รายงานการวิจัยฉบับสมบูรณ์

การพัฒนาวิธีการเพาะเลี้ยงเซลล์เม็ดเลือดกุ้งอย่างง่ายสำหรับการศึกษาไวรัสกุ้งและการพัฒนาเป็นอนุภาคสมบูรณ์แบบของไวรัสหัวเหลืองในเซลล์เม็ดเลือดของกุ้ง

Establishment of a routine primary hemocyte cell culture system for shrimp virus research and envelopment process of yellow head virus (YHV) within cultured cells

โดย ดร. ชุมพร สุวรรณยาน และคณะ

ธันวาคม 2556

สัญญาเลขที่ TRG5480022

รายงานการวิจัยฉบับสมบูรณ์

การพัฒนาวิธีการเพาะเลี้ยงเซลล์เม็ดเลือดกุ้งอย่างง่ายสำหรับการศึกษาไวรัสกุ้งและการพัฒนาเป็นอนุภาคสมบูรณ์แบบของไวรัสหัวเหลืองในเซลล์เม็ดเลือดของกุ้ง

Establishment of a routine primary hemocyte cell culture system for shrimp virus research and envelopment process of yellow head virus (YHV) within cultured cells

	คณะผู้วิจัย	สังกัด
1.	ดร. ชุมพร สุวรรณยาน	ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
2.	ศ. ดร. ทิม เฟลเกล	ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
3.	นายมงคล พันธุระ	หน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพกุ้ง
4.	นายสุริยพงศ์ แจ่มใส	หน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพกุ้ง

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

Acknowledgements

The author would like to thank the Thailand research fund, The Thai National Center for Genetic Engineering and Biotechnology and Mahidol University for funding this research project. I’m grateful to Prof. Paisarn Sithigorngul of Srinakarinwirote University for providing monoclonal antibodies against all shrimp viruses, to Drs Kallaya Dangtip and Saengchan Senapin for providing polyclonal antibodies against Pm Rab7 and Laminin receptor protein. My thanks also go to Mongkhol Phanthura, Suriyapong Chaemsai and Warachin Gangnonngiw for all technical assistance. To my mentor Prof. Tim Flegel, I’m thankful for all his comments, suggestions and encouragement.

บทคัดย่อ

ในโครงการนี้ผู้วิจัยได้พัฒนาระบบการเพาะเลี้ยงเซลล์เม็ดเลือดกุ้ง (*Penaeus monodon*) ขั้นปฐมภูมิ (Primary shrimp hemocyte cell culture) ที่สามารถนำมาใช้ได้ทุกเวลาที่ต้องการ (Routine) โดยที่ไม่ต้องฆ่ากุ้ง โดยทำได้โดยใช้อาหารเลี้ยงเซลล์ 3 สูตรได้แก่ 2 x L-15, Grace's Insect medium, Sf900 ที่มีการเติมด้วยอาหารเสริม จากนั้นได้ทำการทดสอบว่าเซลล์เพาะเลี้ยงนี้อ่อนแอต่อเชื้อไวรัสที่สำคัญๆต่อกุ้งสามชนิดได้แก่เชื้อไวรัสหัวเหลือง ตัวแดงดวงขาวและเชื้อไวรัสทอร่าซินโดรม พบว่าเซลล์เพาะเลี้ยงที่ได้อ่อนแอต่อเชื้อไวรัสทั้งสามชนิด ในกรณีของไวรัสหัวเหลืองนั้นพบว่าในเซลล์ติดเชื้อมีการแสดงออกของโปรตีนโครงสร้างทั้งสามชนิดของเชื้อไวรัสอยู่ แสดงว่าไวรัสสามารถเพิ่มจำนวนได้และสามารถสร้างอนุภาคไวรัสที่สมบูรณ์ได้ จากนั้นได้ทำการศึกษาคุณสมบัติของเซลล์โดยเฉพาะการมีโปรตีนตัวรับของเชื้อไวรัส (Viral receptors) ที่มีรายงาน 2 ชนิดได้แก่ laminin receptor protein และ PM Rab 7 protein พบว่าทุกชนิดของเซลล์เม็ดเลือดมีการแสดงออกของโปรตีนทั้งสอง โดยที่เซลล์ชนิดที่มี granule ขนาดใหญ่ (large granular cells) มีการแสดงออกของ โปรตีน Pm Rab7 มากกว่า เซลล์ชนิดที่มี granule ขนาดเล็ก (small granular cells) และเซลล์ที่ไม่มี granule (non-granular หรือ hyaline cells) ตามลำดับ สำหรับโปรตีน laminin receptor protein นั้นเซลล์ทุกชนิดมีการแสดงออกเท่าๆกัน

ในเซลล์ที่ติดเชื้อตัวแดงดวงขาวนั้นพบว่า laminin protein มักจะเจอในบริเวณเดียวกับ Vp19 ซึ่งเป็นโปรตีนหุ้มผิว (envelope protein) ของไวรัสตัวแดงดวงขาว ในขณะที่ความเข้มของสัญญาณของโปรตีน VP28 กับ Pm Rab7 นั้นมีความสัมพันธ์กันกล่าวคือทั้งสองโปรตีนมีการแสดงออกมากที่สุดในเซลล์ชนิดที่มี granule ขนาดใหญ่และแสดงออกน้อยที่สุดใน เซลล์ชนิดที่มี granule ขนาดเล็ก แต่ทั้งสองโปรตีนนี้ไม่ได้อยู่ตรงบริเวณเดียวกันทั้งหมด

