



## Regular Article

# Primary hemocyte culture of *Penaeus monodon* as an in vitro model for white spot syndrome virus titration, viral and immune related gene expression and cytotoxicity assays

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## ABSTRACT

Immortal cell lines have not yet been reported from *Penaeus monodon*, which delimits the prospects of investigating the associated viral pathogens especially white spot syndrome virus (WSSV). In this context, a method of developing primary hemocyte culture from this crustacean has been standardized by employing modified double strength Leibovitz-15 (L-15) growth medium supplemented with 2% glucose, MEM vitamins (1×), tryptose phosphate broth (2.95 g l<sup>-1</sup>), 20% FBS, N-phenylthiourea (0.2 mM), 0.06 μg ml<sup>-1</sup> chloramphenicol, 100 μg ml<sup>-1</sup> streptomycin and 100 IU ml<sup>-1</sup> penicillin and hemolymph drawn from shrimp grown under a bio-secured recirculating aquaculture system (RAS). In this medium the hemocytes remained viable up to 8 days. 5-Bromo-2'-deoxyuridine (BrdU) labeling assay revealed its incorporation in 22 ± 7% of cells at 24 h. Susceptibility of the cells to WSSV was confirmed by immunofluorescence assay using a monoclonal antibody against 28 kDa envelope protein of WSSV. A convenient method for determining virus titer as MTT<sub>50</sub>/ml was standardized employing the primary hemocyte culture. Expression of viral genes and cellular immune genes were also investigated. The cell culture could be demonstrated for determining toxicity of a management chemical (benzalkonium chloride) by determining its IC<sub>50</sub>. The primary hemocyte culture could serve as a model for WSSV titration and viral and cellular immune related gene expression and also for investigations on cytotoxicity of aquaculture drugs and chemicals.

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## 1. Introduction

*Penaeus monodon*, one of the most widely cultured crustaceans in the world, is under threat from viral pathogens especially white spot syndrome virus (WSSV). White spot disease (WSD) is the most contagious viral infection of decapod crustaceans that can cause 100% mortality in cultured shrimp at any age and size within 3–7 days from the onset (Lightner, 1996). Since its first outbreak in 1992–1993, the disease has caused serious economic loss and still remains without any valid remedial measures. The causative agent is a large, non-occluded, enveloped, and rod-shaped to elliptical DNA virus with a tail-like extension at one end (Takahashi et al., 1994; Chou et al., 1995; Wang et al., 1995). WSSV multiplies in the nucleus and has a very broad host range among cultured crustaceans (Wang et al., 1998; Chen et al., 2000). Based on genetic analyses and morphological features, WSSV has been classified as the sole species of a new monotypic family called *Nimaviridae*; genus *Whispovirus* (<http://www.ictvdb.iacr.ac.uk/index.htm>; Mayo, 2002). Lack of

shrimp cell lines has been hampering progress of research in WSSV in vitro in terms of elucidation of pathogenesis, morphogenesis and also determination of virus titer.

First reported cell culture from penaeid shrimp was from *P. monodon* (Chen et al., 1986). Cell cultures from different penaeid species including *Penaeus stylirostris* (Luedeman and Lightner, 1992; Tapay et al., 1995; Shike et al., 2000; Shimizu et al., 2001), *Penaeus japonicus* (Chen and Wang, 1999; Lang et al., 2002, 2004; Maeda et al., 2003), *Penaeus chinensis* (Tong and Miao, 1996; Fan and Wang, 2002; Gao et al., 2003; Jiang et al., 2005), *Penaeus penicillatus* (Chen et al., 1989; Chen and Wang, 1999), *Penaeus indicus* (Toullec et al., 1996; Kumar et al., 2001), *Penaeus vannamei* (Nadala et al., 1993; Toullec et al., 1996) and non-penaeids such as *Macrobrachium rosenbergii* (Frerichs, 1996) were reported. The tissues selected for culture included lymphoid (Chen et al., 1989; Tapay et al., 1995; Chen and Wang, 1999; Lang et al., 2002), ovary (Chen et al., 1986; Luedeman and Lightner, 1992; Shimizu et al., 2001; Maeda et al., 2003), hepatopancreas (Ghosh et al., 1995), heart (Tong and Miao, 1996; Chen and Wang, 1999) and embryos (Frerichs, 1996; Toullec et al., 1996; Fan and Wang, 2002). Among them Tapay et al. (1995), Frerichs (1996), Fan and Wang (2002)

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and Hu et al. (2007) have reported to have attained the progress of cell cultures up to or more than 10 passages. However, no immortal crustacean cell line has yet been developed.

Circulating hemocytes of crustaceans, including shrimp play a crucial role in cellular defense mechanisms, performing functions such as phagocytosis, encapsulation and lysis of foreign cells (Smith and Söderhäll, 1983; Ratcliffe et al., 1985; Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1992) and release of humoral defense molecules. In spite of the significance of hemocytes in shrimp immunology its *in vitro* culture was attempted only by a few researchers, in *P. vannamei* and *P. aztecus* (Ellender et al., 1992), *P. japonicus* (Itami et al., 1999) and *P. chinensis* (Jiang et al., 2005). Itami et al. (1999) maintained granular hemocytes for 10 days while Ellender et al. (1992) and Jiang et al. (2005) maintained total hemocytes for 3–4 weeks and 20 days respectively. However, demonstration of the viability or metabolic activity of the cells over a period by employing any standard procedures has not been cited in literature; meanwhile determination of the functionality or viability of hemocytes of other crustaceans, such as *Liocarcinus depurator* (L) and *Carcinus maenas* (L) (Walton and Smith, 1999) was accomplished by means of phagocytosis assay and that of *Panulirus argus* by means of trypan blue assay (Li and Shields, 2007).

Multiplication of WSSV in primary cell cultures generated from lymphoid organ (Lu et al., 1995; Wang et al., 2000) ovarian tissue (Maeda et al., 2004) and hemocytes (Jiang et al., 2005) has been documented. However, shrimp cell culture has not been proposed as a model for investigating viral gene expression and host cell response. This requirement prompted us to standardize primary hemocyte culture of *P. monodon* as an *in vitro* model for expressing WSSV and shrimp immune-related genes, and assaying cytotoxicity of health management chemicals in aquaculture.

