures. Thus, researchers have attempted to apply insect cell lines for the study of these viruses. WSSV replication was reported in the Sf9 insect cell line (Chien, Hang, Van, Hanh, Xuan, Minh, Linh & Binh 2006; Nupan, Phongdara, Saengsakda, Leu & Lo 2011) and the persistent expression of viral proteins of WSSV and yellow head virus (YHV) in infected cells of Sf9 and mosquito cell line, C6/36, for up to several passages was described (Sriton, Kanthong, Gangnonngiw, Sriurairatana, Ubol & Flegel 2009). Recently, successful propagation of YHV was reported in C6/36, and whole-cell homogenates from passage 5 were shown to induce yellow head disease in *P. monodon* (Gangnonngiw, Kanthong & Flegel 2010). Genome replication and expression of viral capsid protein of *Macrobrachium rosenbergii* nodavirus (*Mr*NV), which is the causative agent of white tail disease in freshwater prawn, was detected in the SSN-1 fish cell line (Hernandez-Herrera, Chappe-Bonnichon, Roch, Widada & Bonami 2007). Replication of *Mr*NV was also reported in C6/36 cells by Sudhakaran, Parameswaran & Hameed (2007).

Shrimp primary cell cultures were reported by several researchers from various tissues such as lymphoid (Chen & Kou 1989; Tapay, Lu, Brock, Nadala & Loh 1995; Chen & Wang 1999; Lang, Nomura, Wang & Matsumura 2002), ovary (Chen, Chi, Kou & Liao 1986; Leudeman & Lightner 1992; Shimizu, Shike, Klimpel & Burns 2001; Maeda, Mizuki, Itami & Ohba 2003), hepatopancreas (Ghosh, Ray & Dasmahapatra 1995), heart (Tong & Miao 1996; Chen & Wang 1999) and embryos (Frerichs 1996; Toullec, Crozat, Patrois & Porcheron 1996; Fan & Wang 2002). Primary cell cultures of lymphoid organ (Lu, Tapay, Loh, Brock & Gose 1995; Wang, Yang, Tang, Lu, Kou & Lo 2000), ovary (Maeda, Saitoh, Mizuki, Itami & Ohba 2004) and haemocytes (Jiang, Zhan, Wang & Xing 2005; Jose, Mohandas, Philip & Bright Singh 2010) were used for the study of WSSV *in vitro*. Nevertheless, no attempt has been made, except by Jose *et al.* (2010) in haemocyte culture, to employ primary cell cultures as tools for investigating the expression of virus and shrimp immune-related genes *in vitro* during WSSV infection which would throw light on the pathogenesis of the virus and the immune response of shrimp.

In this study, an attempt was made to develop primary cell cultures from lymphoid tissues of *P. monodon* and to demonstrate them as a platform for WSSV and WSSV-induced immune-related gene expression.

Materials and methods

Experimental animals

White spot syndrome virus (WSSV) and monodon baculovirus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a recirculating aquaculture system for shrimp with 15 g L^{-1} salinity integrated with nitrifying bioreactors (Kumar, Achuthan, Manju, Philip & Bright Singh 2009). Detritus in the system was managed by the addition of Detrodigest ™ [National Centre for Aquatic Animal Health (NCAAH), Kochi] and Enterotrophotic ™ (NCAAH) to control *Vibrio* at the rate of 100 mL m^{-3} of water. The larvae were fed with commercially available pelleted feed (Higashimaru). They were confirmed WSSV-negative by nested PCR (WSSV detection kit, Genei) after a period of 3 months when they grew to 8–12 g and used as the donor animals for lymphoid organs.

Surface sterilization of the animals

The animals were sacrificed by immersion in crushed ice and disinfected in 800 mg L^{-1} sodium hypochlorite solution prepared in ice cold sea water (salinity 15 g L^{-1}) for 10 min. Subsequently, they were washed five times in sterile ice cold sea water, dipped in 70% alcohol prepared in distilled water, rinsed again in ice cold sea water, and used for the removal of tissue aseptically.

Development of primary cell culture of lymphoid organ

The lymphoid organ consists of two distinct lobes located ventro-anterior to the hepatopancreas. Five

shrimps were dissected to remove the organ and the tissue pieces were immediately immersed in Hanks balanced salt solution. Before seeding, tissue pieces were washed three times, cut into 1 mm^3 pieces, pipetted vigorously and seeded into 25 cm^2 flasks. One of the media used was Grace's Insect Medium (Sigma Aldrich) supplemented with magnesium chloride hexahydrate (2 g L^{-1}), 10% aqueous sodium bicarbonate (3.5 mL L^{-1}), L-proline (20 mg L^{-1}), sucrose (200 mg L^{-1}), trehalose (200 mg L^{-1}), foetal bovine serum (10%), fish muscle extract (4%), shrimp muscle extract (4%), shrimp haemolymph (8%), 0.06 μg mL^{-1} chloramphenicol, 100 μg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin. Osmolality was 720 ± 10 mOsm. Fish muscle extract was prepared from *Clarias gariepinus* muscle tissue. Tissue (10%, w/v) was macerated in PBS, centrifuged at 10 000 ***g*** for 20 min, incubated at 56 °C for 30 min, centrifuged to remove the coagulated proteins, passed through polyvinylidene fluoride membrane (PVDF, 0.22 μm) (Millipore) and maintained at −20 °C until use. For preparing the shrimp muscle extract (10%, w/v), muscle tissue was isolated from *P. monodon*, after removing the cephalothorax region. The same protocol for the preparation of fish muscle extract was followed. Haemolymph was withdrawn asceptically from healthy *P. monodon* using capillary tubes containing 100 μL anticoagulant (Tris–HCl 0.01 M; pH 7, sucrose 0.25 M, tri sodium citrate 0.1 M) from the rostral sinus. After diluting the haemolymph with PBS (1:1), the same protocol for the preparation of fish muscle extract was followed. Cultures were incubated at 25 °C as a closed system and observed daily under an inverted phase contrast microscope (Leica Microsystems). Leibovitz's L-15 (Sigma Aldrich) at double strength supplemented with 2% glucose, 20% FBS, 0.06 μg mL^{-1} chloramphenicol, 100 μg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin (osmolality: 720 ± 10 mOsm was also used). Given the better performance of cells in L-15-based medium than in Grace's Insect Medium, the former was used for all further experiments. Performance of the primary cell culture in 2X L-15 basal medium and the modified 2X L-15 medium supplemented with MEM Vitamins (1X) (Biochrom AG) and tryptose phosphate broth (2.95 g L^{-1}) was compared. Comparative analysis of cellular metabolic activity was accomplished through MTT reduction assay on 3- and 5-day-old lymphoid cell cultures grown in 96-well plates (Greiner Bio One) in triplicates. Lymphoid tissues from five shrimps were pooled to seed a microwell plate.

