

NOTE

Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of *Penaeus monodon*

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ABSTRACT: Yellow head virus is a highly pathogenic agent that can cause a fatal disease in several species of penaeid shrimps. Using a primary cell culture and an *in vitro* quantal assay (TCID₅₀), this study sought to determine the propagation profile of yellow head virus after inoculation at a low multiplicity of infection in the lymphoid tissue (oka organ) of *Penaeus monodon*. Detectable levels of virus were present as early as 24 h post-inoculation. Maximal viral yields were reached by 4 d post-infection, approximately 24 h after the onset of a detectable cytopathic effect, which was normally observable at 3 d post-inoculation. The methodology provides a useful tool for studying yellow head virus–host-cell interactions.

KEY WORDS: *Penaeus monodon* · Yellow head virus · YHV · Cell culture · Oka organ

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Yellow head virus (YHV) is a highly pathogenic agent that causes an acute and often fatal disease in a variety of penaeid shrimps. Susceptible species include *Penaeus monodon* (Boonyaratpalin et al. 1993), *P. aztecus*, *P. duorarum*, *P. merguensis*, *P. setiferus* (Flegel 1997, Flegel et al. 1997, Lightner et al. 1998) as well as *P. stylirostris* and *P. vannamei*, although in the latter 2 species the characteristic yellow discoloration of the cephalothorax is not observed (Lu et al. 1994). Infection of *P. monodon* shrimp stocks with YHV has been reported in the SE Asian and Indo-Pacific countries of Thailand (Boonyaratpalin et al. 1993), India (Mohan et al. 1998) and Taiwan (Wang & Chang 2000), and has caused significant economic loss.

The virus particle is enveloped and contains a positive-sense single-stranded RNA (Tang & Lightner 1999). The gill and lymphoid oka organ tissues are primary targets for YHV replication (Lu et al. 1995a), and YHV will infect cells of both ectodermal and mesoder-

mal origin (Boonyaratpalin et al. 1993). Cellular necrosis has been reported to be widespread in connective tissues, hematopoietic organs and the lymphoid organ, and massive necrosis of circulating hemocytes is characteristic of YHV infection (Boonyaratpalin et al. 1993).

Currently there are no established penaeid cell lines, and therefore primary cell culture techniques have been developed for the growth and analysis of penaeid shrimp viruses, including YHV (Lu et al. 1995b, Chen & Wang 1999). Explant cell cultures have been obtained from various tissues and organs of the penaeid shrimp, including the lymphoid (oka) organ (Chen & Kou 1989, Nadala et al. 1993, Hsu et al. 1995, Lu et al. 1995b, Tapay et al. 1995, 1997, Chen & Wang 1999, Itami et al. 1999, Kasornchandra et al. 1999, Owens & Smith 1999, West et al. 1999, Wang et al. 2000), the heart (Chen et al. 1986, Chen & Wang 1999, Owens & Smith 1999), nerve cord (Nadala et al. 1993, Owens & Smith 1999), gut (Nadala et al. 1993), hepatopancreas (Owens & Smith 1999) and gonads (Chen et al. 1986, Luedeman & Lightner 1992, Nadala et al. 1993, Chen & Wang 1999, Fraser & Hall 1999, Owens & Smith 1999). Primary cell cultures of the lymphoid organ are susceptible to YHV (Lu et al. 1995b, Chen & Wang 1999) and to white spot syndrome virus (WSSV: Tapay et al. 1997), both of which produce cytopathic effects (CPE) that include 'roundings' of cells and their detachment from the substrate, which occurs as early as 3 d post-inoculation for YHV (Lu et al. 1995b) and 2 d post-inoculation for WSSV (Tapay et al. 1997). The present study aimed at determining the virus propagation profile of YHV in a primary cell culture of the oka organ by means of an *in vitro* quantal assay (TCID₅₀) (Reed & Muench 1938).

Materials and methods. Cell culture, virus purification, and analyses were carried out as follows:

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Primary lymphoid (oka) cell culture: Primary cell cultures from the lymphoid (oka) organ of *Penaeus monodon* were established as described by Kasornchandra et al. (1999), except that we used the growth medium (minus shrimp-meat extract) as holding medium and supplemented both growth medium and holding medium with 5% (w/v) lactalbumin.