## 2. Materials and methods

### 2.1. Experimental animals

WSSV and monodon baculovirus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a recirculating shrimp rearing system integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having salinity 15 g l<sup>-1</sup>. Water quality was maintained by the addition of a probiotic Detrodigest™ (National Centre for Aquatic Animal Health (NCAAH), India) to manage detritus, and Enterotrophic™ (NCAAH, India) to control *Vibrio*. The larvae were fed with commercially available pelleted feed (Higashimaru, India). They were confirmed to be free of WSSV (WSSV detection kit, Genei, India) and MBV (Belcher and Young, 1998) by nested PCR when they grew to 8–12 g, and used for all experiments.

### 2.2. Development of primary hemocyte culture

The animals were sacrificed by immersing in crushed ice and disinfected by maintaining in 800 mg l<sup>-1</sup> sodium hypochlorite solution prepared in ice cold sea water (salinity 15 g l<sup>-1</sup>) for 10 min. Subsequently they were washed 5 times in sterile ice cold sea water, dipped in 70% alcohol and rinsed in ice cold sea water. Hemolymph was withdrawn aseptically 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 rostral sinus, diluted to obtain 5 × 10<sup>5</sup> cells ml<sup>-1</sup> using modified 2 × L-15 (Leibovitz) medium, aliquots of 200 µl were dispensed into 96 well plates (Greiner Bio-One) and incubated at 25 °C. For detection of WSSV infection by immunofluorescence, hemocyte cultures were prepared by adding 1 ml of cell suspension (5 × 10<sup>5</sup> cells) prepared in growth medium

into Leighton tubes with cover slips (10 × 22 mm; Micro-Aid) and incubated at 25 °C. The medium used was Leibovitz's L-15 (Sigma Aldrich) at double strength supplemented with 2% glucose, MEM vitamins (1 ×), tryptose phosphate broth (2.95 mg ml<sup>-1</sup>), 20% FBS, 0.2 mM N-phenylthiourea, 0.06 µg ml<sup>-1</sup> chloramphenicol, 100 µg ml<sup>-1</sup> streptomycin and 100 IU ml<sup>-1</sup> penicillin. The hemocyte response was examined by measuring the metabolic activity through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for 8 days. Replicates were maintained.

### 2.3. MTT assay

The assay is a colorimetric method for determination of cell viability based on 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-colored solution (Mosmann, 1983).

After replacing the medium, 50 µl of MTT (Sigma Aldrich) solution (5 mg ml<sup>-1</sup> in PBS; 720 mOsm) was added to each well and incubated for 5 h in dark. The control consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200 µl dimethylsulfoxide. Absorbance was recorded immediately at 570 nm in a micro plate reader (TECAN Infinite Tm).

### 2.4. 5-Bromo-2'-deoxyuridine (BrdU) assay

A 20 µl sample of 10 mM BrdU solution was added to each well of 96 well plate hemocyte cultures with 200 µl medium and the assay was conducted after 2, 24 and 48 h. Six replicate wells were maintained for each hour. The medium was removed; cells fixed with acidified ethanol for 30 min and blocked using 3% bovine serum albumin in PBS. Peroxidase conjugated anti BrdU solution (Amersham Biosciences) of 100 µl was added to each well and incubated for 90 min at room temperature. Wells were rinsed thrice with PBS, and diaminobenzidine (0.6 mg ml<sup>-1</sup> in 0.05 M tris buffer (pH 7.6) with 0.03% hydrogen peroxide) substrate was added to the wells immediately. After 20 min wells were rinsed with PBS and counterstained with Meyer's hematoxylin for 30 s. Cells without BrdU were kept as control. Cells were observed under inverted microscope, and the total and differential counts (brown colored positive cells) were taken.

### 2.5. Preparation of WSSV lysate from gill and hemolymph

Gill tissue (500 mg) from WSSV infected shrimps weighing 8–12 g under laboratory conditions was macerated in 10 ml cell culture medium with mortar and pestle kept in an ice bath. The extract was centrifuged at 10,000g for 10 min at 4 °C and the supernatant passed through 0.22 µm polyvinylidene fluoride (PVDF) membrane (Millipore). Frozen hemolymph from a WSSV infected shrimp was thawed, centrifuged at 10,000g for 10 min at 4 °C, diluted 10 times with cell culture medium and passed through 0.22 µm PVDF membrane. Similarly, extracts from healthy *P. monodon* adults were used for examining cytotoxicity for gill tissue and hemolymph.

### 2.6. Titration of WSSV suspension prepared from infected gill and hemolymph

The method of MTT<sub>50</sub> was adopted here for the titration of WSSV. A 96 well culture of hemocytes was generated employing the modified 2 × L-15 medium. A double dilution series of WSSV

suspension from gill and hemolymph extracts was prepared in a deep well plate (Axygen). The medium was removed from the wells and 200  $\mu$ l WSSV inoculum was added to each well maintaining quadruplicate for each dilution. For comparison uninfected controls were also maintained. On the 3rd day the wells were observed under phase contrast microscope (Carl Zeiss) for CPE, and subsequently MTT assay was conducted. Similarly, gill tissue and hemolymph extracts from apparently healthy *P. monodon* were applied as controls to hemocyte culture to check the cytotoxicity.

The 50% infectious dose,  $MTT_{50}$ , was calculated according to the method of Heldt et al. (2006). This was determined by plotting the optical density of each well against the  $-\log_{10}$  of the dilution factor. From the plot, 50% optical density was determined and the value was converted per milliliter basis and stated as the  $MTT_{50}$  titer. The optical density was defined as:

$$\text{Optical density} = \frac{\text{Blank absorbance} - \text{Well absorbance}}{\text{Blank absorbance}} \times 100\%$$

The 'blank absorbance' is the average absorbance of 12–48 wells with uninfected cells and the 'well absorbance' is the average absorbance of wells with infected cells.

### 2.7. Immunofluorescence assay for detection of WSSV

A 300-fold dilution of WSSV suspension from gill tissue was used for infecting hemocytes. The medium was removed and 1 ml WSSV inoculum prepared in the growth medium was added to the Leighton tubes with cover slip cultures and incubated at 25 °C. After 72 h cover slips were removed, washed twice in PBS (720 mOsm) and immersed in 10% paraformaldehyde and maintained in 70% ethanol at  $-20$  °C until use. 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/Tween 20 (0.01%) and WSSV C 38 monoclonal antibody (Anil et al., 2002) was added to the slide, incubated for 1 h and washed 3 times in wash buffer. It was again incubated for 1 h after addition of rabbit anti mouse FITC conjugate, 1:40 dilution (Sigma Aldrich) and washed. After incubation with general nuclear stain DAPI (10  $\mu$ l, 0.2  $\mu$ g  $\text{ml}^{-1}$ ) for 3 min, the slides were rinsed with distilled water, air dried, mounted (Vectashield, USA) and observed under fluorescent microscope (Olympus, Germany). DAPI and FITC were viewed under filters with excitation wavelength 360–370 nm and 470–490 nm respectively. The slides were compared with uninfected hemocytes (negative control). The images were processed and merged using the "Image pro-express" software (Media Cybernetics Inc., MD, USA).