MTT assay

The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilized yielding a purple-coloured solution (Mosmann 1983; Berridge & Tan 1992).

After replacing the medium, a sample of 50 μL of MTT (Sigma Aldrich) solution (5 mg mL^{-1} in PBS; 720 mOsm) was added to each well and incubated for 5 h in the dark. The control consisted of the medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200 μL dimethylsulphoxide. Absorbance was recorded immediately at 570 nm in a micro plate reader (TECAN Infinite Tm, A-5082).

Effect of attachment factors on primary lymphoid cell culture

Attachment factors tested were laminin (20 μg mL^{-1}; Sigma Aldrich), fibronectin (20 μg mL^{-1}; Sigma Aldrich) and poly-L-lysine (200 μg mL^{-1}; Sigma Aldrich). Stock solutions were prepared in PBS and aliquots of 34 μL per well of each solution were added to 96-well plates. The plates were incubated at 37 °C for 2 h and the excess fluid removed. Two hundred microlitres of the growth medium (2X L-15, modified) containing explants of uniform size was introduced to the coated wells and incubated at 25 °C. The MTT assay was conducted after 48 h. Triplicates were maintained and the control wells were devoid of attachment factors.

Effect of growth factors on primary lymphoid cell culture

Growth factors tested were fibroblast growth factor-basic (bFGF, from bovine pituitary glands), epidermal growth factor (EGF, from mouse sub

maxillary glands), insulin-like growth factor-1 (IGF-1, human, recombinant expressed in *E. coli*), insulin-like growth factor-2 (IGF-2, human, recombinant expressed in *E. coli*) and transforming growth factor-β1 (TGF-β1, from porcine platelets). All growth factors were purchased from Sigma Aldrich. Primary stock solutions of bFGF and EGF were prepared in the growth medium, while IGF-1 was prepared in distilled water, IGF-2 in 10 mM acetic acid containing 10 μg mL^{-1} bovine serum albumin (BSA) and TGF-β1 in 4 mM HCl containing 1 μg mL^{-1} BSA. Primary stocks were diluted to the required concentrations with growth medium. Ninety-six-well plate cultures of lymphoid organ were prepared and growth factors added to obtain final concentrations of 10, 20 and 40 ng mL^{-1} for bFGF, 1, 10 and 30 ng mL^{-1} for EGF and 10, 25 and 50 ng mL^{-1} for IGF-1, IGF-2 and TGF-β1. The MTT assay was performed after 48 h to assess the effect of growth factors on the primary culture. Triplicates were kept for each concentration of growth factors, and the control wells were without the growth factors.

5-bromo-2′-deoxyuridine (BrdU) assay of lymphoid cell culture

A sample of 20 μL of 10 mM BrdU solution was added to each well of 96-well plate lymphoid cell cultures with 200 μL medium. Wells containing cells without BrdU were kept as controls. After 24 h incubation, the medium was removed, washed with PBS, fixed with 4% paraformaldehyde for 15 min and washed again with PBS. Two molar HCl was added to each well, incubated for 20 min, neutralized with 0.1 M sodium borate (pH 8.5) for 2 min and washed with PBS. Cells were permeabilized with PBS containing 0.2% triton X-100 and 3% BSA for 5 min. After blocking with 3% BSA in PBS for 1 h, a 1:1000 dilution (in 3% BSA) of the mouse monoclonal anti-BrdU antibody (Sigma Aldrich) was added and incubated for 1 h. Cells were washed three times with PBS for 5 min each and incubated for 1 h with rabbit anti-mouse FITC conjugate at 1:40 dilution (Sigma Aldrich). Wells were washed with PBS, stained with DAPI (0.2 μg mL^{-1}) and observed under an inverted fluorescent microscope (Leica). DAPI and FITC were viewed under filters with excitation wavelengths of 360–370 and 470–490 nm, respectively. Test wells were compared with those without BrdU (negative control). The images were processed and merged using the 'Leica Application Suite' software (Leica Microsystems).

Preparation of WSSV lysate from WSSV-infected gill tissue and infection of lymphoid cell culture

A 500 mg gill tissue sample from WSSV-infected shrimps (under laboratory conditions) weighing 8–12 g was macerated using mortar and pestle and kept in an ice bath, in 10 mL of modified 2X L-15 medium, with glass fibre wool. The extract was centrifuged at 10 000 ***g*** for 10 min at 4 °C and the supernatant passed through a 0.22 μm PVDF membrane (Millipore). The extract obtained was diluted 30 times and added to the wells with lymphoid cell culture and observed for cytopathic effects (CPE).