Virus purification: To obtain a source of fresh virus, previously purified YHV was injected directly into the muscle of healthy *Penaeus monodon*, which were then maintained in seawater at 26 to 28°C. When the shrimp became moribund, hemolymph was drawn and YHV was purified by urografin gradient ultracentrifugation (Wongteerasupaya et al. 1995).

Nucleic acid extraction and PCR: Nucleic acids were extracted from either 30 µl of growth medium or 200 µl of hemolymph using TRIzol[®] LS reagent (Gibco-BRL) according to the manufacturer's recommendations. The presence of YHV was detected by reverse transcriptase-PCR (RT-PCR) using the methodology and primers of Wongteerasupaya et al. (1997), which produce a 135 bp amplicon in the presence of YHV. The presence of WSSV was detected by PCR using the SEMTEST MINI KIT (Ezee Gene, SBBU, Thailand) according to the manufacturer's protocol. This reaction produces a 232 bp amplicon in the presence of WSSV. A positive control for WSSV is included in the kit.

SDS-PAGE and Western blot analysis: Either 40 µl of growth medium (after centrifugation at 1500 × *g* for 15 min at 4°C) or 1 µl of purified virus were loaded onto parallel sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Following electrophoresis, 1 gel was stained with Coomassie Brilliant Blue R-250, while the other was blotted onto a nitrocellulose membrane which was then incubated with a mouse monoclonal antibody specific to the p116 protein of yellow head virus (YHV-monoclonal detection kit; Ezee Gene, SBBU, Thailand) at a dilution of 1:3000 for 2.5 h at room temperature (25°C) with gentle agitation. After rinsing 3 times with Tris-buffered saline, the membrane was incubated with a horseradish peroxidase labeled goat anti-mouse (IgG) antibody (Sigma Chemical) at a dilution of 1:3000 at room temperature with gentle agitation on a shaker for 1.5 h. The signal was developed using the ECL Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech) and exposure to X-ray film for 5 min.

Electron microscopy: Transmission electron microscope examination of the viral stock was essentially as described by Wongteerasupaya et al. (1995), and grids were examined with a JOEL Model JEM-200CX transmission electron microscope with an accelerating voltage of 100 kV.

Propagation of YHV in primary lymphoid cell culture: Primary cell cultures from lymphoid organs were established in 25 cm² culture flasks. When confluent monolayers were formed, the cell cultures were infected with YHV. Briefly, purified YHV was diluted from 2 × 10⁹ TCID₅₀ ml⁻¹ to 2 × 10³ TCID₅₀ ml⁻¹ (determined by TCID₅₀ assay: see following subsection) with basal medium, passed through a 0.45 µm filter and inoculated onto confluent monolayers (containing 2 × 10⁶ cells) at a multiplicity of infection (MOI) of 0.001. After infection, 4.5 ml of basal medium was added. Flasks were then incubated at 28 ± 1°C for 8 d and duplicate 100 µl aliquots of culture medium were sampled daily during this time. Each 100 µl aliquot was subjected to TCID₅₀ analysis, allowing duplicate determination of viral levels at each time point. The experiment was undertaken in duplicate. Control flasks were inoculated with basal medium only.

In vitro quantal assay (TCID₅₀): Confluent monolayers of primary cell cultures of the lymphoid organ, established in 96-well tissue-culture plates were used for TCID₅₀ analysis. Half of the 100 µl of the aliquots containing YHV was diluted by 10-fold serial dilutions with basal medium, 50 µl of each dilution was inoculated into each of 8 parallel wells and plates and incubated at room

