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In vitro propagation of hepatopancreatic parvo-like virus (HPV) of shrimp in C6/36 (*Aedes albopictus*) cell line

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ABSTRACT

Hepatopancreatic parvovirus (HPV) which causes infection in many species of penaeid shrimp is a serious viral pathogen in the young life stages of shrimp. An attempt was made to develop an *in vitro* system using C6/36 subclone of *Aedes albopictus* cell line for propagation of HPV. The results revealed that C6/36 cells were susceptible to this virus and the infected cells showed CPE in the form of vacuole formation. The results of PCR, immunocytochemistry and Western blot revealed the HPV-infection in C6/36 cell line. The RT-PCR analysis confirmed the replication of HPV in C6/36 cell line. The HPV load was quantified at different time intervals by ELISA and real time PCR, and the results showed the increase of viral load in C6/36 cell line in time course of infection. HPV propagated in C6/36 cell line was used to infect post-larvae of shrimp and the results showed that the twentieth passage of HPV propagated in C6/36 cell line caused 100% mortality in post-larvae after 6 weeks post infection (d.p.i.). The infected post-larvae showed clinical signs of reduced growth, reduced preening, muscle opacity and atrophy of hepatopancreas. The HPV-infection was confirmed by PCR. The results of the present study showed that C6/36 cell line can be used as an *in vitro* model for HPV replication instead of whole animal.

1. Introduction

Disease outbreaks have been recognized as a major constraint to aquaculture production and trade, since it is responsible for severe economic loss in aquaculture industry worldwide. Diseases of viral and bacterial origin have been found to be responsible to a great extent for severe economic loss in shrimp culture industry in different parts of the world. Till date more than twenty viruses have been reported to be associated with shrimp although only some viruses are pathogenic and pose a threat to shrimp farming (Flegel, 2012). In India, the most serious viral pathogen is white spot syndrome virus (WSSV). Other viruses reported in shrimp farming system are monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic virus (HPV) or *Penaeus monodon* densovirus (PemoDNV) (Sahul Hameed et al., 1998; Rai et al., 2009; Otta et al., 2003; Safeena et al., 2010). The occurrence of HPV was first reported in *Penaeus merguiensis* and *Penaeus semisulcatus* with clinical signs of poor growth rate, anorexia, reduced preening activity and opaqueness of tail muscle (Lightner and Redman, 1985). Since its report in 1985, very few studies have been carried out on the HPV due to various reasons including the lack of proper animal models or HPV-susceptible shrimp cell lines. Catap and Travina (2005) have successfully transmitted HPV-infection horizontally by feeding post-larvae of *P. monodon* with virus-infected post-larvae.

Attempts have been made by different workers to make use of primary cell culture of shrimp for propagation of viruses. Lu et al. (1995) have developed a convenient assay protocol using primary culture of shrimp lymphoid organ to titre yellow head virus. Primary cell cultures derived from the lymphoid organ of *P. monodon* were used as an *in vitro* model for propagation of WSSV (Wang et al., 2000). Propagation of WSSV has also been reported in ovarian primary cell culture (Maeda et al., 2004) and primary hemocyte culture (Jiang et al., 2005). Efforts have been made by different workers to make use of mosquito cell line (C6/36) for *in vitro* propagation of viruses of shrimp and prawn. Sudhakaran et al. (2007) have successfully propagated *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in C6/36 cell line. Subsequently, Sriton et al. (2009) have tried two insect cell lines such as immortal mosquito and lepidopteran cell lines for propagation of WSSV and YHV, respectively and viral propagation was confirmed by immunohistochemistry using labelled monoclonal antibodies to WSSV and YHV antigens. Arunrut et al. (2011) have reported the successful production of Taura syndrome viral antigens in C6/36 cells and also the successful infection of *Penaeus vannamei* with homogenates of TSV-infected insect cells. In the

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present study, an attempt was made to develop an *in vitro* system using C6/36 cell line for propagation of HPV. The replication of HPV in C6/36 cells was confirmed by PCR, RT-PCR, Western blot, immunocytochemistry, ELISA and real time PCR. Further, bioassay test was conducted to determine the virulence of HPV propagated in C6/36 cells.

2. Materials and methods

2.1. Collection and maintenance of experimental animals

Healthy post-larvae (PL-15) of *P. monodon* were collected from a hatchery near Chennai with no record of HPV infection. They were randomly selected and screened for HPV by PCR assay prior to challenge experiments. The PL were washed with sterile freshwater to remove food and other materials adhering to the body. After washing, the PL were maintained in glass aquaria (25 L) containing aerated sea water at a temperature of 27–30 °C with salinity between 20 and 25 parts per thousand (ppt). Natural seawater used in all the experiments was pumped from the adjacent sea, near Chennai and allowed to sediment to remove the sand and other suspended particles. The seawater was first chlorinated by treating it with sodium hypochlorite at a concentration of 25 ppm (ppm) and then dechlorinated by vigorous aeration. It was then passed through a sand filter and used for the experiments. The animals were fed with *Artemia* nauplii twice a day.

2.2. Preparation of viral inoculum

HPV-infected post-larvae of *P. monodon* were collected from hatcheries in Kakinada, Andhra Pradesh, India. They were frozen and transported to the laboratory in ice. To prepare the inoculums, the frozen infected post-larvae were thawed and homogenized in a sterile homogenizer. A 10% (w/v) suspension was made with NaCl–Tris–EDTA (NTE) buffer (0.2 M NaCl, 0.02 M Tris–HCl and 0.02 EDTA, pH 7.4). The homogenate was centrifuged at 4000 g for 20 min at 4 °C. The supernatant was collected and re-centrifuged at 10,000 g for 20 min at 4 °C. Then the final supernatant collected was filtered through a 0.22 μm pore membrane. The presence of HPV in the extract was confirmed by PCR using the primer specific to HPV (Phromjai et al., 2002). The homogenate was also tested for other shrimp viruses by PCR, as multiple viral infections are common in India (Manivannan et al., 2002). The filtrate was stored at −80 °C until further use.