Immunofluorescence assay for detection of WSSV in lymphoid cell culture

For immunofluorescence detection of WSSV, lymphoid cell cultures were prepared in Leighton tubes with cover slips (10 × 22 mm; Micro-Aid) at 25 °C. After 48 h, 500 μL WSSV lysate was added to each tube and incubated for 12 h. Subsequently, cover slips were removed from Leighton tubes, washed twice in PBS (720 mOsm), immersed in 10% paraformaldehyde and maintained in 70% ethanol at −20 °C until used. These fixed cover slip cultures were used for the immunofluorescence detection of WSSV. The cover slips were attached to a glass slide, free sites were blocked using 3% BSA in PBS and incubated in a humidified chamber for 1 h. The slides were washed in PBS containing (0.01%) Tween-20 and WSSV monoclonal (C 38) antibody (Anil, Shankar & Mohan 2002) on the slide, incubated for 1 h and washed three times in wash buffer. They were again incubated for an hour after addition of rabbit anti-mouse FITC conjugate at 1:40 dilution (Sigma Aldrich) and subjected again to washing with wash buffer. After incubation with general nuclear stain DAPI (10 μL, 0.2 μg mL^{-1}) for 3 min, the slides were rinsed with distilled water, air-dried, mounted (Vectashield) and observed under a fluorescent microscope (Olympus). DAPI and FITC were viewed under filters with excitation wavelengths of 360–370 and 470–490 nm, respectively. The slides were compared with uninfected lymphoid organ cell cultures (negative control). The images were processed and merged using the 'Image pro-express' software (Media Cybernetics Inc.).

RNA isolation from WSSV-infected lymphoid cell culture

Lymphoid cell cultures prepared in 24-well plates in modified 2X L-15 medium were used for RNA extraction. After 48 h incubation, the medium was removed and 500 μL WSSV suspension added. Wells added with heat-inactivated (56 °C for 1 h) WSSV were kept as controls. RNA isolation was performed after 8 and 24 h from 10 wells each and from the control wells after 24 h. For RNA isolation, the medium was removed, wells washed with ice cold PBS, explants detached by repeated pipetting and removed, and 200 μL TRI reagent (Sigma Aldrich) added to each well containing the cell monolayer. Complete lysis of cells was allowed to take place by repeated pipetting, and the suspension was collected in microcentrifuge tubes. RNA extraction was accomplished according to the manufacturer's protocol (Sigma Aldrich). An aliquot of 0.2 mL chloroform was added to TRI reagent (1 mL), shaken vigorously for 15 s and allowed to stand for 15 min at room temperature. The resultant mixtures were centrifuged at 12 000 ***g*** for 15 min at 4 °C. The colourless upper aqueous phase was separated from the three layers formed, transferred to a fresh tube, 0.5 mL isopropanol added, stored for 10 min at RT and centrifuged at 12 000 ***g*** for 10 min at 4 °C. The supernatant was discarded and the pellet washed twice with 75% ethanol. The pelleted RNA was air-dried and dissolved in 20 μL DEPC-treated sterile water by repeated pipetting at 55 °C. These RNA samples were subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs), accomplished by adding aliquots of 0.2 units of the enzyme per microgram RNA and incubating at 37 °C for 10 min. The enzyme was inactivated at 75 °C for 10 min. Concentration and quality of RNA were measured from the absorbance at 260/280 nm in a UV-Visible spectrophotometer.

RT-PCR of WSSV and WSSV-induced shrimp immune-related genes

One microgram RNA was subjected to cDNA synthesis with 20 μL reaction mix containing M-MuLV reverse transcriptase (80 U), RNase inhibitor (8 U), Oligo $(dT)_{12}$ primer (40 pmoles), dNTP mix (1 mM), RTase buffer (1X) and $MgCl_2$ (2 mM) at 42 °C for 1 h. All reagents were purchased from New England Biolabs. Expressions of eight WSSV genes and six immune-related genes were examined. Shrimp β actin gene was also amplified as a reference. PCR using 2 μL cDNA was performed with specific primer sets as given in Table 1. Twenty-five microlitre PCR reaction mix contained 0.5 U Taq DNA polymerase, 200 μM dNTP mix, 10 pmoles each of forward and reverse primer and 1X PCR buffer. The hot start PCR program used for WSSV genes was 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing for 30 s, extension at 68 °C for 30 s followed by final extension at 68 °C for 10 min. Annealing temperatures were 50 °C for endonuclease (*endnu*), 53 °C for latency 1 and ribonucleotide reductase 1 (*rr*1), 54 °C for DNA polymerase (*dnapol*), immediate early gene 1 (*ie*1), Vp 28 and thymidine kinase and thymidilate kinase (*tk-tmk*) and 55 °C for protein kinase 1(*pk*1) and β actin. For immune genes, the hot start PCR was programmed with 94 °C for 2 min, followed by cycles of 94 °C for 2 min, annealing for 1 min, extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min. Annealing temperature and number of cycles employed for PCR of astakine and peroxinectin were 56 °C and 30 cycles, for prophenoloxidase (Pro PO) 56 °C and 35 cycles, for alpha 2 macroglobulin 65 °C and 30 cycles, and for crustin and penaeidine 55 °C and 35 cycles. A 10-μL sample of each PCR product was analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light.