การทดสอบความเกี่ยวข้องของ actin polymerization กับ replication ของไวรัส พบว่าในเซลล์ติดเชื้อที่ได้รับ cytochalasin D ซึ่งเป็นสารที่มีฤทธิยับยั้งการสร้างเส้นใยของ actin (inhibits actin polymerization) นั้นมีการแพร่ของไวรัสที่น้อยกว่าในเซลล์ติดเชื้อทีไม่ได้รับสารดังกล่าว นอกจากนี้ยังพบว่าในเซลล์ที่มีการติดเชื้อจะมีการสร้างส้นใยของ actin มากกว่าเซลล์ที่ไม่ติดเชื้อ

นอกจากนี้แล้วยังได้ศึกษาบทบาทของ N-linked glycosylation ต่อการเพิ่มจำนวนและการสร้างอนุภาคของไวรัสหัวเหลืองพบว่า ในเซลล์ติดเชื้อที่ได้รับ tunicamycin ที่มีฤทธิยับยั้งการ N-linked glycosylation ในระดับ 0.5 μg/ml ในอาหารเลี้ยงพบว่าเซลล์ติดเชื้อในอาหารนี้ยังมีการสร้างอนุภาคของไวรัสได้แม้จะน้อยกว่าในเซลล์ติดเชื้อที่ไม่ได้รับสารนี้ เป็นไปได้ว่าสารนี้อาจมีผลกระทบในเชิงลบแต่เซลล์ที่เพาะเลี้ยงหากเลี้ยงไว้นาน ผลที่ได้ต่างจากการศึกษาในตัวกุ้งที่พบว่ากุ้งติดเชื้อที่ได้รับสารดังกล่าวจะไม่มีการสร้างอนุภาคไวรัสที่สมบูรณ์ อย่างไรก็ดีในโครงการนี้โดยสรุปผู้ใจได้วิธีเพาะเลี้ยงเซลล์เม็ดเลือดกุ้งที่ดีและสามารถนำไปใช้ศึกษาไวรัสกุ้งได้อย่างน้อยสามชนิด ซึ่งขณะนี้การศึกษาก็ยังดำเนินอยู่เพื่อยืนยันผล

Abstract

The lack of a reliable immortal shrimp cell line is a major constraint for shrimp virus research. Although several attempts have been made to establish shrimp cell lines, none have been successful. Insect cell lines such as Sf9 cells have been used as an alternative for shrimp cells. Although susceptible to many shrimp viruses, detailed studies with yellow head virus (YHV) revealed that the cells were not as permissive as originally hoped since the cells failed to produce mature, enveloped virions. In this study, primary hemocyte cell culture systems were successfully established for both *P. monodon* and *P. vannamei*. Double strength-L-15 or single strength grace's insect medium was used as the basal medium. This was supplemented with 15% fetal bovine serum, 2x antibiotics (penicillin and streptomycin) and 0.5% NaCl. Cells cultured in this medium could be maintained for several weeks in an incubator set at 30 ºC. These cells were found to be susceptible to white spot syndrome virus (WSSV), Taura syndrome virus and YHV in infection experiments where viral envelope (Vp19 of WSSV) and nucleocapsid proteins (p20 and Vp1 of YHV and TSV, respectively) were detected in the cells when monoclonal antibodies against these proteins were used. It was also found that in WSSV and YHV infection experiments, large granular cells were the first cell type to be infected. To study the role of *N*-linked glycosylation in the YHV replication cycle, tunicamycin (an inhibitor of *N*-linked glycosylation) was used. YHV-infected culture cells treated and not treated with tunicamycin (0.5 μg/ml) were tested for the presence of all three YHV structural proteins (gp116, gp64 and p20) using monoclonal antibodies (MAb) specific to each protein. Although positive immuno reactions for all three antibodies were observed in both treated or not treated cells, the immuno reactive signals in tunicamycin-treated cells were much less intense than those in untreated cells. The results indicated that the cultured cells could support full YHV virus replication and that tunicamycin reduced YHV replication under the experimental conditions used. Despite reduced replication in the tunicamycin-treated cells, all three structural proteins were still present and some mature, enveloped viral particles were formed.

Keywords: Shrimp, hemocyte, cell culture, yellow head virus, white spot syndrome virus,

Introduction

The lack of an established immortal cell line is one of the major obstacles for shrimp virus research. Although several attempts to produce such cell lines have made by many researchers around the globe using different tissue sources, different cell culture media, different culture conditions and even various mutation techniques, none have been successful (Crane and Benzie, 1999). Thus, to study shrimp viruses, many researchers opt to use primary cultures of shrimp cells, and especially cells that originate from the shrimp lymphoid organ (LO) (Chen and Kou, 1989; Itami et al., 1999). However, since the LO is associated with pathogen clearance and since it is among the first organs to be infected with many viruses and bacteria, it is difficult to obtain uncontaminated lymphoid organ tissue. In cultured shrimp, the LO often shows evidence of pathogen contamination in the form of spheroidal clumps of cells called spheroids that form in response for foreign material. Insect immortal cell lines such as Sf9 cells and C6/36 mosquito cells have sometimes been used as alternatives to shrimp cells to study shrimp viruses. These cells were found to be susceptible to many shrimp viruses including yellow head virus (YHV), white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) (Arunrut et al., 2011; Gangnonngiw et al., 2010; Sriton et al., 2009). Detailed study on YHV replication in Sf9 cells revealed that the cells failed to produce mature enveloped particles or that the cells were not as permissive to the virus as originally thought (Sriton et al., 2009).