2.3. Cell culture and maintenance

Mosquito cell line (C6/36) was obtained from the National Centre for Cell Science (NCCS, Pune, India). The cells were grown in Leibovitz L-15 medium (Gibco, BRL, USA) containing 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} fungizone supplemented with 2% foetal bovine serum (FBS). The flasks containing cells were incubated at 28 °C. The cell culture medium was replaced every 5 days. After the formation of monolayer, the cells were sub-cultured. For sub-culturing, the old medium was removed and the cell sheets were washed with phosphate buffered saline (PBS) twice. The cells were dispersed with 0.25% trypsin/EDTA solution (0.25% trypsin and 0.2% EDTA in PBS). The cells were then re-suspended in 10 ml of growth medium and were taken in two flasks. These cells were used for infectivity studies.

2.4. Viral susceptibility

The susceptibility of C6/36 cells to HPV was tested. The cells were seeded in a 6-well plate and incubated for 12–24 h at 28 °C to give a confluence of 60–70%. After removing the medium, 0.1 mL of virus suspension was inoculated into the cells and allowed to adsorb for 1 h. Then, 1 mL of maintenance medium containing 2% FBS was added. The inoculated cells were incubated at 28 °C and examined daily under an inverted microscope for cytopathic effect (CPE). The infected cells were harvested along with medium at different day intervals. The collected samples were freeze thawed thrice and filtered through a 0.22 μm membrane. The filtrate samples were subjected to various analyses for HPV infection and replication.

2.5. Confirmation of HPV infection by PCR

PCR analysis was carried out to confirm the HPV-infection in C6/36 cells using the primers specific to HPV (Phromjai et al., 2002). DNA was extracted from C6/36 cells exposed to HPV following the protocol described by Lo et al. (1996). The infected C6/36 cells were removed from the wells of the plate and homogenized. The homogenized samples were centrifuged at 3000*g* at 4 °C, after which the supernatant fluid was placed in another centrifuge tube together with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, and 0.1 mg/ml proteinase K). After incubation at 65 °C for 2 h, the digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation and dried. The dried DNA pellet was resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). For PCR, the primers specific to HPV were used to amplify the PCR product of 441 bp. The primer sequences used in the PCR are given in Table 1. Each PCR reaction was carried out in a 25 μl volume containing both forward and reverse primers (10 μM, 0.5 μl each), $MgCl_2$ (25 mM, 1.5 μl) dNTPs (2 mM, 2.0 μl), PCR buffer (10 × 2.5 μl), Taq-DNA (1 U, 0.5 μl), template DNA (100 ng) and nucleic acid free water (16.5 μl). PCR cycle consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, annealing temperature of 60 °C for 1 min, 72 °C for 30 s, and a final extension of 10 min at 72 °C. The PCR products were analysed by electrophoresis in 1.8% agarose gel stained with ethidium bromide, and visualized by ultraviolet transillumination.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out to study the expression of HPV capsid gene in C6/36 cell line exposed to HPV. The cells exposed to HPV were collected and pelleted by centrifugation at 500*g* for 5 min and suspended in Trizol to extract total RNA as per the manufacturer's instructions (Gibco BRL, Life Technologies, Rockville, USA). The extracted RNA was dissolved in nuclease free water. The contaminating DNA from the sample was removed by treating with DNase I (Bangalore Genei, India) at 37 °C for 30 min. The integrity of RNA was verified by electrophoresis using ethidium bromide staining. The purity of RNA was checked by measuring the ratio

Table 1
Primers used in the present study.

Primers	Product size (bp)	Sequence (5′–3′)	Annealing temp (°C)
HPVF	441	GCATTACAAGAGCCAAGCAG	60
HPVR		ACACTCAGCCTCTACCTTGT	
qHPVF	147	CGCGGATCCAGGTAGAGGCTGAGTGTAA	55
qHPVR		CGCGAATTCCAGGTAGTGACGCCGAAA	
β-ActinF	686	GAYGAYATGGAGAAGATCTGG	57
β-ActinR		CRGGGTACATGGTGGTRCC	

PCR primer.
Real time PCR primer.

of OD_{260} nm/OD_{280} nm. The DNase-treated total RNA was denatured by heating at 85 °C for 10 min in 10 μl DEPC-water containing 100 pmol oligo-dT primers (Bangalore Genei, India). The first strand cDNA was synthesized by the addition of 3 μl 5 × M-MuLV buffer, 1 μl 100 mM DTT, 1 μl 10 mM dNTPs, 10 U RNasin (Bangalore 200 Genei, India) making a total volume of 10 μl including 100 U M-MuLV reverse transcriptase (New England Biolabs, Beijing, China). The reaction was allowed to proceed at 37 °C for 1 h. The cDNA reaction products were subjected to PCR as described above with the primers specific to capsid gene of HPV and β-actin. The sequences of primers used are given in Table 1. The β-actin served as an internal control for RNA quality and amplification efficiency (Tsai et al., 2000; Zhang et al., 2002). A NoRT control reaction for PCR was performed with total RNA without M-MuLV reverse transcriptase to confirm the lack of genomic DNA contamination in each reaction mixture.