Statistical analyses

The results given are average values of three replicates ± standard deviation. The effects of treatments were statistically analysed by single-factor and two-factor analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

Results

Primary cell culture from lymphoid organ

Performance of the primary cell cultures in 2X L-15 medium was found to be better than in the modified Grace's insect medium. The explants attached to the culture vessel within 2 h of seeding. Once attached, round cells in large numbers were seen to migrate from the explants and continued to remain with the same morphology (Fig. 1). Apart

Table 1 Primer sequences of WSSV genes, shrimp immune-related genes and β actin used in this study

Gene	Primer sequence (5′–3′)	Size (bp) of PCR product	References
WSSV genes			
Immediate early gene (*ie*1)	F-GACTCTACAAATCTCTTTGCCA R-CTACCTTTGCACCAATTGCTAG	502	Liu, Chang, Wang, Kou & Lo 2005
Protein kinase (*pk*1)	F-TGGAGGGTGGGGACCAACGGACAAAAC R-CAAATTGACAGTAGAGAATTTTGCAC	512	Liu *et al.* 2005
Thymidine kinase and thymidylate kinase (*tk-tmk*)	F-GAGCAGCCATACGGGTAAAC R-GCGAGCGTCTACCTTAATCC	412	Liu *et al.* 2005
Ribonucleotide reductase (*rr*1)	F-ATCTGCTAGTCCCTGCACAC R-AAAGAGGTGGTGAAGGCACG	408	Liu *et al.* 2005
DNA polymerase (*dnapol*)	F-TGGGAAGAAAGATGCGAGAG R-CCCTCCGAACAACATCTCAG	586	Liu *et al.* 2005
Endonuclease (*endnu*)	F-TGACGAGGAGGATTGTAAAG R-TTATGGTTCTGTATTTGAGG	408	Liu *et al.* 2005
Vp 28	F-CTGCTGTGATTGCTGTATTT R-CAGTGCCAGAGTAGGTGAC	555	Liu *et al.* 2005
Latency 1	F-CTTGTGGGAAAAGGGTCCTC R-TCGTCAAGGCTTACGTGTCC	647	Liu *et al.* 2005
Shrimp immune-related genes and β actin			
Prophenoloxidase (pro PO)	F-TGGCACTGGCACTTGATCTA R-GCGAAAGAACACAGGGTCTCT	590	Jiravanichpaisal *et al.* 2007
Astakine	F-GTCGCGCATTTAACAAGGAG R-CCCTGTGGATTGAGCTCACT	455	Jiravanichpaisal *et al.* 2007
Peroxinectin	F-CGAAGCTTCTTGCAACTACCA R-GCAGGCTGATTAAACTGGCTT	547	Jiravanichpaisal *et al.* 2007
Alpha 2 macroglubilin	F-ATGGCCAATCCCGAGAGGTACCTACTG R-TGTTGCTGCAGAAGTTTGTTATCCTCAT	345	Lin *et al.* 2007
Crustin	F-GCACAGCCGAGAGAAACACTATCAAGAT R-GGCCTATCCCTCAGAACCCAGCACG	430	Jiravanichpaisal *et al.* 2007
Penaeidin-3	F-AGGATATCATCCAGTTCCTG R-ACCTACATCCTTTCCACAAG	240	Jiravanichpaisal *et al.* 2007
β Actin	F-CTTGTGGTTGACAATGGCTCCG R-TGGTGAAGGAGTAGCCACGCTC	520	Zhang *et al.* 2007

WSSV, white spot syndrome virus.

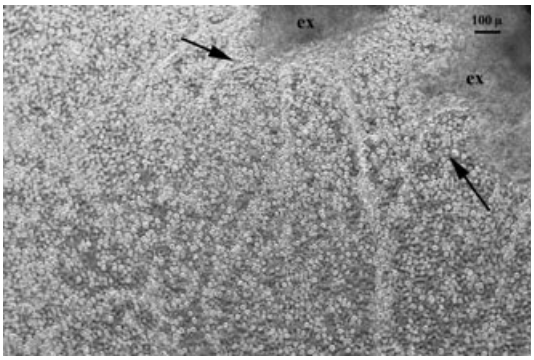

Figure 1 Round cells (arrows) migrating from explant (ex) of lymphoid tissue from *Penaeus monodon*, after 4 h incubation.

from these cells, fibroblastic (Fig. 2) and epithelioid cells (Fig. 3) were also found developing as part of the primary cell culture system. Cells in the mitotic phase were frequently seen among the epithelioid cells (Fig. 4), while mitotic events were rare in round and fibroblastic cells. Epithelioid cells which were released to the medium were transferred to another flask where they attached and formed a monolayer (Fig. 5). Attempts to subculture the fibroblastic cells did not yield satisfactory results as only a few cells were able to reattach on passage. The fibroblastic cells formed monolayers quickly while the epithelioid cells did not.

Performance of lymphoid cell cultures in the basal 2X L-15 and modified 2X L-15 media was compared through MTT assay. No significant ($P > 0.05$) difference was found on the 3rd day of the culture between the two media (Fig. 6). However, on the 5th day, significant differences were obtained ($P < 0.05$) with higher MTT values for modified 2X L-15 medium. Furthermore, in the modified medium, metabolic activity of the cells was significantly higher on the 5th day ($P < 0.05$) which, in contrast, was declining in the basal

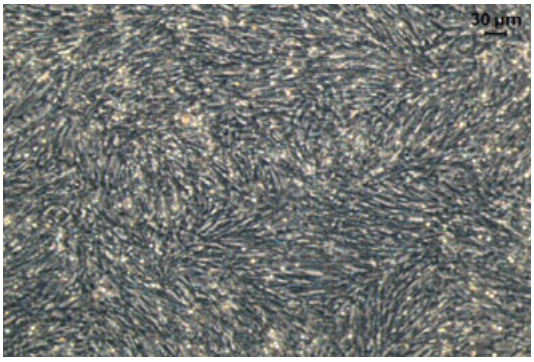

Figure 2 Monolayer of primary cell culture (fibroblastic) developed from lymphoid tissue of *Penaeus monodon*, after 48 h incubation.