Relatively little is known about the detailed replication cycles of shrimp and other crustacean viruses. Mostly, this is due to lack of immortal shrimp cell lines for laboratory experiments. As an alternative, we successfully established a repeatable, primary cell culture model based on easily-obtained shrimp blood cells (called hemocytes) from both the giant tiger shrimp *Penaeus monodon* and the whiteleg shrimp *P. vannamei* and we used them to test the effect of the anti-glycosylation agent tunicamycin and the inhibitor of actin filament polymerization cytochalasin D on yellow head virus (YHV) infection.

Methods:

1. Hemocyte cell preparation:

Hemolymph containing hemocyte cells was withdrawn from the ventral sinus of shrimp into a 1 ml syringe containing an equal volume of shrimp salt solution (SSS). The hemolymph was thoroughly mixed with SSS before approximately 100 µl was dropped onto cover glass that was placed in a well of a 24 well-plate. Then, 2x L-15 medium (500 µl) was added to each well and the culture plate was placed on top of an orbital shaker set at low speed (5 rpm) for approximately 1 h to allow the cells to spread and attached to the cover glass.

After that the cells were incubated in an incubator set at 30°C. Chamber slides (4-8 chambers/slide) were also used to culture cells with similar results.

2. Crude virus preparation

Crude yellow head virus (YHV), white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) were prepared from hemolymph of shrimp experimentally infected with each virus. The hemolymph of infected shrimp (2, 3 and 7 days post injection for YHV, WSSV and TSV, respectively) was withdrawn into a sterile syringe containing 1:1 volume of SSS. The hemolymph and SSS were mixed by moving the syringe plunger up and down a few times before the mixture was filtered through a 0.2 µm membrane filter. The filtrates (stock viruses) were kept at -80°C until used. The original stocks of YHV and WSSV were obtained from outbreak farms in Thailand in 2000 and 1999, respectively. The stock of TSV was kindly provided by Dr. Kullaya Somboonwiwat of the National Center for Genetic Engineering and Biotechnology (BIOTEC) and Chulalongkorn University.

3. Virus inoculation

To inoculate a virus, approximately 80% of the culture medium (2x L15 base) in the culture plate was removed. The diluted stock virus 1:100 in culture medium (100µl) was placed or dropped onto the cells and the culture plate was shaken on an orbital shaker set at low speed (5 rpm) to ensure that the virus was spread evenly in the culture wells. This was done for at least 15 min before the cells were either fixed or cultured further in an incubator set at 30°C for various times (30 min, 2 h, 12 h, 24 h, 48 h, 72 h or longer). For those cells that were cultured further, approximately 500 µl of culture medium was added before further incubation. Cells cultured in Grace's insect medium were also tested for their susceptibility to yellow head virus infection, but this failed to initiate infections.

4. Cell fixation and immunohistochemical staining

To fix the cells, culture medium was removed from the culture wells. The cells were then fixed with 4% paraformaldehyde for 10 min before they were washed by incubation three times in PBS (10 min each). To permeabilize the cultured cells, the cells were treated with 0.1% triton x 100 in PBS for 5 min before they were washed with PBS 3 times 5 min each. The cells were then blocked with 10% FBS in PBS before the first antibody (antibody specific for each virus) was added followed by incubation at 37°C for 1 h. Unbound antibodies were removed by washing 3 times with PBS (10 min each). Fluorochrome labeled secondary antibody (Alexa Fluor 488, 594 or 633, from Invitrogen) (1:500- 1:1000) was used and the cells were incubated for 1 hour at 37°C together with Topo 3 nuclear stain (1:500) and Phalloidin-Fluorescein isothiocyanate (1:500) stain for actin filaments (Both from Sigma). Then the unbound secondary antibody and dye were washed by incubation three times with PBST (PBS with 0.05% tween20) for 10 min each. The cells were mounted using Prolong gold anti fade reagent and studied by confocal microscopy (Olympus).

5. Tunicamycin and cytochalasin D treatment of cultured cells

Treatment of cultured cells with an *N*-linked glycosylation inhibitor, tunicamycin and an inhibitor of actin polymerization cytochalasin D were either done at the same time, prior to or after virus infection. To do this the culture medium (approx. 80%) was removed from the culture wells and replaced with new culture medium containing either tunicamycin (0.5 μg/ml) or cytochalasin D (from *Zygosporium mansonii*, Sigma, 1 μM). The culture plates were then placed on top of an orbital shaker set at approximately 5 rpm speed for 15 min before the culture plates were incubated in an incubator set at 30°C before they were collected and processed for immuno-histochemical staining.