2.7. Quantitation of HPV by real time PCR

The C_T (Cycle Threshold) value of HPV in C6/36 cell line exposed to HPV was determined by quantitative real time PCR at different day intervals (d.p.i.) based on the method described by Pfaffl (2001) and Dhar et al. (2002). Viral DNA was isolated from HPV-infected C6/36 cells by the method described above. DNA was quantified using the NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, USA) at 260 nm. The primers specific to capsid gene (Table 1) of HPV were used to amplify a fragment of 147 bp. The viral load was estimated by StepOnePlus™ system (Applied Biosystems, Singapore) using DyNAmo™SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). Reaction was carried out in triplicate with a volume of 10 μl in each reaction. Each PCR had a negative control in which uninfected C6/36 DNA was added as template. The PCR parameters consisted of 40 cycles of denaturation at 94 °C for 10 s, annealing at 56 °C for 5 s, and extension at 72 °C for 10 s. Known concentrations of Plasmid pHCP (a small stretch of HCP gene of HPV cloned into pRSET B vector) were used as standards in triplicates wells to estimate DNA concentrations from the C6/36 cell line exposed to HPV.

2.8. Indirect ELISA analysis

Indirect ELISA was carried out to detect HPV in post-larval samples and infected C6/36 cells exposed to HPV at different day intervals of post inoculation (p.i.) according to the method of Schurrs and van Weemen (1977). The ELISA plates were coated with samples (approximately 500 ng per well) in coating buffer (0.1 M sodiumcarbonate, 0.25 M sodiumbicarbonate, pH, 9.6) and kept overnight at 4 °C. After coating, the plates were washed once with PBS and blocked with 2% BSA in PBS for 1 h at 37 °C. The assay was done as described by Huang et al. (2001), where the normal mice serum and purified HPV were used as negative and positive controls, respectively. The plates were incubated with 100 μl of primary antibodies (final dilution 1:1000) for 3 h at 37 °C, obtained from mice sensitized against purified whole virus. Then, the plates were washed with PBS/T (0.05% Tween 20) three times for 2 min each and incubated with 100 μl of anti-mouse IgG alkaline phosphatase conjugate (final dilution 1:5000) for 1 h. The plates were finally washed with PBS/T three times for 2 min each and developed with the substrate p-nitrophenyl phosphate in substrate buffer. The optical density was measured at 405 nm using an ELISA reader.

2.9. Western blot analysis

Western blot analysis was carried out to detect the HPV in virus-inoculated C6/36 cells by the method of Talbot et al. (1984). Naturally infected PL samples were used as positive control and uninfected cells as negative control. After SDS–PAGE separation of samples, the gel was transferred to nitrocellulose paper (NCP) at 300 mA for 2 h at 4 °C. After transfer, the NCP was blocked for 1 h with 3% skimmed milk in PBS. The NCP was incubated in primary antiserum raised in mouse against purified whole virus at a dilution of 1/10,000 in PBS containing 0.2% BSA for 3 h. Subsequently, the membrane was then incubated in ALP-conjugated goat anti-mouse IgG (Sigma–Aldrich, Bangalore, India) at a dilution of 1:1000 for 1 h and the presence HPV was detected with a substrate solution of 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma–Aldrich, Bangalore, India) in substrate buffer.

2.10. Cytological study

C6/36 cell line was grown on cover slips for 24 h, exposed to HPV, incubated at 28 °C and examined daily under an inverted microscope for cytopathic effect (CPE). When 80% of the cells formed CPE, the cells were washed and fixed with 3.7% formalin in PBS. Cells were then stained with hematoxylin and eosin.

2.11. Immunocytochemical study

C6/36 cell line was grown on cover slips for 24 h, exposed to HPV (10 days) and fixed with 3.7% p-formaldehyde for 10 min at 4 °C, washed with 5× PBS, permeabilized with 0.2% Triton X-100 and blocked in PBS containing 1% bovine serum albumin (BSA). Polyclonal antibodies raised against purified HPV were diluted at 1:100 in PBS with 1% BSA and directly added to the fixed cells and then kept for 2 h at room temperature. Cells were again washed with wash buffer, followed by addition of the appropriate secondary antibody. Secondary antibodies, anti-mice IgG fluorescein iso-thio cyanate (FITC) at a dilution of 1:100 in the same solution as the primary antibody were applied for 45 min at room temperature. Cells were again washed with wash buffer, and the cover slips were mounted with antifade 1, 4-diazobicyclo-2, 2, 2-octanex (DABCO) in mounting medium (Sigma). The cover slips were observed under a fluorescent microscope (Carl Zeiss, Germany).

2.12. Virus virulence study

Experimental pathogenicity was carried out in healthy post-larvae of *P. monodon* by immersion method to determine the virulence of HPV propagated in C6/36 cells. The HPV propagated in twenty successive passages in C6/36 cells were also used in the infectivity studies. The inoculum of HPV was prepared from HPV-infected C6/36 cells using the protocol described earlier. The post-larvae (30 per beaker) were divided into four groups and maintained separately in 5 L beakers at 27–30 °C with salinity ranging between 20 and 25 ppt. The beakers were covered with a lid to prevent contamination. The viral inoculum was added to the medium at a concentration of 0.1% as described by Venegas et al. (1999) and Chen et al. (2000). In groups I and II, the post-larvae were treated with viral inoculum prepared from infected and uninfected post-larvae, respectively. In groups III and IV, the animals were exposed to the viral suspension prepared from infected and uninfected C6/36 cells, respectively. The experiment was conducted in triplicate. The animals were observed twice daily and cumulative mortality was calculated at different time intervals. The collected PL samples was washed three times with PBS, and stored at −20 °C for PCR analysis.