### 2.8. RNA isolation from WSSV infected hemocyte culture

Twenty four well plate cultures of hemocytes were prepared in modified 2 $\times$  L-15 medium by seeding each well with 1 ml hemolymph suspension containing  $8 \times 10^5$  cells. After 6 h incubation at 25 °C, the medium was removed and 500  $\mu$ l WSSV suspension from gill tissue (diluted 300 times) added. Control wells were maintained without WSSV and with heat inactivated (56 °C for 1 h) WSSV. After 2, 12, 24, 36, and 48 h of incubation, total RNA was extracted from 10 wells each, which were restricted to 48 h in the case of controls. Wells were washed with ice cold PBS and 200  $\mu$ l TRI reagent was added (Sigma Aldrich) to each well. 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, and allowed to stand for 15 min. The resultant mixture was centrifuged at 12,000g for 15 min at 4 °C. To the separated upper aqueous phase, 0.5 ml iso-propanol was added, and centrifuged at 12,000g for 10 min at 4 °C. The pellet obtained was washed twice with 75% ethanol, air dried and dissolved in 20  $\mu$ l DEPC treated sterile water. RNA samples were subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs). Aliquots of 0.2 units of the enzyme were added per  $\mu$ g of RNA and incubated at 37 °C for 10 min. The enzyme was inactivated at 75 °C for 10 min. Concentration and quality of RNA was measured by measuring the absorbance at 260/280 nm in a UV-Visible spectrophotometer.

### 2.9. RT-PCR of WSSV and immune-related genes

One microgram of RNA was subjected to cDNA synthesis with 20  $\mu$ l of reaction mix containing M-MULV reverse transcriptase (80 U), RNase inhibitor (8 U), Oligo (dT)<sub>12</sub> primer (40 pmoles), dNTP mix (1 mM), and RTase buffer (50 mM tris-HCl (pH 8.3), 75 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM DTT) at 42 °C for 1 h. All reagents were purchased from New England Biolabs. Subsequently, eight WSSV genes and six immune-related genes were amplified by PCR using 2  $\mu$ l cDNA with specific primer sets as given (Table 1). Shrimp beta actin gene was also amplified as a reference. Twenty-five microliter PCR reactions contained 0.5 U of Taq DNA polymerase, 200  $\mu$ M dNTP mix, 10 pmoles of each forward and reverse primer and 1 $\times$  PCR buffer (New England Biolabs). The hot start PCR programme 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, 53 °C for latency 1 and ribonucleotide reductase (rr1), 54 °C for DNA polymerase (dnapol), immediate early gene1 (ie1), VP28 and thymidine kinase and thymidilate kinase (tk-tmk), and 55 °C for protein kinase 1 (pk1) and beta actin. For immune-related genes also hot start PCR was performed 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 crustin and penaeidin 55 °C and 35 cycles and for alpha 2-macroglobulin 65 °C and 30 cycles. Ten microliter of each PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented.

### 2.10. Cytotoxicity of benzalkonium chloride (BKC)

BKC is selected, as a representative of health management chemical used in aquaculture, to assess the suitability of primary hemocyte culture for testing cytotoxicity. A 96 well plate culture of hemocytes was developed and incubated at 25 °C for 12 h. Different concentrations of BKC were added to the wells to attain final strength ranging from 0.012 to 25  $\text{mg l}^{-1}$ , and after 14 h of incubation MTT assay was performed maintaining quadruplicates. Cells in the growth medium were kept as control. Percentage inhibition of cells at each concentration of BKC was calculated based on the formula, percentage inhibition of hemocytes = [100 – (average absorbance (MTT assay) of hemocytes at a particular concentration of the compound/average absorbance of control hemocytes without the compound)  $\times$  100].

### 2.11. Statistical analysis

The results in the figures are average values of 3–6 replicates  $\pm$  standard deviation. Data were tested for normality and equality of variance

**Table 1**  
Primer sequences of WSSV genes, shrimp immune-related genes and beta actin.

Gene	Primer sequence (5'–3')	Size (bp) of PCR product	Reference
<i>WSSV genes</i>			
Immediate early gene (ie1)	F-GACTCTACAAATCTCTTTGCCA R-CTACCTTTGCACCAATTGCTAG	502	Liu et al., 2005
Protein kinase (pk1)	F-TGGAGGGTGGGACCAACGGACAAAAC R-CAAATTGACAGTAGAGAATTTGCAC	512	Liu et al., 2005
Thymidine kinase and thymidylate kinase (tk–tmk)	F-GAGCAGCCATACGGGTA AAC R-GCGAGCGTCTACCTTAATCC	412	Liu et al., 2005
Ribonucleotide reductase (rr1)	F-ATCTGCTAGTCCCTGCACAC R-AAAGAGGTGGTGAAGGCACG	408	Liu et al., 2005
DNA polymerase (dnapol)	F-TGGGAAGAAAGATGCGAGAG R-CCCTCCGAACAACATCTCAG	586	Liu et al., 2005
Endonuclease	F-TGACGAGGAGGATTGTAAG R-TTATGGTCTGTATTTGAGG	408	Liu et al., 2005
VP28	F-CTGCTGTGATTGCTGTATT R-CAGTGCCAGAGTAGGTGAC	555	Liu et al., 2005
Latency 1	F-CTGTGGGAAAAGGTCCTC R-TCGTCAAGGCTTACGTGTC	647	Liu et al., 2005
<i>Shrimp immune-related genes and beta actin</i>			
Prophenol oxidase (pro PO)	F-TGGCACTGGCACTTGATCTA R-GCGAAAGAACACAGGTCTCT	590	Jiravanichpaisal et al., 2007
Astakine	F-GTCGCGCATTTAACAAGGAG R-CCCTGTGGATTGAGCTCACT	455	Jiravanichpaisal et al., 2007
Peroxinectin	F-CGAAAGCTTCTTGCAACTACCA R-GCAGGCTGATTAACCTGGCTT	547	Jiravanichpaisal et al., 2007
Alpha 2 macroglobulin0	F-ATGCCAATCCCAGAGGTACTACTG R-TGTTGCTGCAGAAAGTTTGTTATCCTCAT	345	Lin et al., 2007
Crustin	F-GCACAGCCGAGAGAAACTATCAAGAT R-GGCCTATCCCTCAGAACCCAGCAGC	430	Jiravanichpaisal et al., 2007
Penaeidin-3	F-AGGATATCATCCAGTTCCTG R-ACCTACATCCTTTCCACAAG	228–243	Jiravanichpaisal et al., 2007
Beta actin	F-CTGTGGTTGACAATGGCTCCG R-TGGTGAAGGAGTAGCCACGCTC	520	Zhang et al., 2007