6. Culture media formulas

ลำดับที่	ชื่อสูตรอาหาร	strength	Supplements/antibiotics	pH
1	Grace’s Insect medium	1x	15%FBS, 2% pen strep (stock Penicillin and streptomycin, 5000 units and 5000 ug/ml), 0.5% NaCl	6.9-7.0
2	Leibovitz-L-15	1x, 2x	15%FBS, 2% Pen strep, 0.5% NaCl	7.2
3	Sf-900 III SFM	1x	2% Pen strep	UD

Osmolality of the 2x L-15 used was approximately 720 mOs /kg.

Results

1. Routine methods for primary hemocyte cell culture were successfully established

Using three different culture media, namely double strength (2x) Leibovitz’s-L15 with L- glutamine (Gibco), Grace’s insect medium and Sf900 medium as basal media (supplemented with 15% fetal bovine serum, 2% penicillin and streptomycin (stock Penicillin and streptomycin, 5000 units and 5000 μg/ml) and 0.5% NaCl), we successfully established repeatable, primary cell cultures based on easily-obtained shrimp blood cells (called hemocytes) from both the giant tiger shrimp *Penaeus monodon* and the white leg shrimp *P. vannamei*. The cultured cells could be maintained in an incubator set at 30°C or at room temperature for up to 9 months in Grace’s insect medium and approximately 3 weeks in 2x L15-based medium in an incubator set at 30°C with media changes every 3-4 days. Cytoplasm of some cultured cells in 2x L15 started to retract towards the cell body and the cells dislodged from the culture flask surface after day 10, with the majority of the cells gradually dislodging from the flasks and dying in the fallowing weeks. Cells cultured in 1x L15 media were not as good as those in 2x L16 as most of the cells contained vacuoles and dislodged from the culture flasks after 24 h. Cells cultured in Sf900 medium appeared to have diverse morphologies when compared to those in 2x L15 and in Grace's insect medium. Examples of the cultured cells in different media are shown in Figs. 1 and 2.

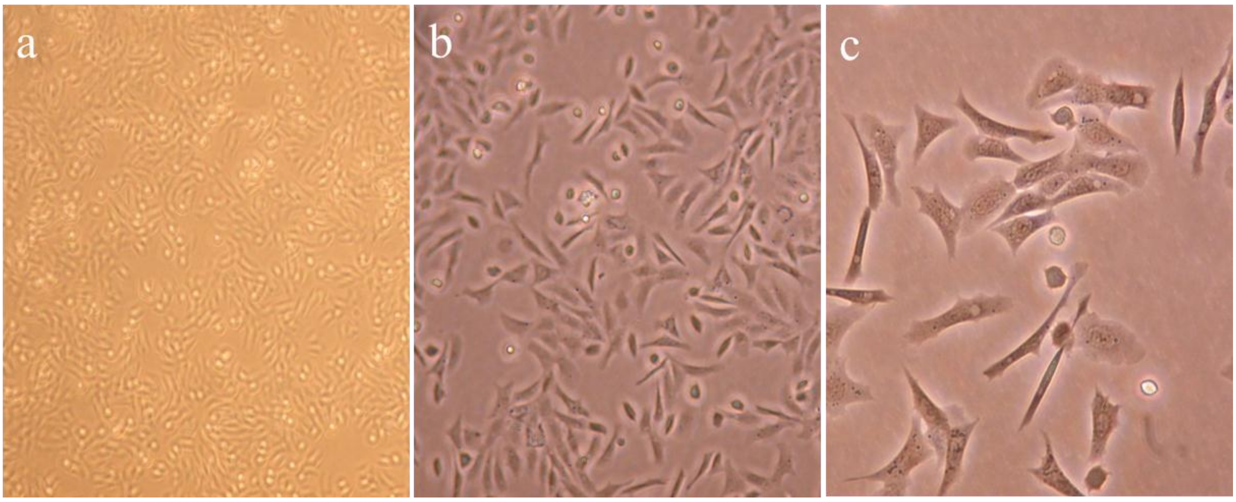

Fig.1 Examples of cultured hemocyte cells of *Penaeus monodon* in 2x L-15 medium with supplements at different magnifications