3. Results

The viral inoculum used to infect C6/36 cells was initially confirmed by PCR (Fig. 1). CPE observed in HPV-infected cells includes typical multiple vacuolation, shrinkage of cells and degeneration of monolayer, no CPE was observed in uninfected C6/36 cells (Figs. 2 and 3). Initially, CPE developed as localized areas of rounded and refractile cells which later spread over the monolayer after 5 days of post infection and formed a network of degenerating cells. The monolayer disintegrated completely after 7 days. The CPE was also observed in the cells exposed to HPV propagated after five successive passages in C6/36 cells.

The PCR analysis was carried out to confirm the HPV-infection in C6/36 cells exposed to HPV and the results are shown in Fig. 4. The PCR results revealed a distinct band of 441 bp specific to HPV after electrophoresis of the PCR product while no band was observed in the control group (Fig. 4). The replication of HPV in C6/36 cells was confirmed by RT-PCR analysis with reference to capsid gene of HPV and the results are shown in Fig. 5. The results revealed that the messenger RNA specific to capsid gene of HPV could be clearly detected in the HPV exposed C6/36 cells at different d.p.i. The β-actin mRNA was detected in all the samples tested (Fig. 5B) and NoRT controls without reverse transcriptase enzyme were also run (Fig. 5C).

The HPV propagated in C6/36 cells was quantified at different time intervals by real time PCR and the results are presented in Fig. 6A. The viral load increased during the course of infection in the cell line exposed to HPV and a lower C_T value of HPV was observed in the cell line exposed to virus for 12 days when compared to those cells which were exposed to the virus for 4 days. The coefficient of variation (CV) for the C_T values of HPV capsid protein gene was found to be less than 5%. This indicated that the assay was highly reproducible.

The replication of HPV in C6/36 cells was also quantified by indirect ELISA using the polyclonal antiserum raised against purified whole virus of HPV. The viral protein was quantified in C6/36 cell line at different day intervals and the results are shown in Fig. 6B. The amount of viral protein was found to be higher in C6/36 cell line exposed to HPV for 12 days when compared to early days of exposure. Western blot analysis using the antiserum raised against the purified whole virus detected the HPV in C6/36 cell line exposed to different days and the results are shown in Fig. 7. A distinct band corresponding to 54 kDa molecular marker protein was observed in the HPV exposed C6/36 cell line whereas no background coloration was observed in the uninfected cell line.

Further investigation performed by immunocytochemical study using antiserum raised against purified whole virus also supported

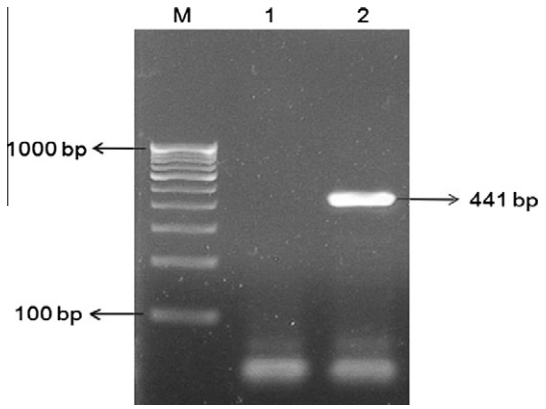

Fig. 1. PCR Confirmation of naturally infected *P. monodon* by HPV specific primers. M – marker; 1 – negative control; 2 – positive control (infected PL).

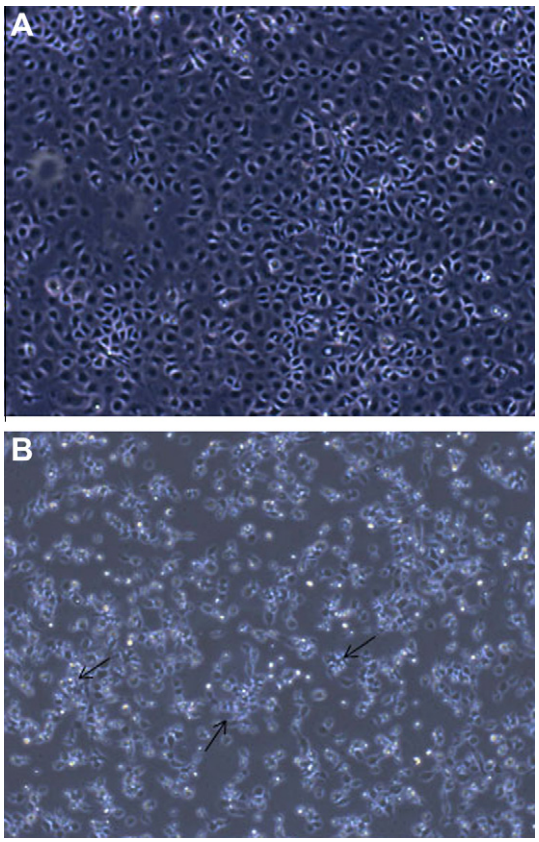

Fig. 2. Phase-contrast photomicrographs of C6/36 cell line exposed to HPV. (A) Normal C6/36 cell line, (B) C6/36 cell line exposed to HPV. Note vacuolations in the cells exposed to HPV (arrow).