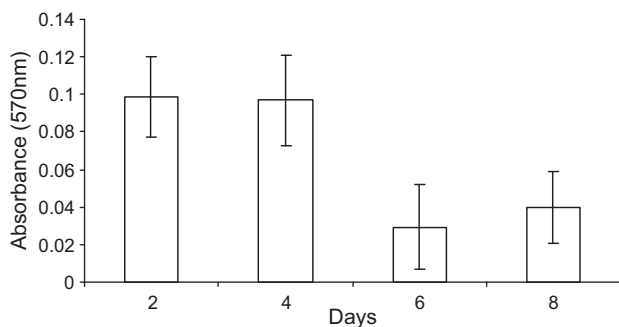
and the effect of treatments were statistically analyzed by analysis of variance (ANOVA). Differences were considered significant at  $P < 0.05$ . The results of the cytotoxicity assays were subjected to probit analysis using the SPSS software (SPSS Inc., USA).

### 3. Results

#### 3.1. Primary hemocyte culture

In the modified  $2 \times L-15$  medium cells started declining at 6th day and remained viable up to 8 days (Fig. 1a). Hemocytes in culture appeared spherical or elliptical initially, and within a few hours two distinct morphological types such as round to elliptical and small and large spindle shaped cells, with granules, could be seen (Fig. 1b).

The BrdU assay showed that maximum labeling had taken place at 24 h, recording  $22 \pm 7\%$  positive cells (Fig. 2). Dividing cells were not observed under the microscope.



**Fig. 1a.** Viability (in terms of MTT assay) of primary hemocyte culture during an incubation period of 8 days ( $n = 3$ ) in the modified  $2 \times L-15$  medium.

#### 3.2. Titration of WSSV

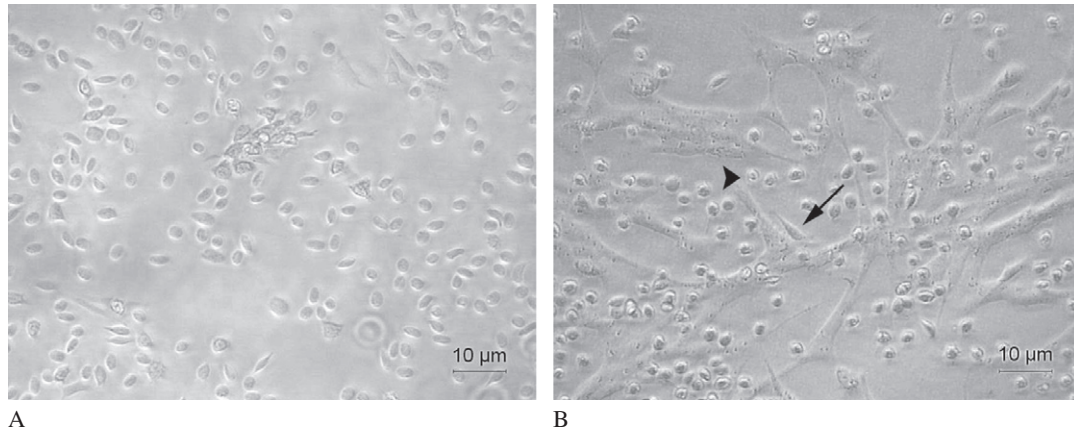
Sigmoid curves were obtained on plotting optical density against  $-\log_{10}$  of the dilution factor, and 50% optical density was determined from the curve and converted to per milliliter basis. The MTT titer of infected gill extract and hemolymph were  $2.9 \log_{10}$  (MTT<sub>50</sub>/ml) and  $3.2 \log_{10}$  (MTT<sub>50</sub> ml<sup>-1</sup>) respectively (Fig. 3a). The gill extract from healthy animals proved non-toxic to hemocyte culture whereas the hemolymph exhibited 5% inhibition (MTT values) at 10 times dilution (Fig. 3b).

#### 3.3. Cytopathic effect (CPE)

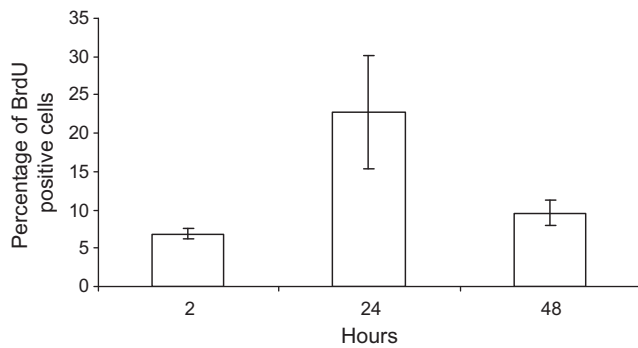
On inoculating with hemolymph and gill extracts from shrimp infected with WSSV (lowest dilutions) the hemocytes in culture were found disintegrated within a few hours (Fig. 4). At lower dilutions the cells were round to elliptical and in still higher dilutions there were both round to elliptical and small and large spindle shaped cells; beyond which a mixture of larger number of both cell types could be noticed. At higher dilutions the cells appeared the same as that of the control. This sequence of changes suggested that both types of cells were susceptible to WSSV; the spindle shaped cells were the most. Cytopathic effect (CPE) included shrinkage and disintegration of hemocytes, which was visible from 1st day onwards in lower dilutions of WSSV and 2nd to 3rd day in higher dilutions.