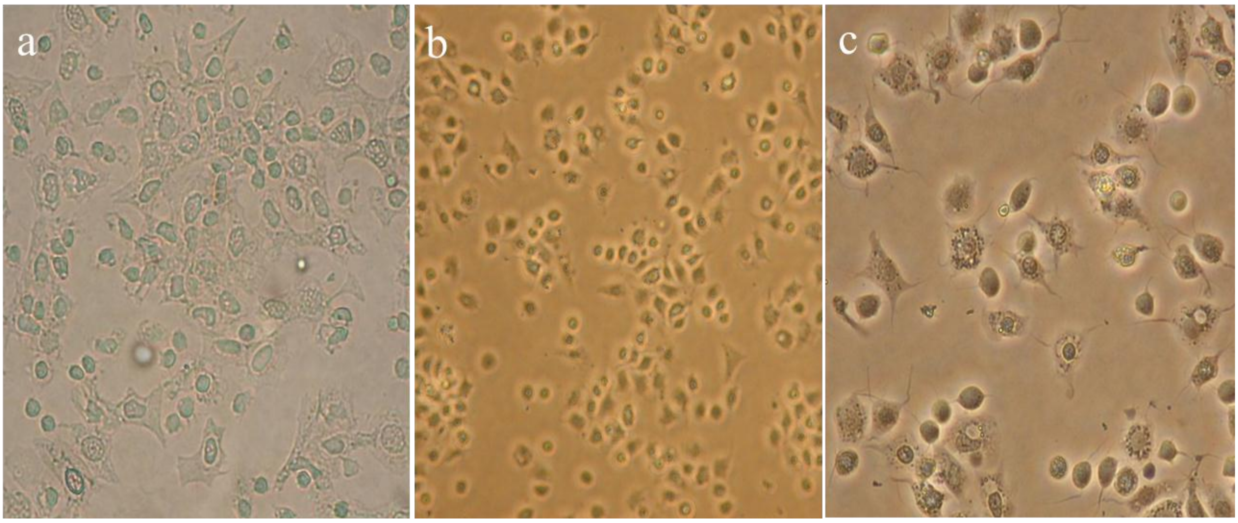

Fig. 2 Examples of cultured hemocytes of *Penaeus monodon* at different magnifications in (a) Grace's insect medium with supplements and (b and c) in Sf900 medium

2. The cultured hemocyte cells were susceptible to yellow head virus (YHV), white spot syndrome virus (WSSV), and Taura syndrome virus (TSV)

In this study we tested weather the cultured hemocyte cells were susceptible to three important shrimp viruses [white spot syndrome virus (WSSV), yellow head virus (YHV) and Tuara syndrome virus (TSV)]. To do this, 1 hour or overnight cultured cells in 2 x L15 were exposed to all three viruses and the viruses were allowed to infect the cells for one hour before the cells were washed with fresh medium to remove excess, unbound virus. Some of the infected cells were also fixed at 5, 15 and 30 min post infection. The treated cells were cultured further in an incubator set at 30°C before the culture medium was removed and the cells were fixed with fixative and processed for immunocytochemical assay using MAb specific for each virus' nucleocapsid protein [monoclonal antibodies against WSSV envelope protein Vp19, YHV nucleocapsid protein or p20, and TSV nucleocapsid (N) protein]. Positive immunoreactions were observed for all three antibodies tested (See Figures 3 b, 4 and 6 for YHV p20, TSV N and WSSV Vp19 respectively), indicating that the cultured cells were susceptible to the three viruses.

To determine if these cells are permissive to YHV infection (or to determine if the cultured cells could support the full replication cycle of YHV), YHV infected cells were also stained with monoclonal antibodies against its two envelope glycoproteins, gp116 and gp64. It was found that the infected cells were positive with both tested antibodies (Figures 3c and 3d). These results indicated that the cultured cells were not only susceptible but also permissive to the virus, i.e. the cells could support virus replication and the formation of enveloped viral particles formation and the cultured cells could be used to study other aspects of virus replication.

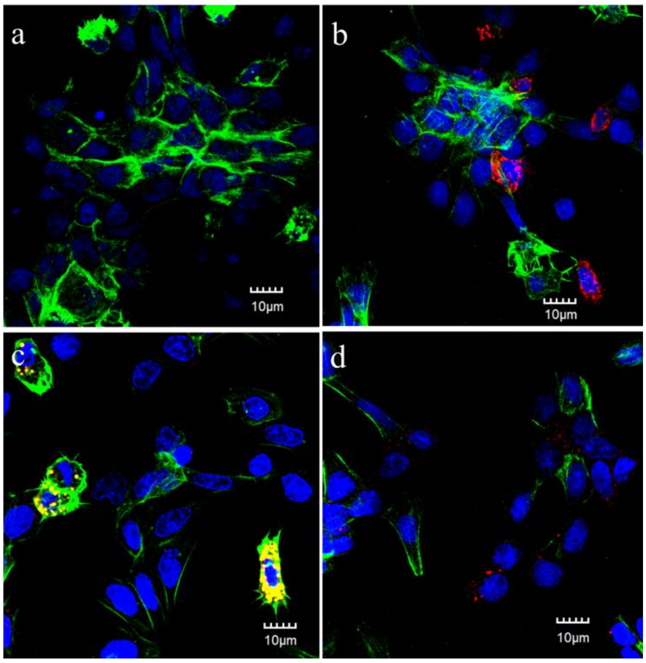

Fig. 3 Cultured hemocyte cells infected and not infected with YHV immuno-stained with Mabs against all three structural proteins of YHV. a) un-infected control cells labeled with anti gp116, b) anti nucleocapsid protein, c) anti envelope protein (gp64) and d) anti envelope protein gp116. All Mabs were detected with the secondary antibody labeled with Alexa Fluor 633 (Red). The cell nuclei were stained with DAPI (blue) and their actin filaments with FITC labeled phalloidin (green). YHV infected cells were positive with all three Mabs.