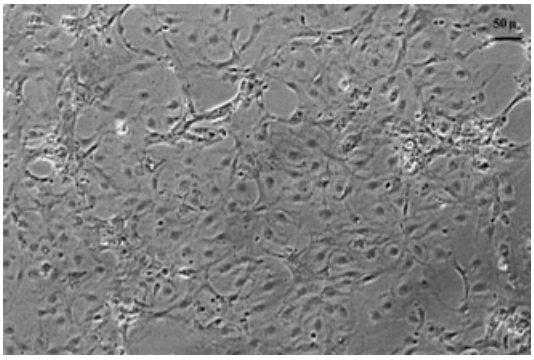

Figure 3 Monolayer of primary cell culture (epithelioid) developed from lymphoid tissue of *Penaeus monodon*, after 48 h incubation.

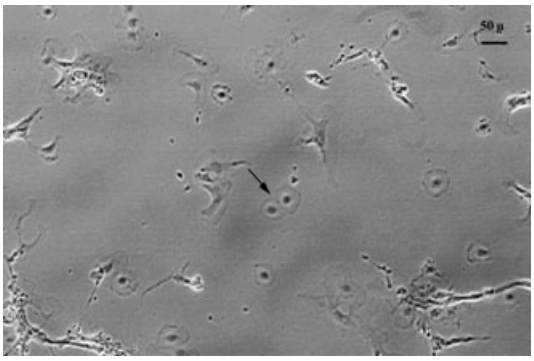

Figure 4 Epithelioid cells in division (arrow head) generated from primary lymphoid cell culture of *Penaeus monodon*, after 12 h incubation.

medium during the same period. Accordingly, the modified medium containing Leibovitz's L-15 at double strength supplemented with 2% glucose,

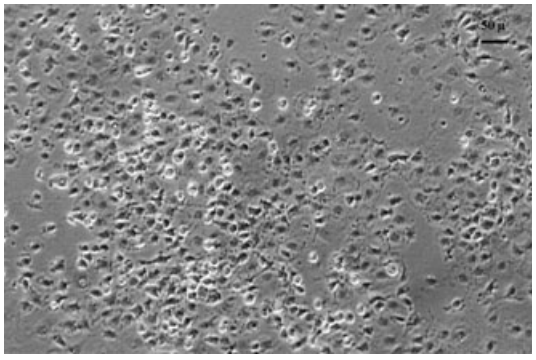

Figure 5 First passage of epithelioid cells, after 24 h incubation, from a primary cell culture generated from lymphoid tissue of *Penaeus monodon.*

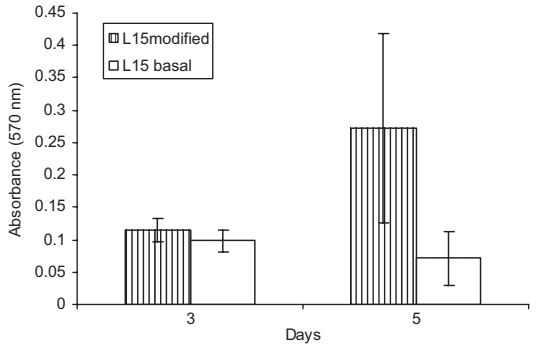

Figure 6 Effect of 2X L-15 basal medium and modified 2X L-15 medium on the growth of lymphoid primary cell culture from *Penaeus monodon* from MTT readings (n = 3).

MEM Vitamins (1X), tryptose phosphate broth (2.95 g L^{-1}), 20% FBS, 0.06 µg mL^{-1} chloramphenicol, 100 µg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin was used for all subsequent studies.

Effect of attachment factors on primary lymphoid cell culture

Efficacy of attachment factors such as laminin, fibronectin and poly-L-lysine was evaluated in terms of MTT readings by growing lymphoid cells on microwell plates similarly coated. Accordingly, in laminin (20 µg mL^{-1})-coated wells, negative absorbance was recorded after 48 h incubation (Fig. 7). Absorbance was not significantly ($P > 0.05$) different from the control with respect to fibronectin (20 µg mL^{-1}) coated wells, while absorbance for poly-L-lysine (200 µg mL^{-1})-coated wells was significantly higher than that of the control.

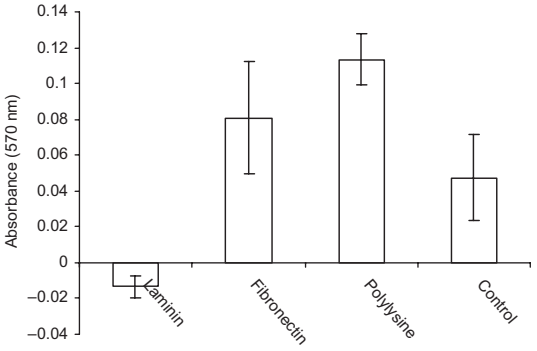

Figure 7 Effect of attachment factors on the growth of primary cell culture from lymphoid tissue of *Penaeus monodon* from MTT readings (n = 3).

Effect of growth factors on primary lymphoid cell culture

Among the growth factors tested, bFGF, EGF, IGF-2 and TGF β did not have any significant effect ($P > 0.05$) on growth and multiplication of the cells, as measured by MTT assay when compared to that of the control (Table 2). However, IGF-1 at 10 ng mL^{-1} supported better growth when compared to the control ($P < 0.05$).

5-bromo-2′-deoxyuridine (BrdU) assay of primary lymphoid cell culture

Green fluorescence of FITC was observed only at the edge instead of whole nuclei in a few cells (Fig. 8), while in the remaining cells, negative blue signals from DAPI alone could be visualized.