#### 3.4. Immunofluorescence detection of WSSV

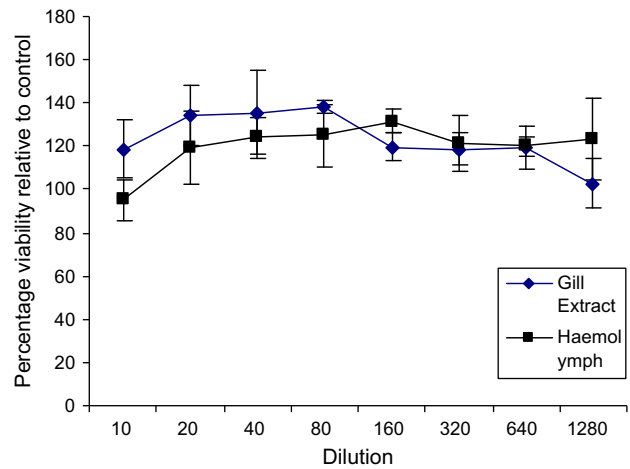
Blue fluorescence of DAPI was observed in the cell nuclei in all preparations including the control. Green positive signals with FITC conjugated monoclonal antibodies against WSSV were observed



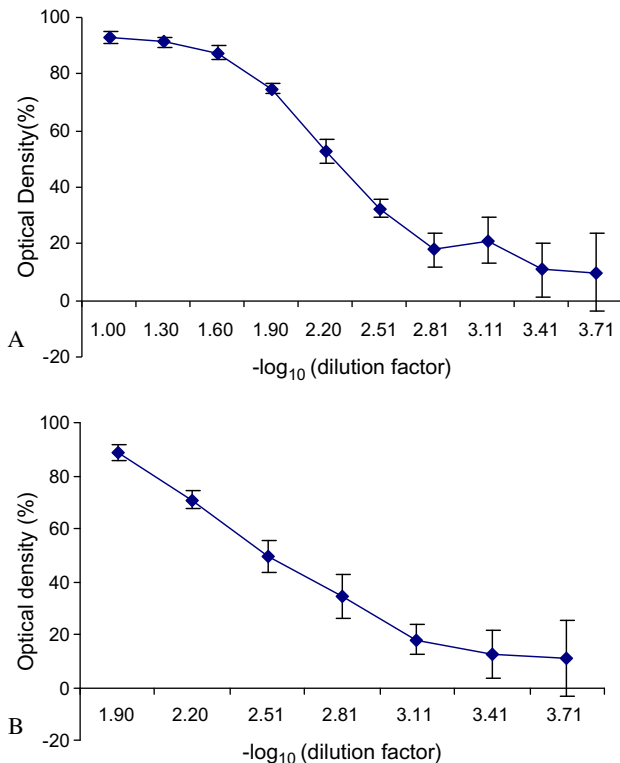
**Fig. 1b.** Morphology of primary hemocyte culture. (A) 0 h, (B) 24 h (arrow head: rounded to elliptical cells; arrow: small to large spindle shaped cells).



**Fig. 2.** Incorporation of BrdU in cultured hemocytes (n = 6).



**Fig. 3b.** Effect of gill extract and hemolymph of healthy *P. monodon* in primary hemocyte culture (n = 4) in terms of MTT assay.



**Fig. 3a.** Determination of WSSV titer in (A) gill extracts (B) hemolymph of infected shrimp in terms of MTT<sub>50</sub> using primary hemocyte culture.

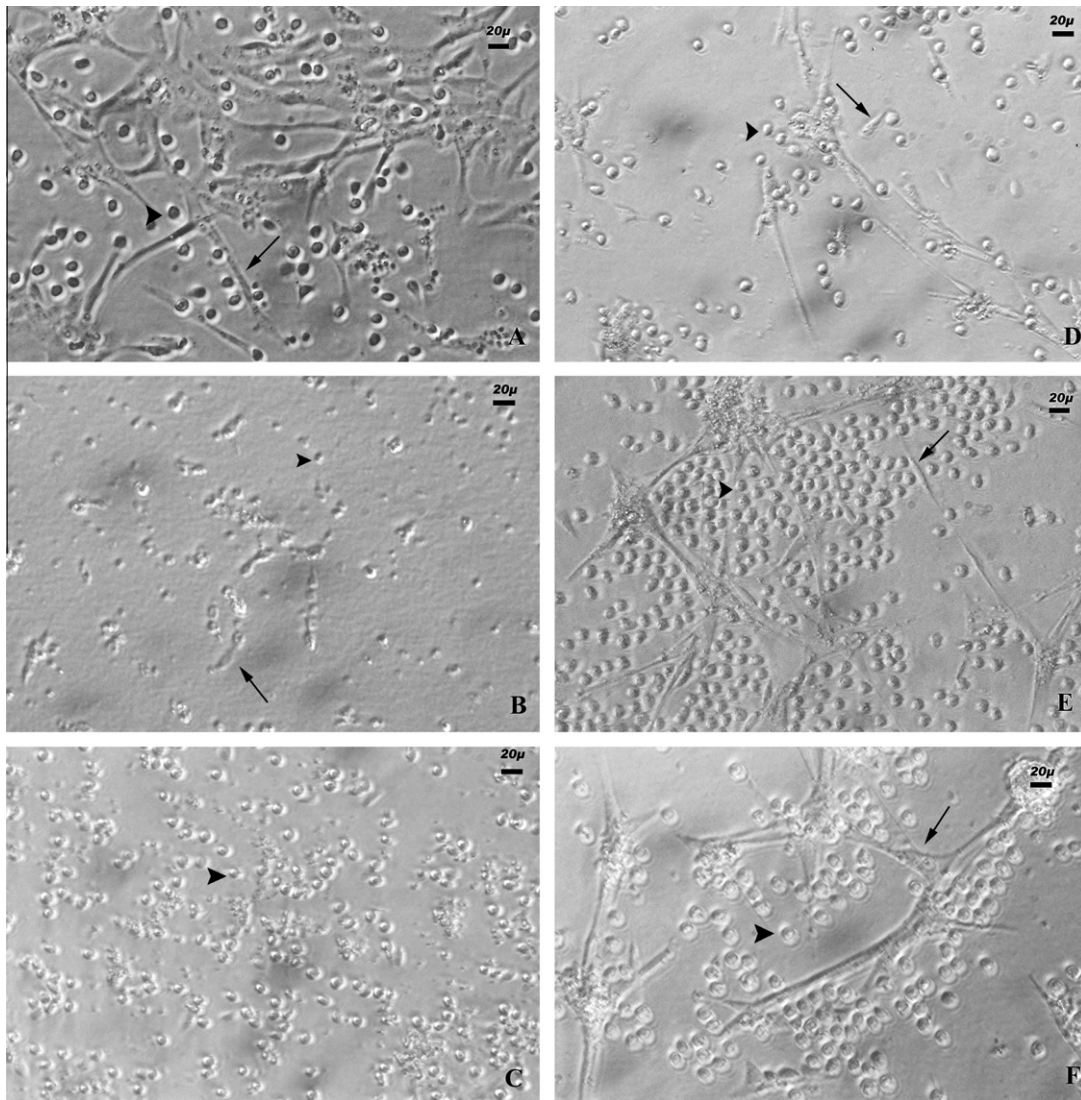
from the nuclei of cells assayed after 72 h of exposure to the virus (Fig. 5).