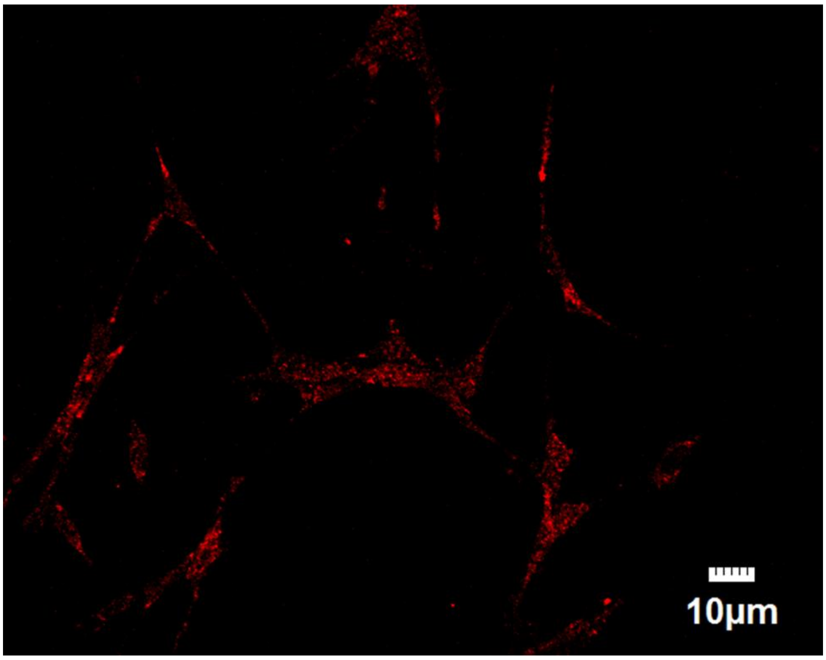

Fig. 4 Cultured hemocyte cells infected with TSV (15 h P.I.), the cells were immuno-stained with a Mab against the virus nucleocapsid protein and detected with a secondary antibody labeled with Alexa Fluor 633 (Red). Positive signals for the protein were observed primarily in the cytoplasm of most cells.

3. Expression and colocation of two WSSV envelope proteins, Vp19 and Vp28 with two known receptor proteins (Pm Rab 7 and laminin receptor protein) in cultured cells

In this project, the expression patterns of two known shrimp receptor proteins, the laminin receptor protein and Pm Rab7 in cultured hemocyte cells were also investigated. Laminin receptor protein which is a mammalian cell receptor for several arthropod-borne viruses (arboviruses) has been reported to interact with many virus proteins including nucleocapsid proteins of Taura syndrome virus Vp1 (TSV) (Senapin and Phongdara, 2006) and infectious myonecrosis virus (IMNV) and gp116 envelope protein of YHV (Busayarat et al., 2011). But whether this protein is a sole receptor protein for these three shrimp viruses is still not known. In this study a polyclonal antibody against the protein was used to stain cultured cells either infected or not infected with WSSV and TSV. It was found that the laminin protein was expressed or present in all three hemocyte cell types (i.e., large granular cells containing two different sizes of granules, large and small, small granular containing only small size granules and hyaline cells or non-granular cells containing no granules). The protein was found primarily in vesicles of the granular cells which are distributed somewhat evenly in the cell cytoplasm (Fig. 5). These locations were similar to the locations where positive signals for TSV Vp1 were observed (Fig. 4). It is interesting to note that some positive signals for the protein were also outside of the cells. Thus, it is possible this protein may also be excreted.

Pm Rab 7 protein is a VP28-binding protein involved in WSSV infection (Sritunyalucksana et al., 2006). Using a polyclonal antibody preparation against Pm Rab7 protein to stain cultured cells, it was found that the protein, similar to laminin receptor protein, was expressed in all hemocyte cell types. However, unlike the laminin protein, Pm Rab 7 was also expressed in both the cell nucleus and cytoplasm.

4. Co-location of WSSV Vp19 and laminin receptor protein

Immunocytochemical analysis of WSSV infected cultured cells (2 h p.i) using a monoclonal antibody against WSSV Vp19 and a polyclonal antibody against laminin receptor protein revealed that all hemocyte cell types expressed both proteins in their cytoplasm (Figures 6 and 7). The locations of the immunoreactions for both proteins were found to be similar and in the same area. These results suggested that the two proteins were co-located in the cytoplasm of infected cells (Fig. 7). It is interesting to note that the immunoreactive signals for the viral Vp19 protein were relatively stronger in the cytoplasm of granular cells than in the cytoplasm of hyaline cells. These results suggested that the virus either infects granular cells before hyaline cells or that the replication rate of the virus in granular cells is higher than in the hyaline cells as (See Fig. 6).