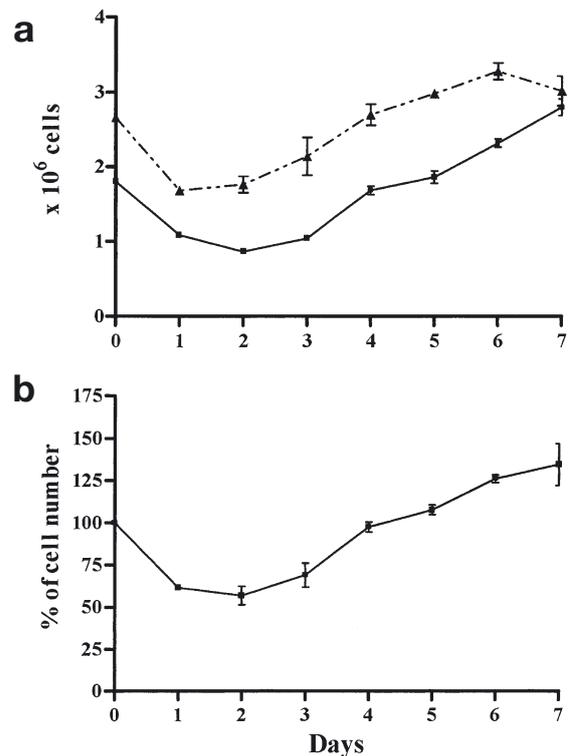


Fig. 1. *Penaeus monodon*. Growth curves of primary cell cultures of oka organ. (a) Mean (\pm SD, $n = 2$) cell numbers in 2 independent experiments; (b) mean (\pm SEM, $n = 2$) growth of primary cell cultures as percentage of cells initially seeded