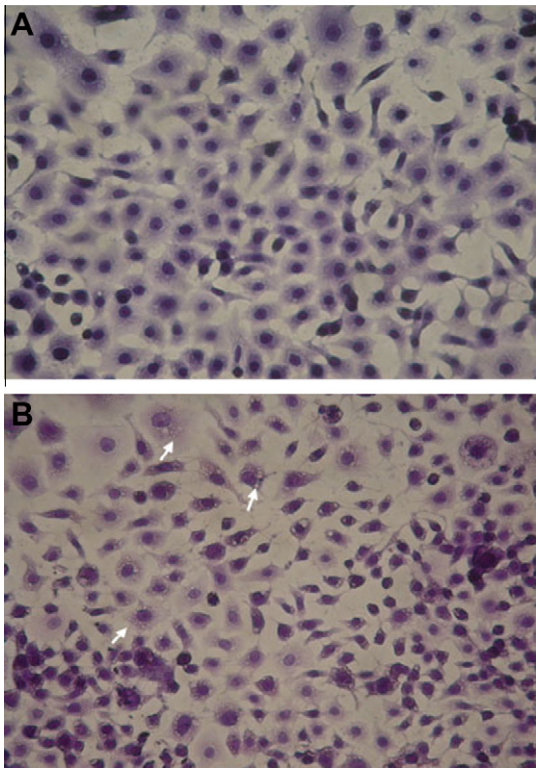

Fig. 3. Photomicrographs of C6/36 cell line stained with hematoxlyin and eosin. (A) Normal C6/36 cell line, (B) C6/36 cell line exposed to HPV showing multiple vacuolations (arrow).

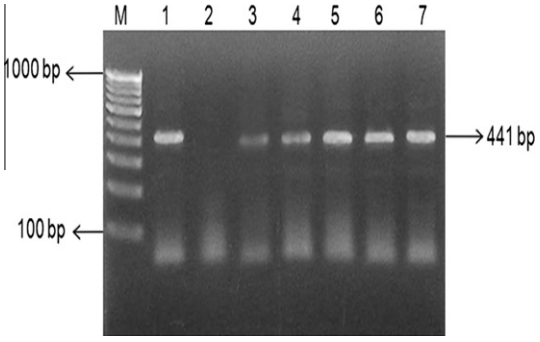

Fig. 4. PCR detection of HPV in C6/36 cell line exposed to HPV at different time intervals. M – marker; Lane 1 – positive control (HPV-infected shrimp PL); Lane 2 – uninfected C6/36 cell line; Lanes 3, 4, 5, 6 and 7 – cell line of 4, 6, 8, 10 and 12 days of post infection respectively.

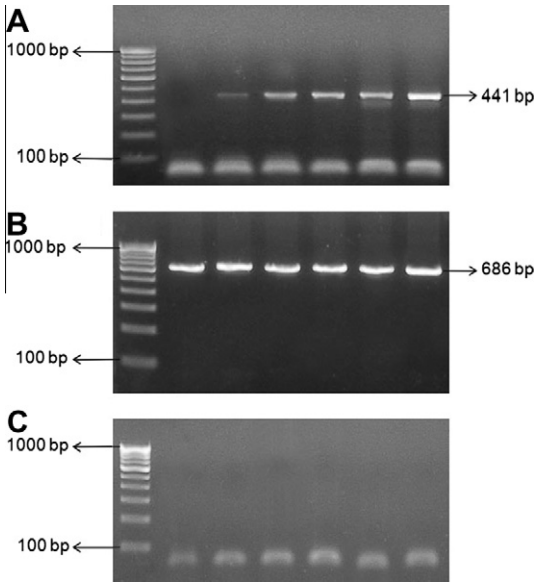

Fig. 5. (A) RT-PCR confirmation of replication of HPV in C6/36 cell line. M – marker; 1 – uninfected C6/36 cell line; Lanes 2, 3, 4, 5 and 6 – cell line of 4, 6, 8, 10 and 12 days culture period, (B) RT-PCR results of the same samples for the β-actin gene. (C) NoRT control reactions without reverse transcriptase.

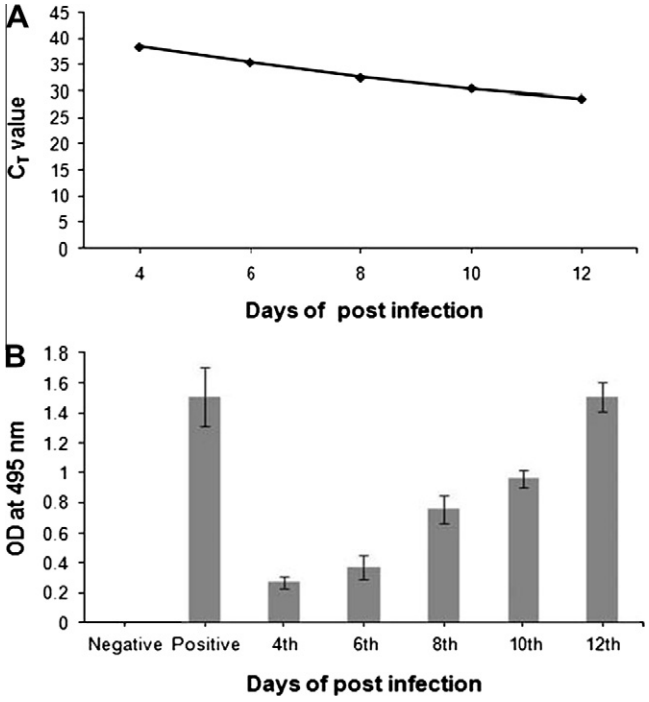

Fig. 6. (A) Mean values of cycle threshold (C_T) of HPV per micro litre of total DNA from HPV exposed to C6/36 cell line at different day intervals. (B) Quantification of capsid protein of HPV in C6/36 cell line exposed to HPV by ELISA using anti-HCP of HPV and positive PL is used as control.