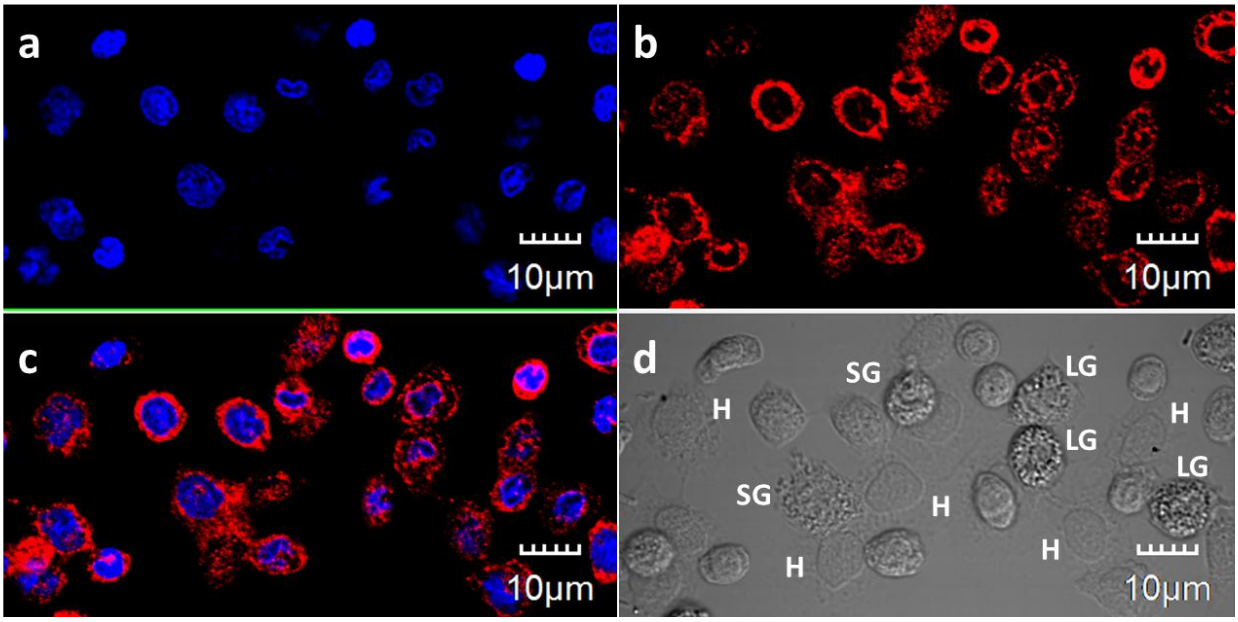

Fig. 5 Examples of laser confocal micrographs of *Penaeus monodon* cultured hemocytes in 2x L15 medium immuno stained with a specific polyclonal antibody against laminin protein. (a) Cell nuclei (blue) stained with TOPO 3. (b) Laminin protein (red) stained with a specific polyclonal antibody and with an anti-rabbit antibody conjugated with Alexa Fluor 546. (c) Merged images of a and b. (d) Bright field photomicrograph of the same area revealing the different hemocyte cell types: large granular (LG), small granular (SG) and hyaline (H) cells. The immuno-reactive signal for the laminin protein was found in cytoplasm of all three hemocyte types.