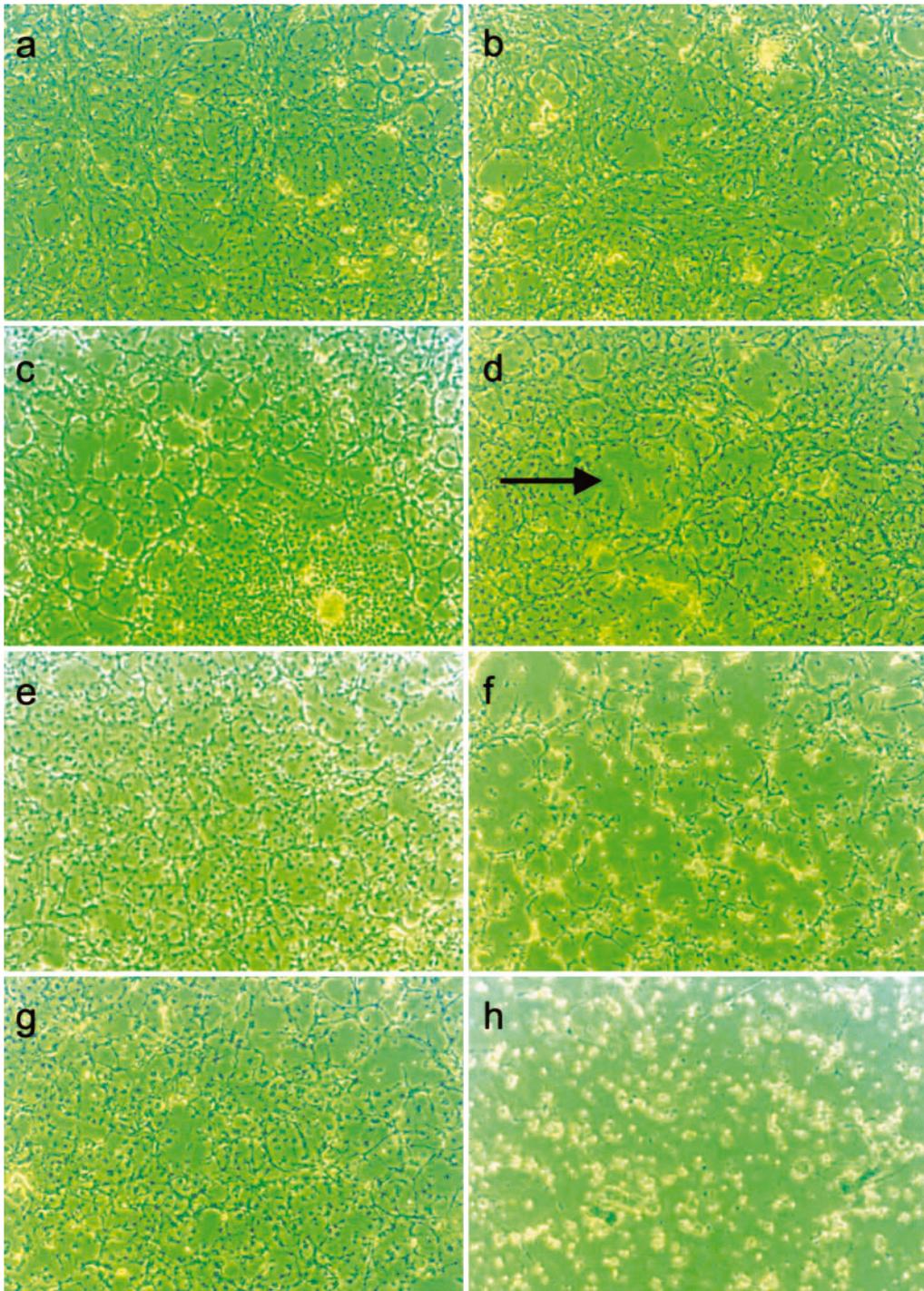


Fig. 2. *Penaeus monodon*. Propagation of yellow head virus (YHV) in primary cell culture of oka organ. (a,c,e,g) Confluent monolayers of cells inoculated with control medium, and (b,d,f,h) inoculated with YHV; all incubated for 7 d. (a,b) Culture at 1 d, (c,d) at 3 d, (e,f) at 5 d, (g,h) at 7 d post-inoculation. Cytopathic effects (arrow in d) were first observed at 3 d post-inoculation magnification (a–h) $\times 40$

temperature for 90 min. After incubation, 150 μ l of basal medium was added. The inoculated plate was then incubated at $28 \pm 1^\circ\text{C}$ for 7 d. TCID₅₀ calculations were made as described by Reed & Muench (1938).

Results and discussion. While several studies have reported the establishment of primary cell cultures from the oka organ (Chen & Kou 1989, Nadala et al. 1993, Hsu et al. 1995, Lu et al. 1995b, Tapay et al.

1995, 1997, Chen & Wang 1999, Itami et al. 1999, Kasornchandra et al. 1999, Owens & Smith 1999, Wang et al. 2000), only 1 (West et al. 1999) has provided a quantitative analysis of their growth to confluence. We therefore undertook to determine the growth profile of oka primary cell cultures from initial seeding to confluence.

Primary lymphoid (oka) cell culture: Given that a loss of cell viability has been reported following trypsinization (Itami et al. 1999, Owens & Smith 1999), the experiment was performed by seeding parallel flasks from a single starting preparation and counting duplicate flasks at each time point. Viable cell number was determined by trypsinizing the cells followed by trypan blue staining and counting cells with a hemocytometer. As the counted cells were not further propagated, the deleterious effect of trypsinization on cell viability was avoided. Fig. 1a shows the growth profiles of 2 independent experiments ($n = 2$ for each experiment), Fig. 1b the combined data of both experiments, with cell number calculated as a percentage of initial number of cells seeded. Both experiments showed very close agreement when numbers were calculated as a percentage of initial numbers of cells. There was an initial drop in cell numbers in both experiments (39.95 and 36.83%, respectively) followed by a relatively constant increase. After confluence, the cells remained viable (with a change of media) for up to 21 d (data not shown).

Virus amplification and purification: A small amount of viral stock of the yellow head virus was subjected to amplification by passage through healthy prawns. The prawns were pre-screened as negative for both YHV (RT-PCR) and WSSV (PCR). Analysis of the resultant viral stock revealed it to be positive for YHV (RT-PCR) and negative for WSSV (PCR) (data not shown). The stock was further analyzed by SDS-PAGE gel electrophoresis and Western blotting with an antibody directed against the p116 protein of YHV, and by transmission electron microscopy (data not shown). The stock was analyzed for viral titer by an *in vitro* quantal assay (TCID₅₀).

YHV propagation profile: We infected 25 cm² flasks of confluent oka primary cell culture with the purified YHV stock at an MOI of 0.001. We chose this low MOI because it may more accurately reflect the actual exposure of shrimp to the virus *in vivo*. The cell cultures were examined daily under the microscope, and the onset of CPE was recorded at 3 d post-inoculation. The CPE progressively increased, reaching almost 100% by Day 7 (Fig. 2h). Aliquots of cell growth-media were taken immediately after inoculation and at daily intervals thereafter. Viral levels were determined by the *in vitro*