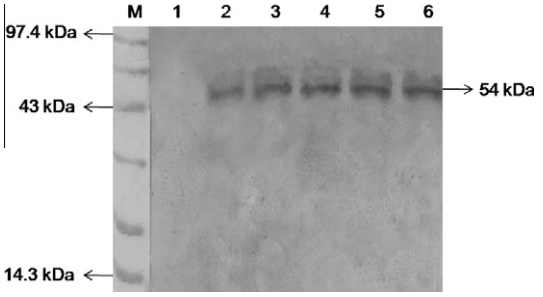

Fig. 7. Detection of HPV by Western blot analysis using anti-HCP of HPV in C6/36 cell line of different day exposed to HPV. M – marker; Lane 1 – uninfected C6/36 cell line; Lanes 2, 3, 4, 5 and 6 – cell line of 4, 6, 8,10 and 12 days of post infection respectively.

the replication of HPV in C6/36 cell line. Immuno fluoresce was observed in C6/36 cells infected with HPV (Fig. 8) while uninfected cells did not show fluorescence. This confirms that HPV specific antibody reacts with HPV. As described above, immersion challenge experiments were conducted with post-larvae of *P. monodon* in four groups. Groups I and II were treated with inoculum prepared from infected and uninfected PL homogenates, respectively and groups III and IV with infected and uninfected cell culture supernatants respectively. No mortalities were observed in groups II and IV whereas 100% mortality was observed in groups I and III after 6 weeks of post-infection (Fig. 9). The dead and moribund post-larvae collected from the groups I and III were analyzed by PCR and results showed the presence of HPV whereas the PCR results were negative in post-larvae belonging to the groups II and IV (Fig. 10).

4. Discussion

In the present study, an attempt was made to propagate HPV in C6/36 cell line. The results of PCR, RT-PCR, Western blot, ELISA and real time PCR analysis revealed that the HPV could easily be propagated in mosquito cell line (C6/36 cell line). Currently, there is no host specific cell culture for studying crustacean viruses, thus presenting a need to find an appropriate model. A promising *in vitro* system has not been established either from penaeid shrimp or other crustacean hosts for HPV propagation. In the present study, C6/36 cells have been used to propagate the HPV and this virus has been successfully propagated in C6/36 cells.

Initial attempts have been made to make use of mosquito cell lines for propagation of crustacean viruses (Sudhakaran et al., 2007; Sriton et al., 2009; Gangnonngiw et al., 2010; Arunrut et al., 2011). The *Mr*NV and XSV of freshwater prawn have been successfully propagated in C6/36 cell line by serial passaging of the cell culture supernatant (Sudhakaran et al., 2007). The *Mr*NV/XSV have been isolated successfully from RT-PCR positive insect carriers collected from ponds which experienced white tail disease (WTD) outbreak (Sudhakaran et al., 2008). This clearly indicates the suitability of C6/36 cell line for isolation of *Mr*NV and XSV from

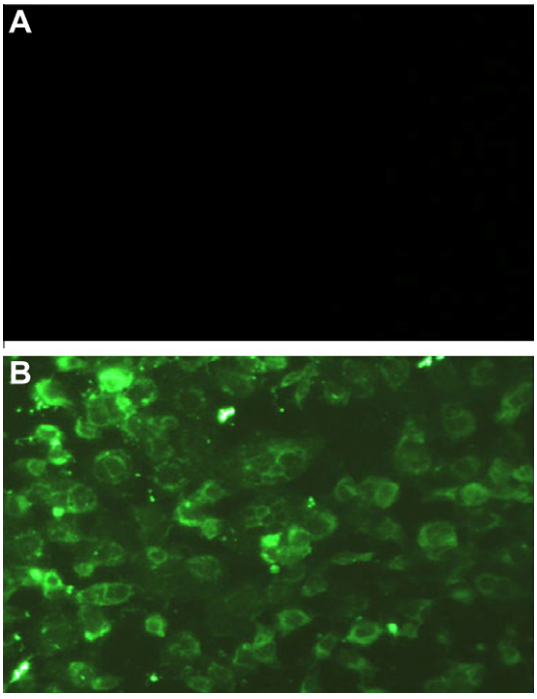

Fig. 8. Detection of HPV in HPV exposed to C6/36 cell line by immunofluorescence using antiserum raised against capsid protein of HPV. (A) Normal control, (B) HPV exposed to C6/36 cell line.

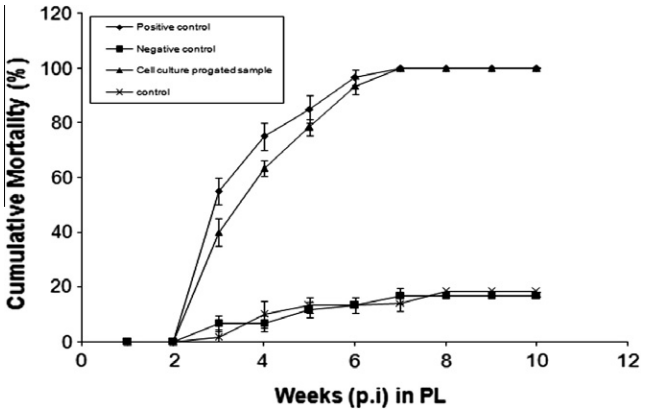

Fig. 9. Cumulative percent mortality of post-larvae of *P. monodon* exposed to HPV propagated in C6/36 cell line at different time intervals.