**3.5. Expression of viral genes in WSSV infected hemocyte culture**

Expression of an immediate early gene (ie1), 5 early genes (pk1, tk-tmk, rr1, dnapol, endonuclease), one of the late genes (VP28) and a latency gene (latency 1) were examined at 2, 12, 24, 36 and 48 h post infection (Fig. 6a). The results were categorized as (a) those which were expressed and maintained more or less uniformly through out the study period of 48 h: ie1, pk1, tk-tmk, rr1, endonuclease, (b) genes which were not expressed after 24th hour: dnapol, VP28 and latency 1. When VP28 was found upregulated the latency 1 was down regulated. None of the WSSV genes were expressed in control hemocytes without WSSV and control cells with heat inactivated WSSV. Shrimp beta actin gene was expressed in all the samples.

**3.6. Expression of immune-related genes in WSSV infected hemocyte**

Expression of immune-related genes examined at the event of WSSV infection included prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, crustin and penaeidin (Fig. 6b). Expression of these genes could be categorized as, (a) those with increased expression at 2nd hour: prophenoloxidase, peroxinectin, alpha 2-



**Fig. 4.** Cytopathic effect of WSSV in primary hemocyte culture. (A) Control without WSSV, (B) 1/10 dilution: hemocytes were found disintegrated within a few hours of inoculation, (C) 1/40 dilution: only round to elliptical cells were present, (D) 1/160 dilution: round to elliptical and small to large spindle cells were present, (E) 1/320 dilution: a mixture of larger number of round to elliptical and small to large spindle shaped cells noticed, (F) 1/2560 dilution: cells appeared the same as that of the control, (arrow heads: rounded to elliptical cells; arrows: small to large spindle shaped cells).

macroglobulin, crustin and penaeidin, (b) those which were maintained the expression in more or less same order at 12th hour: peroxinectin, crustin and penaeidin, (c) those which were slightly upregulated on administering heat inactivated WSSV for 48 h when compared to the negative control without WSSV: prophenoloxidase, peroxinectin, alpha 2-macroglobulin and penaeidin. In general, the expression of most immune-related genes decreased after initial hours of infection.

### 3.7. Cytotoxicity of benzalkonium chloride (BKC)

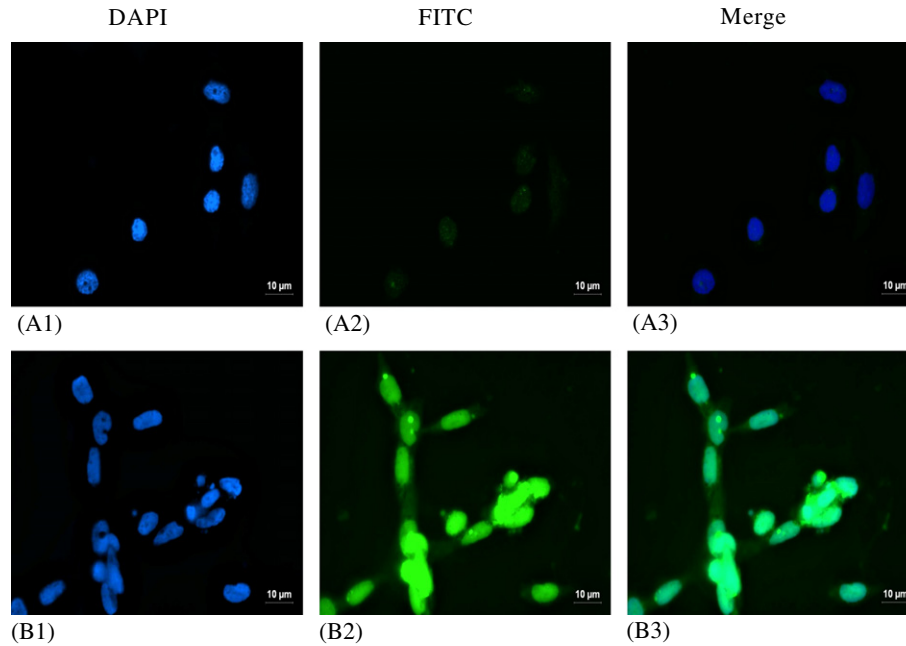
Morphological changes in the cells such as clumping and shrinkage were visualized microscopically after exposure to higher concentrations (above  $3.13 \text{ mg l}^{-1}$ ) of BKC (Fig. 7a) with an  $\text{IC}_{50}$  of  $0.49 \pm 0.13 \text{ mg l}^{-1}$  (Fig. 7b).