WSSV infection in lymphoid cell culture and immunofluorescence detection

Cytopathic effect was visible within 12 h of inoculation with WSSV and was more prominent at 24 h. Infected cells were shrunken and accumulated refractile granules (Fig. 9). Finally, the cells detached from the culture plate and lysed. Green positive signals with FITC-conjugated monoclonal antibodies against WSSV were observed (Fig. 10) on the nuclei of infected cells. No such positive signals were obtained from the control.

Expression of WSSV genes in virus-infected lymphoid cell culture

Expressions of immediate early gene (*ie*1), five early genes (*pk*1, *tk-tmk*, *rr*1, *dnapol*, *endnu*), a late gene (Vp 28) and latency gene (latency 1) were investigated at 8 and 24 h post-infection (Fig. 11). All the genes, except the latency 1 gene, were expressed at 8 and 24 h, the latter only at 8 h. However, downregulation of *pk*1 and *dnapol* at 24 h post-infection was noticed. None of the viral genes was

Table 2 Effect of growth factors (bFGF, EGF, IGF-1, IGF-2 and TGF β) on the primary cell culture from lymphoid tissue of *Penaeus monodon* as MTT readings

Growth factors, ng mL^{-1}	Absorbance (570 nm)
bFGF – 10	0.34 ± 0.04
bFGF – 20	0.40 ± 0.05
bFGF – 40	0.41 ± 0.13
EGF – 1	0.43 ± 0.06
EGF – 10	0.40 ± 0.08
EGF – 30	0.42 ± 0.12
IGF 1 – 10	0.50 ± 0.11*
IGF 1 – 25	0.34 ± 0.04
IGF 1 – 50	0.31 ± 0.04
IGF 2 – 10	0.38 ± 0.02
IGF 2 – 25	0.31 ± 0.05
IGF 2 – 50	0.38 ± 0.13
TGF β – 10	0.40 ± 0.07
TGF β – 25	0.46 ± 0.14
TGF β – 50	0.35 ± 0.03
Control	0.38 ± 0.02

EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; TGF β, transforming growth factor-β.
Values expressed as mean ± standard deviation (n = 3).
*Significantly higher absorbance ($P < 0.05$) than that of the control.

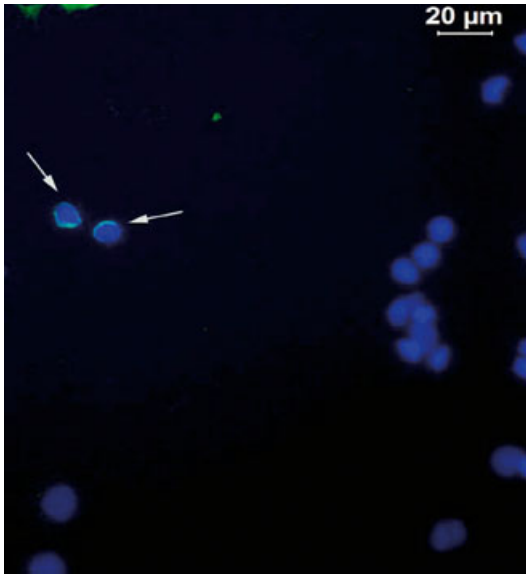

Figure 8 BrdU assay of the primary cell culture from lymphoid tissue from *Penaeus monodon* in 2X L-15 modified medium. Arrows show positive fluorescence at the edges of nuclei.

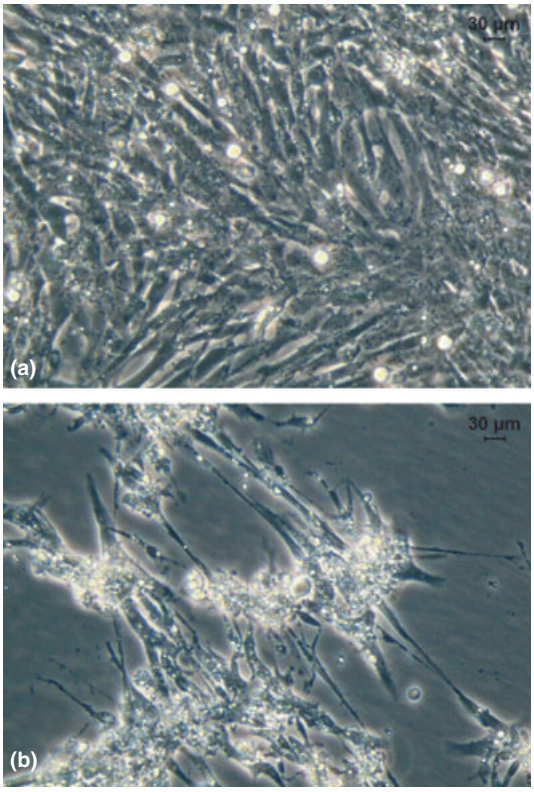

Figure 9 Cytopathic effect of white spot syndrome virus (WSSV) in lymphoid primary cell culture from *Penaeus monodon*, (a) Cells before inoculation with WSSV, (b) cells 24 h after inoculation with WSSV.

found expressed in the control cell cultures exposed to heat-inactivated WSSV.

Expression of immune-related genes in WSSV-infected lymphoid cell culture

Immune-related genes investigated included prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, crustin and penaeidin (Fig. 12). All the genes were amplified at 8 h post-infection and the level of expression of transcripts was higher than that of the control. At 24 h, only alpha 2 macroglobulin and penaeidin were expressed, and the former was weak. In the control exposed to heat-inactivated WSSV, expression of transcripts of peroxinectin, alpha 2-macroglobulin and crustin was low and that of penaeidin was high. In general, the expression of most immune-related genes was either downregulated or there was no expression after the initial hours of infection.