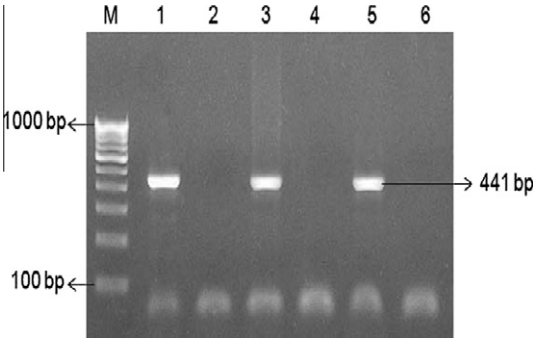

Fig. 10. PCR detection of HPV in infected post larvae. M – marker; Lane 1 – positive control (HPV-infected shrimp PL); Lane 2 – uninfected PL; Lanes 3 – group I sample, Lanes 4 – group II sample, Lanes 5 – group III sample, Lanes 6 – group IV sample.

WTD-infected prawn. Sriton et al. (2009) have challenged mosquito and lepidopteran cell lines with WSSV and YHV, and observed the expression of viral antigens in these cell lines using antibodies to shrimp viral antigens as observed in the present study. RT-PCR analysis revealed the expression of capsid protein in HPV-infected C6/36 cells which was further confirmed by ELISA and Western blot using the antibodies raised against HPV. These results clearly indicate the replication of HPV in C6/36 cells. In the present study, HPV infection in C6/36 cells was confirmed by PCR, RT-PCR, Western blot, immunocytochemistry and bioassay. The HPV load was also quantified by indirect ELISA and real time PCR. It is clear from the results obtained that the C6/36 cells are highly permissive to HPV and that it can be propagated on large-scale in the C6/36 cells. Transcription of capsid gene of HPV observed was a strong evidence for multiplication of HPV in C6/36 cells. Time course studies of HPV protein production assayed by indirect ELISA indicate replication of HPV in C6/36 cells. The observation on production of viral protein in time course of infection in C6/36 cells in the present study concurs with the observation made by Jiravanichpaisal et al. (2006) in haematopoietic stem cells and Nathiga Nambi et al. (2012) in heart explants of freshwater crab on production of VP28 protein in time course infectivity study.

The load of HPV in C6/36 cells was determined by real time PCR at different days intervals and the results revealed a decrease in C_T value of HPV in C6/36 cell line exposed to virus for 12 days when compared to the cells exposed for 5 days. Nathiga Nambi et al. (2012) have quantified WSSV in heart explants of freshwater crab and observed low C_T in heart explants on prolonged viral exposure when compared to short time exposure as observed in the present study.

The pathogenesis of HPV propagated from C6/36 cell culture was successfully carried out in post-larvae of *P. monodon* by immersion method, as ascertained by the presence of the clinical signs of the disease and subsequent confirmation by PCR. The result of experimental pathogenicity agrees with the observation made by Sudhakaran et al. (2007) on virulence of *Mr*NV and XSV propagated in C6/36 cell line but differs from the observation made by Arunrut et al. (2011) on the virulence of YHV propagated in C6/36 cell line. Arunrut et al. (2011) reported that YHV cultivated in C6/36 cells from passage 5 caused mortality with typical clinical sign of YHV infection but the YHV from passage 15 did not result in shrimp mortality. Arunrut et al. (2011) suggested that the YHV that had become attenuated after 15 passages in insect cells and virus from lower passages might cause infection in susceptible shrimp.

The results of the present study reveal the suitability of C6/36 cell line for HPV propagation. The C6/36 cell line of *A. albopictus* is simple and cost-effective to maintain in the laboratory. They can be stored at room temperature (25–28 °C) for approximately 2 weeks without a change of medium. The cells have a high split ratio of 1–8 to 1–10 and grow to a complete monolayer in 3–4 days when seeded at 5×10^5 cells per ml. In conclusion the results of the present study also indicate that the C6/36 cells would be a good alternative to whole animals for *in vitro* replication and production of HPV. This cell line was found to be suitable not only for propagating some viruses of shrimp and prawn, but also for their isolation from infected animals. Further work is needed to test the suitability of C6/36 cell line for propagating other shrimp viruses.

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References

Arunrut, N., Phromjai, J., Gangnonngiw, W., Kanthong, N., Sriurairatana, S., Kiatpathomchai, W., 2011. *In vitro* cultivation of shrimp Taura syndrome virus (TSV) in a C6/36 mosquito cell line. J. Fish. Dis. 34, 805–810.

Catap, E.S., Travina, R.D., 2005. Experimental transmission of hepatopancreatic parvovirus (HPV) infection in *Penaeus monodon* postlarvae. Diseases in Asian Aquaculture V. pp. 415–420.

Chen, L.L., Lo, C.F., Chiu, Y.L., Chang, C.F., Kou, G.H., 2000. Natural and experimental infection of white spot syndrome virus WSSV in benthic larvae of mud crab *Scylla serrata*. Dis. Aquat. Org. 40, 157–161.

Dhar, A.K., Roux, M.M., Klimpel, K.R., 2002. Quantitative assay for measuring the Taura syndrome virus and yellow head virus load in shrimp by real-time RT-PCR using SYBR Green chemistry. J. Virol. Methods 104 (1), 69–82.

Flegel, T.W., 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. J. Invertebr. Pathol. 110 (2), 166–173.

Gangnonngiw, W., Kanthong, N., Flegel, T.W., 2010. Successful propagation of shrimp yellow head virus in immortal mosquito cells. Dis. Aquat. Org. 90, 77–83.