## 4. Discussion

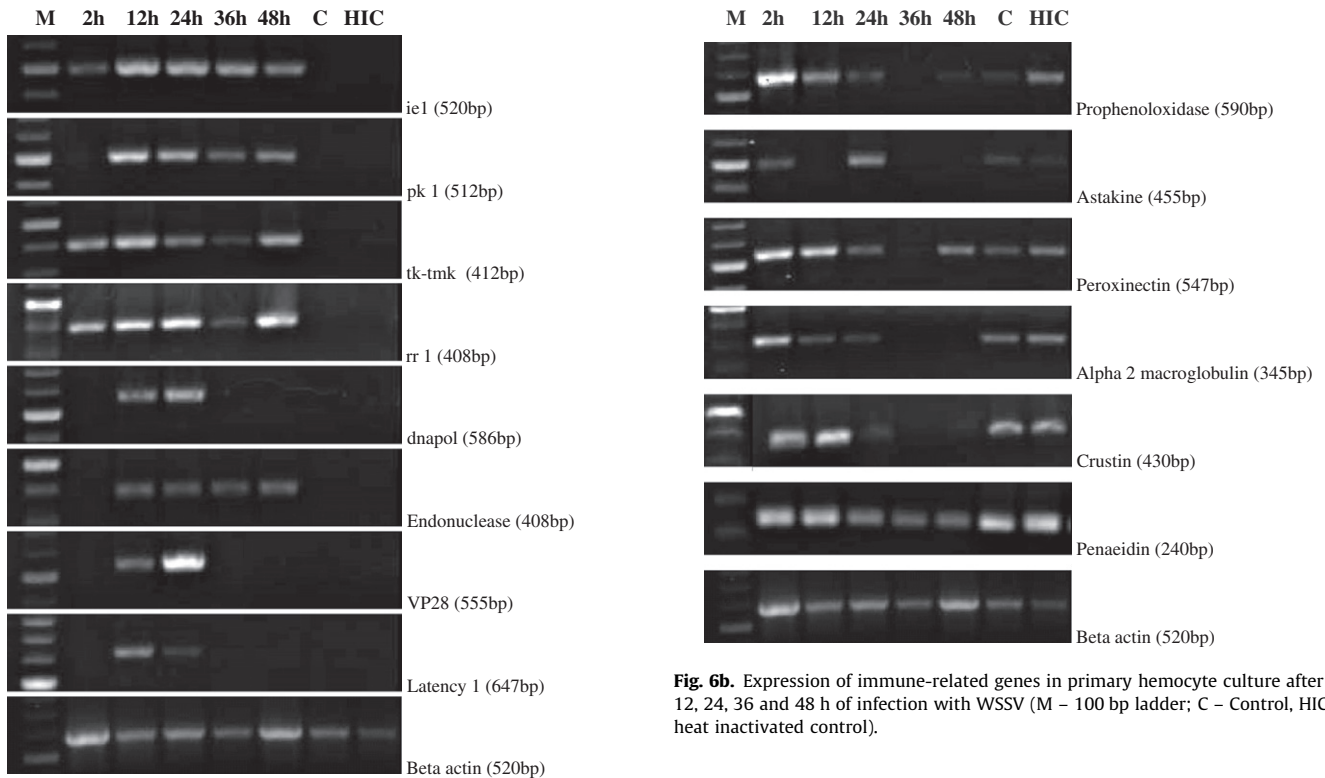
Leibovitz L-15 was chosen as the basal medium based on its strong buffering capacity contributed by the relatively high amino

acid concentration. Over the years L-15 has been employed by several for shrimp cell culture development (Wang et al., 2000; Mulford et al., 2001; Maeda et al., 2003), however, none of the attempts has culminated in establishment of a cell line. In this context it was necessary to improvise the medium by the addition of supplements, and the response of hemocytes was assessed in terms of metabolic activity using MTT assay. Performance of the shrimp cell culture was previously based on microscopic observation and not by quantitative assays. Lone deviation from this normal practice recorded in literature was the attempt of Shimizu et al. (2001) who opted for  $^3\text{H}$ -thymidine uptake assay and  $^{35}\text{S}$ -methionine uptake assay for analyzing performance of ovarian cells in different media.

The present study did not result in the development of a cell line but the data suggested that the metabolic activity of primary hemocyte culture did not vary considerably during the second, third and fourth days in the modified  $2 \times$  L-15 medium and the activity started declining only then onwards. Advantageously, with in this period WSSV titration, viral and immune gene expression and cytotoxicity assays could be completed. Among the types of cells developed half turned out to be spindle shaped within three



**Fig. 5.** Immunofluorescence detection of WSSV in primary hemocyte culture using a monoclonal antibody against the 28 kDa envelope protein of the virus (A) Control (B) 72 h post infection. (B2 and B3) Strong positive signals from nuclei of cells after 72 h of infection.



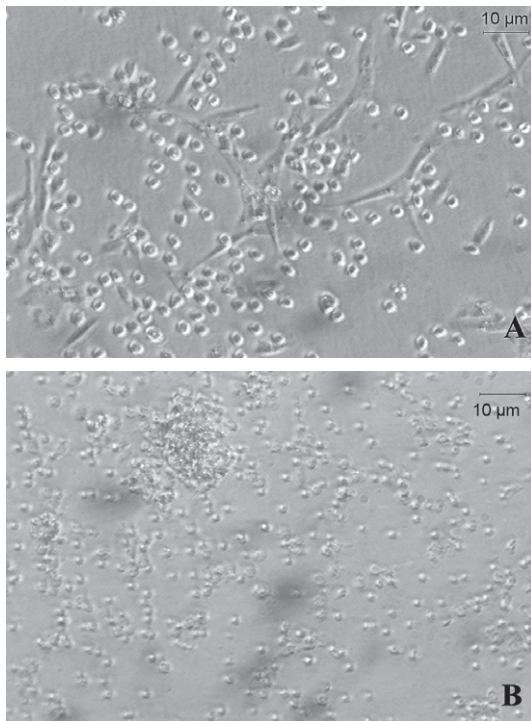
**Fig. 6a.** Expression of WSSV genes in primary hemocyte culture at 2, 12, 24, 36 and 48 h post infection (M – 100 bp ladder; C – Control, HIC – heat inactivated control).

**Fig. 6b.** Expression of immune-related genes in primary hemocyte culture after 2, 12, 24, 36 and 48 h of infection with WSSV (M – 100 bp ladder; C – Control, HIC – heat inactivated control).

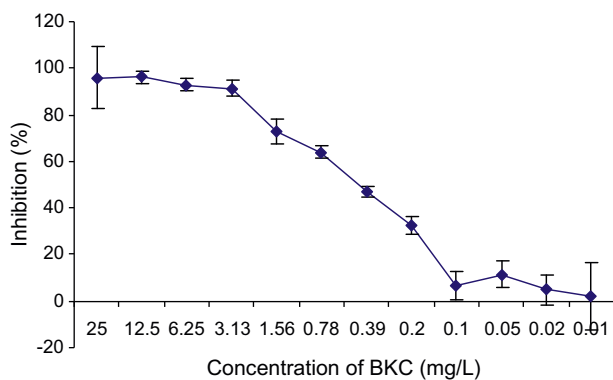
hours of incubation matching with the findings of Roper et al. (2001). L-15 medium was also found to support hemocytes of other crustaceans such as *L. depurator* (L) and *C. maenas* (L) (Walton and Smith, 1999) for 14 days and that of *P. argus* (Li and Shields, 2007) for 18 days.