Discussion

This was an attempt to generate primary cell cultures from lymphoid organs of *P. monodon* and to improve the culture technique to establish them or/and use them for investigations on WSSV. Although explant cell cultures have been obtained from various tissues and organs of penaeid shrimps,

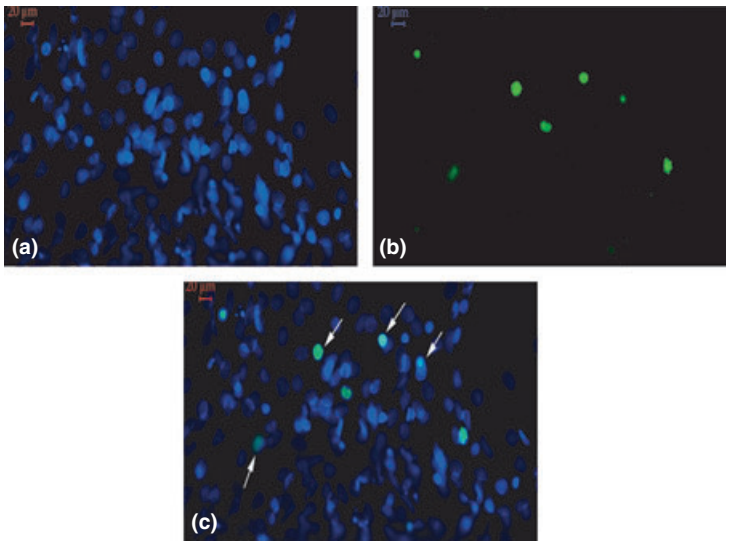

Figure 10 Immunofluorescence detection of white spot syndrome virus in primary lymphoid cell culture from *Penaeus monodon*, after 12 h of incubation, with the virus inoculum. (a) Both infected and uninfected cells as seen under DAPI filter, (b) infected cells with green fluorescence under FITC filter, (c) merger of (a) and (b) showing green positive cells (white arrows) among DAPI stained uninfected cells.

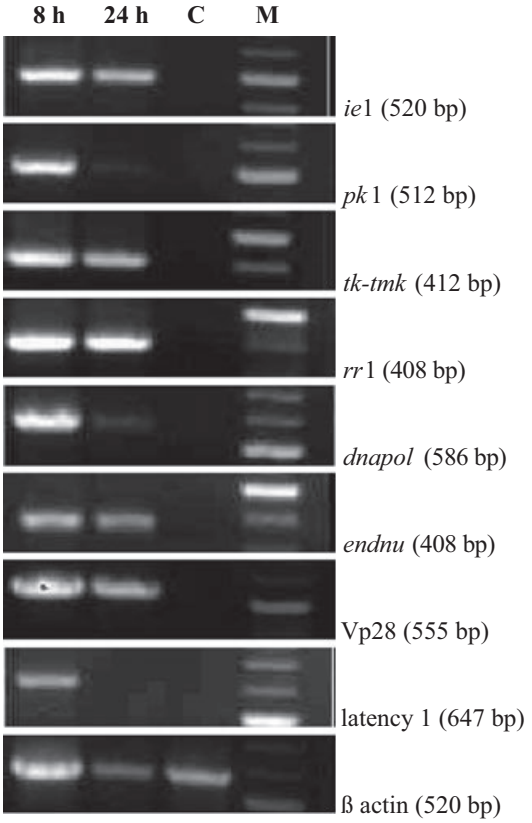

Figure 11 Expression of white spot syndrome virus genes in primary lymphoid culture from *Penaeus monodon* at 8 and 24 h post-infection (M-100 bp ladder; C-Control).

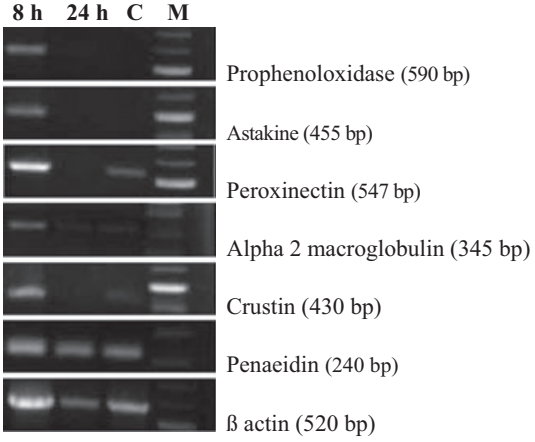

Figure 12 Expression of immune genes in primary lymphoid culture from *Penaeus monodon* at 8 and 24 h post-infection (M-100 bp ladder; C-Control).

the cells from lymphoid organs formed monolayers rapidly with more than 90% confluence and remained stable for longer periods of time (Nadala, Lu & Loh 1993). Thus, researchers have chosen the lymphoid organ as their preferred tissue for cell culture development (Chen, Jong & Kou 1989; Nadala *et al.* 1993; Lu *et al.* 1995; Tapay *et al.* 1995; Tong & Miao 1996; Tapay, Yuanan, Gose, Nadala, Brock & Loh 1997; Chen & Wang 1999; Itami, Maeda, Kondo & Takahashi 1999; Kasornchandra, Khongpradit, Ekpanithanpong & Boonyaratpalin 1999; Owens & Smith 1999; West, Mahony, McCarthy, Watanabe, Hewitt & Hansford 1999; Shike, Shimizu, Klimpel & Burns 2000; Wang *et al.* 2000; Lang *et al.* 2002).). The lymphoid organ or oka organ was first described by Oka (1969) followed by Martin, Hose & Kim (1987) and Hose, Martin, Tiu & McKrell (1992). Although the latter authors attributed a haematopoietic function for the lymphoid organ, van de Braak, Botterblom, Taverne, Van, Muiswinkel, Rombout & van der Knaap (2002) did not support this view because of the limited number of mitotic figures observed and proposed the lymphoid organ as a filter for all foreign materials encountered in the haemolymph.