quantal methodology of Reed & Muench (1938), using 96-well plates seeded with suspensions of oka primary cell culture grown to confluence. Combined results from 2 independent experiments ($n = 2$ for each experiment) are shown in Fig. 3. Infective YHV particles were detectable as early as 24 h post-inoculation, with values of 2.314 and 2.661 $\times \log_{10}$ TCID₅₀ ml⁻¹ calculated for the 2 experiments (mean = 2.4875 $\times \log_{10}$ TCID₅₀ ml⁻¹). Maximal viral levels occurred at 4 d post-inoculation (Fig. 3). The levels of the virus on Day 4 represented a 1000-fold increase over initial virus levels, although actual amplification levels may have been higher, since not all the initial viruses were internalized (evidenced by the titer at Day 0). The presence of YHV and the absence of WSSV in the culture medium was confirmed by RT-PCR for YHV and PCR for WSSV (data not shown) as well as by Western blotting (Fig. 4). The presence of YHV on Day 1 was confirmed by RT-PCR analysis, although viral levels were too low for Western blot detection before Day 2.

This report documents the production of infectious viral particles within 24 h of infection, preceding the appearance of cytopathic effects in the primary cells by 48 h. Currently there is no data on the time from infection to signs of disease in naturally infected shrimp. In experimentally infected shrimp, signs of disease similar to those in naturally infected shrimp have been reported to occur 24 to 48 h after challenge (Boon-yaratpalin et al. 1993, Lu et al. 1995a, Wang & Chang 2000). However, these results were obtained by intramuscular injection of crude preparations of infected organs, and as such possibly represent very high inocula of the virus and may therefore not reflect the situation *in vivo*.

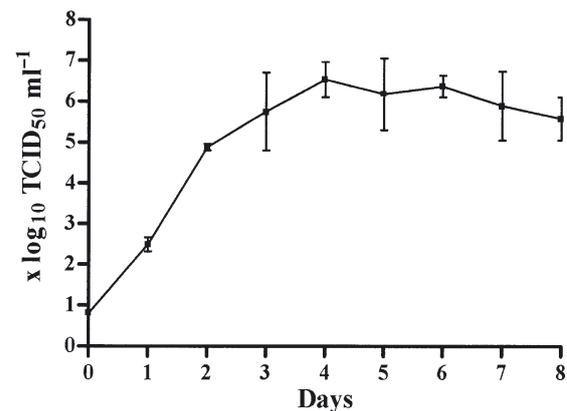


Fig. 3. *Penaeus monodon*. Profile of yellow head virus propagation in primary culture of oka cells. Data are means (\pm SEM, $n = 2$) of 2 independent experiments

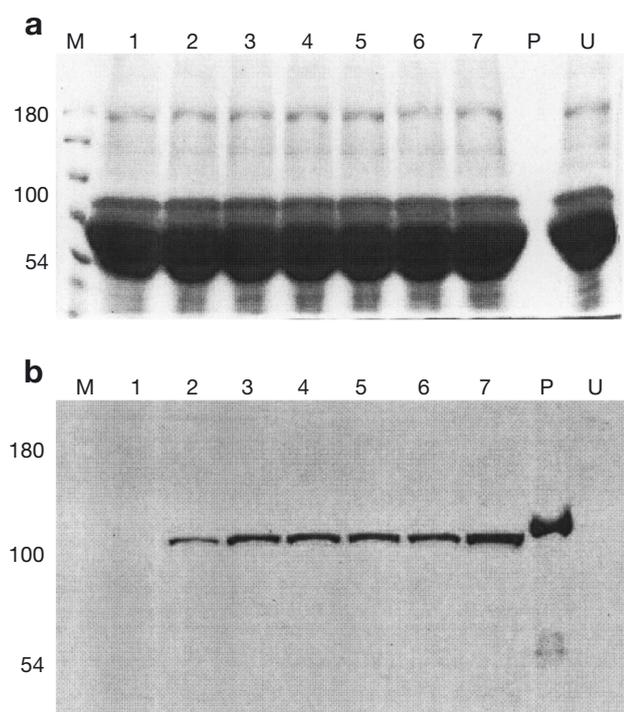


Fig. 4. *Penaeus monodon*. (a) SDS-PAGE and (b) Western blot analyses of daily aliquots of YHV propagation medium kDa (a) SDS-PAGE gel analysis. (b) Western blot analysis using antibody directed against YHV p116 protein. M: Protein marker standard lane (kdaltons) (Fermentas); Lanes 1 to 7: 20 μ l aliquots of daily growth medium from YHV propagation experiment; P: positive control of purified YHV stock virus; U: negative control from uninfected cells

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