Huang, C.H., Zhang, L.R., Zhang, J.H., Xiao, L.C., Wu, Q.J., Chen, D.H., Li, J.K.K., 2001. Purification and characterization of white spot syndrome virus (WSSV) produced in an alternate host: crayfish *Procambarus clarkii*. Virus Res. 76, 115–125.

Jiang, Y.S., Zhan, W.B., Wang, S.B., Xing, J., 2005. Development of primary shrimp hemocyte cultures of *Penaeus chinensis* to study white spot syndrome virus (WSSV) infection. Aquaculture 253, 114–119.

Jiravanichpaisal, P., Lee, B.L., Soderhall, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213–236.

Lightner, D.V., Redman, R.M., 1985. A parvo-like virus disease of penaeid shrimp. J. Invertebr. Pathol. 45, 47–53.

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H., 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimps, crabs and other arthropods. Dis. Aquat. Org. 27, 215–225.

Lu, Y., Tapay, L.M., Loh, P.C., Brock, J.A., Gose, R., 1995. Development of a quantal assay in primary shrimp cell culture for yellow head baculovirus (YBV) of penaeid shrimp. J. Virol. Methods 52, 231–236.

Maeda, M., Saitoh, H., Mizuki, E., Itami, T., Ohba, M., 2004. Replication of white spot syndrome virus in ovarian primary cultures from the kuruma shrimp *Marsupenaeus japonicus*. J. Virol. Methods 116, 89–94.

Manivannan, S., Otta, K.S., Karunasagar, I., Karunasagar, I., 2002. Multiple viral infection in *Penaeus monodon* shrimp postlarvae in an Indian hatchery. Dis. Aquat. Org. 48, 233–236.

Nathiga Nambi, K.S., Abdul Majeed, S., Sundar Raj, N., Taju, G., Madan, N., Vimal, S., Sahul Hameed, A.S., 2012. In vitro white spot syndrome virus (WSSV) replication in explants of the heart of freshwater crab, *Paratelphusa hydrodomous*. J. Virol. Methods 183, 186–195.

Otta, S.K., Karunasagar, Indrani., Karunasagar, Iddaya., 2003. Detection of *monodon* baculovirus (MBV) and White spot syndrome virus (WSSV) in apparently healthy postlarvae from India by polymerase chain reaction. Aquaculture 220, 59–67.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29 (9), 2002–2007.

Phromjai, J., Boonsaeng, V., Withyachumnarnkul, B., Flegel, T.W., 2002. Detection of hepatopancreatic parvovirus in Thai shrimp *Penaeus monodon* by in situ hybridization, dot blot hybridization and PCR amplification. Dis. Aquat. Org. 51, 227–232.

Rai, P., Pradeep, B., Safeena, M.P., Karunasagar, I., Karunasagar, 2009. Simultaneous presence of infectious hypodermal and hematopoetic necrosis virus (IHHNV) and Type A virus-related sequences in *Penaeus monodon* from India. Aquaculture 295, 168–174.

Safeena, Muhammed P., Tyagi, Anuj, Rai, Praveen, Karunasagar, Iddya, Karunasagar, Indrani, 2010. Complete nucleic acid sequence of Penaeus monodon densovirus (PmDNV) from India. Virus Res. 150, 1–11.

Sahul Hameed, A.S., Anilkumar, M., Stephen Raj, M.L., Jayaraman, Kunthala, 1998. Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. Aquaculture 160, 31–45.

Schurrs, A.H.W.M., van Weemen, B.K., 1977. Enzyme immunoassay. Clin. Chim. Acta 81, 1–40.

Sriton, A., Kanthong, N., Gangnonngiw, W., Sriurairatana, S., Ubol, S., Flegel, T.W., 2009. Persistent expression of shrimp – virus antigens in two insect cell lines challenged with two shrimp viruses. Fish Pathol. 44 (2), 86–93.

Sudhakaran, R., Parameswaran, V., Sahul Hameed, A.S., 2007. *In vitro* replication of *Macrobrachium rosenbergii* nodavirus and extra small virus in C6/36 mosquito cell line. J. Virol. Methods 146, 112–118.

Sudhakaran, R., Haribabu, P., Rajesh Kumar, S., Sarathi, M., Ishaq Ahmed, V.P., Sarath Babu, V., Venkatesan, C., Sahul Hameed, A.S., 2008. Natural aquatic insect carriers of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). Dis. Aquat. Org. 79, 141–145.

Talbot, P.V., Knobler, R.L., Buchmeier, M., 1984. Western and dot immunoblotting analysis of viral antigens and antibodies: application to murine hepatitis virus. J. Immunol. Methods 73, 177–188.

Tsai, M.F., Lo, C.F., van Hulten, M.C., Tzeng, H.F., Chou, C.M., Huang, C.J., Wang, C.H., Lin, J.Y., Vlak, J.M., Kou, G.H., 2000. Transcriptional analysis of the ribonucleotide reductase genes of shrimp white spot syndrome virus. Virology 277, 92–99.

Venegas, C.A., Nonaka, L., Mushiake, K., Nishizawa, T., Muroga, K., 1999. Athogenicity of penaeid rod-shaped DNA virus PRDV to kuruma prawn at different development stages. Fish Pathol. 170, 179–194.

Wang, C.H., Yang, H.N., Tang, C.Y., Lu, C.H., Kou, G.H., Lo, C.F., 2000. Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis. Aquat. Org. 41, 91–104.

Zhang, X., Huang, C., Xu, X., Hew, L., 2002. Transcription and identification of an envelope protein gene (p22) from shrimp white spot syndrome virus. J. Gen. Virol. 83, 471–477.