L-15 medium has been described as the most suitable for penaeid shrimp cell culture and has been employed by various workers (Chen, Jong & Kou 1988; Nadala *et al.* 1993; Kasornchandra *et al.* 1999; Owens & Smith 1999). Kasornchandra *et al.* (1999), Itami *et al.* (1999) and Wang *et al.* (2000) reported fibroblastic and epithelioid morphology of cells in lymphoid cell culture; meanwhile, Wang *et al.* (2000) also described round cells. Round cells migrating out of the explant, as observed in the present study, appear to be haemocytes present in the lymphoid organ, the filtering organ of haemolymph. van de Braak *et al.* (2002) reported the presence of granular and semi-granular types of haemocytes within the central haemal lumen of the lymphoid organ.

Difficulty in subculturing cells by either mechanical or enzymatic means has been reported by several researchers (Chen *et al.* 1989; Itami *et al.* 1999; Owens & Smith 1999), while Kasornchandra *et al.* (1999) reported up to three passages. Even though Lang *et al.* (2002) reported cell division in lymphoid organ cultures, no researcher was able to passage the culture beyond a few times. Owens & Smith (1999) attributed the ready formation of monolayers in lymphoid cell cultures to cell migration from the explant rather than by cell division. Hsu, Yang, Chen, Tung, Wu, Engelking & Leong (1995) claimed up to 95 passages, but

Rinkevich (1999) reported, after careful examination of the published figures, this as thraustochytrid contamination. Meanwhile, more than 21 passages of the cultures transfected with simian virus 40 large T antigen employing a pantropic retroviral vector has been reported by Hu, Wang, Wang & Yang (2008). In spite of these efforts, no permanent cell line has been established.

Laminin and fibronectin are extracellular matrix proteins and poly-L-lysine is a positively charged aminoacid, which, at 200 μg mL^{-1}, was found to enhance attachment and proliferation of cells from the explants. However, Frerichs (1996) observed no increase in adhesiveness of embryonic cells of *Macrobrachium rosenbergii* in poly-L-lysine-coated plates. Similarly, Cooke, Graf, Grau, Haylett, Meyers & Ruben (1989) and Braasch, Ellender & Middlebrooks (1999) did not observe any positive results with laminin and fibronectin coating for crab, *Cardisoma carnifex*, and lobster, *Panulirus marginatus*, neurons and *Penaeus vannamei* haemocytes.

Fan & Wang (2002) observed enhanced growth and proliferation of embryonic cells of *Penaeus chinensis* after administration of IGF-2 and bFGF, while Fraser & Hall (1999) reported no stimulation of growth after the application of EGF and bFGF. In the present study, only IGF-1 enhanced cell proliferation at a concentration of 10 ng mL^{-1}.

BrdU, a synthetic analogue of thymidine which is incorporated into DNA during the S-phase of the cell cycle (Gratzner 1982), was used for the analysis of cell proliferation, and the assay has not been reported in the lymphoid cell culture of penaeid shrimp. However, 1–2% cells in haemocyte culture of *P. vannamei* (Braasch *et al.* 1999), 22 ± 7% cells in haemocyte culture of *P. monodon* (Jose *et al.* 2010) and 35% cells in the ovarian primary cell culture of *Penaeus japonicus* (Maeda *et al.* 2003) were observed to be positive. In the present study, the edges of nuclei of a few cells emitted positive fluorescence instead of whole nuclei which supports the findings of van de Braak *et al.* (2002) that only a limited number of mitotic figures were seen in lymphoid cell cultures.

Wang *et al.* (2000) proved the susceptibility of WSSV in lymphoid primary cell culture. Shrinkage, rounding and detachment of infected cells were the common CPE reported (Wang *et al.* 2000; Maeda *et al.* 2004; Jiravanichpaisal, Soderhall & Soderhall 2006). Additionally, in the present study, accumulation of refractile granules was also observed. Jiravanichpaisal *et al.* (2006) employing electron microscopy and *in situ* hybridization demonstrated WSSV infectivity of the cells, while Jiang *et al.* (2005) employed immunodetection with monoclonal antibodies against WSSV as performed here.

Genes of most double-stranded DNA viruses are expressed in the host in a cascade fashion. Immediate early and early genes are expressed before viral DNA replication, while expression of late genes occurs after replication of the viral genome (Honess & Roizman 1974; Friesen & Miller 1986; Blissard & Rohrmann 1990; Blissard 1996). All the tested genes including *ie* 1, *pk*-1, *tk - tmk*, *rr* 1, *endnu*, *dnapol*, Vp 28 and latency 1 genes were expressed in WSSV-infected lymphoid cell culture. This demonstrated the suitability of lymphoid cell culture as a platform for investigating WSSV morphogenesis.

The expression of WSSV-stimulated immune-related genes in lymphoid cell culture as observed in the present study could be attributed to the haemocytes present in the lymphoid explant tissue which migrated to the culture vessel during incubation as reported earlier by van de Braak *et al.* (2002). Our results suggest that immune-related genes were triggered immediately after exposure to WSSV and increase in the expression of transcripts, if any, might result in an enhanced immune response to ward off the pathogens. In contrast, the apparent downregulation of expression of most immune-related genes after the initial hours of infection indicates that the heightened response is temporary or short-lived in the event of WSSV infection. This pattern of expression was observed in *P. japonicus* following peptidoglycan stimulation (Fagutao, Yasuike, Caipang, Kondo, Hirono & Takahashi 2008) and in WSSV-infected haemocyte culture of *P. monodon* (Jose *et al.* 2010).

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