To determine the proliferation of the cultured hemocytes, incorporation of 5-bromo-2'-deoxyuridine (BrdU) was accomplished

(Gratzner, 1982). BrdU, a synthetic analog of thymidine incorporated into DNA during the S-phase of the cell cycle, was used for the analysis of cell proliferation, as monitoring DNA synthesis happened to be an indirect method. Several workers have attempted to study the proliferation of circulating hemocytes in shrimp employing this method. Sequeira et al. (1996) observed that only about 0.6% of circulating hemocytes in *P. japonicus* were in G2 or M phase, and the percentage increased to 3 with the injection of lipopolysaccharide or infection with the fungus *Fusarium*, however, with out any dividing cells under circulation. Lipopolysaccharide



**Fig. 7a.** Cytotoxicity of BKC in primary hemocyte culture. (A) Control. (B) Test. Clumping of cells observed on administering BKC above  $3.13 \text{ mg l}^{-1}$ . Spindle shaped cells disappeared leaving only shrunken rounded cells.



**Fig. 7b.** Toxicity of BKC in primary hemocyte culture in terms of percentage inhibition of cells determined through MTT assay ( $n = 4$ ).

injection was also shown to induce DNA synthesis in a subpopulation of hemocytes of swimming crab, *L. depurator* (Hammond and Smith, 2002). Söderhäll et al. (2003) detected  $<1$ – $2.5\%$  BrdU incorporation in circulating hemocytes of *Pacifastacus leniusculus* after 90 min of labeling. In our study BrdU incorporation was attempted in vitro and  $22 \pm 7\%$  of cells were in S phase after 24 h even though dividing cells were not observed. This enhancement in the percentage of S-phase cells in vitro could be attributed to the efficacy of the modified  $2 \times$  L-15 medium and to the attachment and spreading of hemocytes to the culture vessel.

Because of the presence of  $\beta$ -integrin receptor, which mediates WSSV infection (Li et al., 2007) shrimp hemocytes were selected for its titration. In our study, hemocyte culture was successfully demonstrated as in vitro system for titrating WSSV. Researchers reported quantal assays for yellow head baculovirus (YBV) and non-occluded baculo like virus in primary lymphoid explant cultures (Lu et al., 1995; Tapay et al., 1997). The advantage of using

hemocyte culture over explant cultures for WSSV titration and cytotoxicity studies is the easiness in quantification of cells to be seeded to maintain uniform cell number in micro plates, a prerequisite for quantal assays, and the rapidity in obtaining monolayer of cells. Gill extract and hemolymph from WSSV infected shrimp were used for the virus titration. While doing so the observation on the cytotoxicity (Fraser and Hall, 1999) of hemolymph on shrimp cell culture was considered, and both gill extract and hemolymph from normal animals were proved non toxic to hemocyte culture.

Determining viral titer of an infected tissue is of utmost importance in virology and a common method recommended is the endpoint titration assay, the number of infectious units expressed as  $\text{TCID}_{50} \text{ ml}^{-1}$ . MTT assay employed in the present study is a high throughput assay which doesnot require manual assessment of cytopathic effect; instead metabolic activity of the cells is measured. Even though the MTT assay was successfully used in the titration of vertebrate viruses (Levi et al., 1995; Andersson et al., 2005; Heldt et al., 2006) this had not been employed for titration of WSSV so far. Susceptibility of the hemocyte culture to WSSV could be proved by means of immunofluorescence detection of the virus using MAb C38 (Anil et al., 2002) which reacted strongly with its 28 kDa envelope protein.

In the present study WSSV early genes, a late gene and a latency gene were expressed in infected hemocyte culture. Early genes such as *ie1*, *tk-tmk* and *rr1* were expressed as early as 2 h post infection and the other early genes (*pk1*, *dnapol* and endonuclease) from 12 h post infection. *VP28*, a late gene, involved in the production of envelope protein could be found upregulated from 12 to 24 h while the latency gene was down regulated. This is a clear indication of the virulence of WSSV to hemocytes by not having been entering in to a latent phase. It has been reported that envelope proteins such as *VP28* are highly expressed in infected shrimp relative to latency genes and the reverse is true in the case of asymptomatic carriers of WSSV (Khadijah et al., 2003). Use of in vitro crustacean cultures for analyzing the viral gene expression has been rarely reported in literature. The expression of *VP28* gene demonstrated by Jiravanichpaisal et al. (2006) in crayfish hematopoietic stem cell culture by RT-PCR is note worthy. The study suggested that primary hemocyte culture could be used to delineate the infectious mechanisms of the virus and its morphogenesis.

Understanding the interaction between host and pathogen will be helpful in controlling infectious diseases in shrimp. The expression of immune-related genes was reported in different larval stages of *P. monodon* (Jiravanichpaisal et al., 2007), in *P. japonicus* after exposure to peptidoglycan (Fagutao et al., 2008) and in *P. chinensis* after exposure to WSSV (Wang et al., 2006). Results of the present study suggested that immune-related genes were triggered immediately after exposure to WSSV and the increase in expression might result in an enhanced immune response to ward off the pathogens. In contrast, the apparent decrease in expression of most immune-related genes after initial hours of infection indicates that the heightened response is temporary or short lived in the event of WSSV infection in shrimp primary culture. Similar response was observed in *P. japonicus* following peptidoglycan stimulation (Fagutao et al., 2008). The slight increase in the expression of prophenoloxidase, peroxinectin, alpha 2-macroglobulin and penaeidin in control cells with heat inactivated WSSV than the negative control cells has implications in vaccination of shrimp using inactivated WSSV. Singh et al. (2005) have demonstrated the efficacy of formalin inactivated WSSV in immunizing shrimp for 10–15 days. Crustins and penaeidins are antimicrobial peptides of which crustins act against gram positive bacteria (Rattanachai et al., 2004) while penaeidins have antibacterial and antifungal properties (Destoumieux et al., 1997). It has to be pointed out that expression of both genes decreased with increasing duration of



exposure to WSSV. But initial expression of crustin was higher than that of the control while initial penaeidin expression was similar to that of the control. This suggests the higher level of response of crustin to WSSV infection.

Cytotoxicity is considered an important index for evaluating safety of antimicrobials and management chemicals prior to their administration in aquaculture. In vitro studies can forecast their in vivo effects as well as assisting their optimization for field level application. This is specifically because under field conditions realizing their negative impacts happens to be quite cumbersome (MacGowan et al., 2001). Accordingly, the cytotoxicity of benzalkonium chloride (BKC), a quaternary ammonium compound widely used as broad spectrum disinfectant in shrimp culture ponds, was assessed and found safe at 0.1 mg l<sup>-1</sup> level.

In conclusion, the hemocyte primary culture developed using the modified 2 × L-15 medium could serve as a suitable in vitro model for WSSV titration and viral and immune related gene expression besides assessing cytotoxicity of aquaculture drugs and chemicals.

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