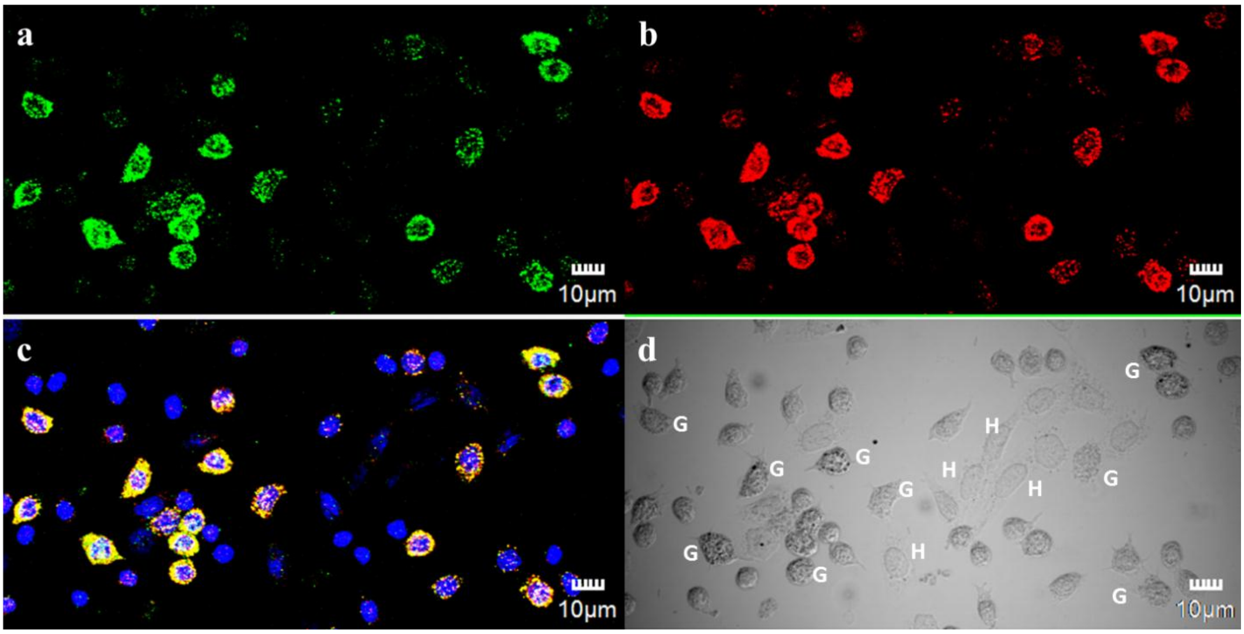

Fig. 6 Examples of laser confocal micrographs of cultured hemocytes of *Penaeus monodon* in 2x L15 medium infected with white spot syndrome virus (WSSV) (2 h. p.i.) and immunostained with specific antibodies. (a) Detection of host laminin protein (green) using a specific monoclonal antibody and anti-mouse antibody conjugated with Alexa Fluor 488 (green). (b) Detection of WSSV envelope protein Vp19 (red) with a specific polyclonal antibody and Alexa Fluor 546. (c) Merged images a and b together with an image of cell nuclei (blue) stained with TOPO 3. (d) Bright field image of the same field. The virus signals are higher in granular cells (G) than hyaline cells (H). The merged image shows that the two proteins are collocated primarily in the cell cytoplasm as red + green = yellow.

Careful observations using higher magnifications clearly showed that the cells that possess the most intense immunoreactive signals for both Vp19 and the laminin protein were the large granular cells. This was followed by decreased intensity in the small granular cells and even more decreased intensity in the hyaline cells. Hyaline cells were found to possess the least intense immunoreactive signals for both proteins (Fig. 7). It is interesting to note that the signals for both proteins in the large granular cells were found mostly at the same positions or that they were perfectly co-located (yellow ring surrounding the cell nuclei) whereas the same was not true for both proteins in small granular cells where some of the red and green fluorescence appeared at different locations (Fig. 7).

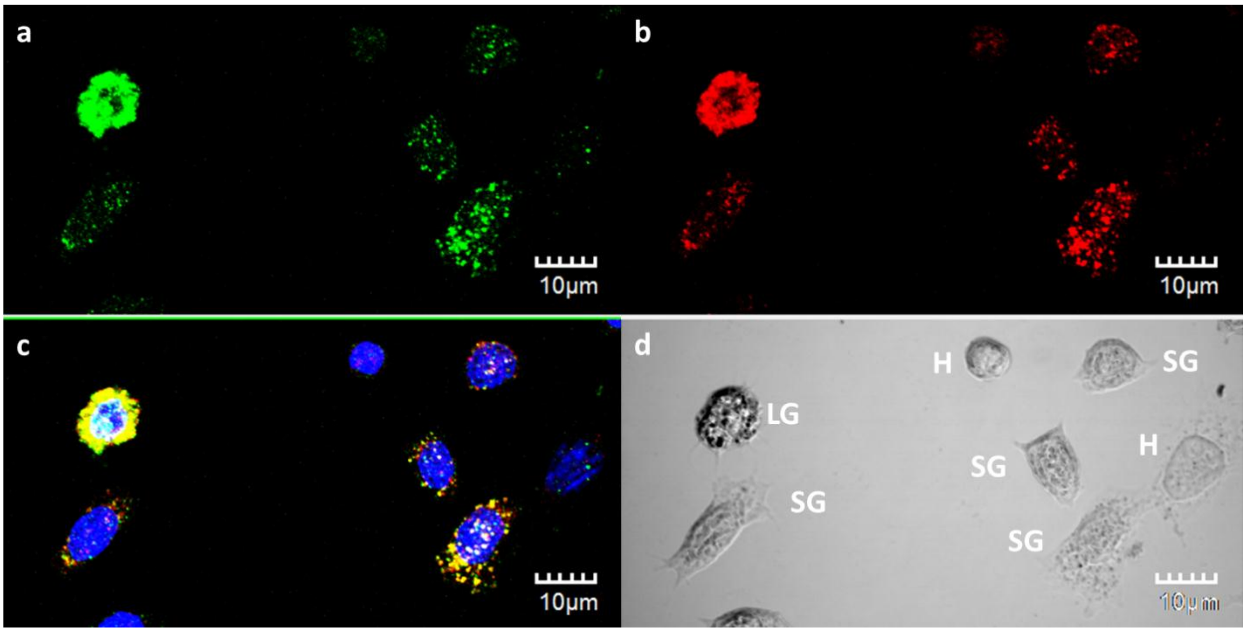

Fig. 7. Examples of laser confocal micrographs of cultured hemocytes of *Penaeus monodon* in 2x L15 medium infected with white spot syndrome virus (2 h. p.i.) and immunostained with specific antibodies against WSSV envelope protein Vp19 which is and host laminin protein. (a) WSSV envelope protein Vp19 (green) stained with a specific MAb and with an anti-mouse antibody conjugated with Alexa Fluor 488. (b) Host laminin protein (red) stained with a specific polyclonal antibody and with anti-rabbit antibody conjugated with Alexa Fluor 546. (c) Merged images a and b together with cell nuclei (blue) stained with TOPO 3. (d) Bright field of the same field as in a and b showing hemocyte types: large granular cells (LG) than in small granular cells (SG) and hyaline cells (H). Strongest immunopositive signals were observed in LG for both WSSV Vp19 and laminin followed by SG and H in decreasing order. The two proteins appear to collocate primarily in the cell cytoplasm as shown in the merged picture as yellow color (i.e., red plus green).

5. Expression of WSSV Vp28 and Pm Rab7 protein in WSSV-infected cultured cells

As with laminin receptor protein, immunoreactions for Pm Rab 7 were also observed in all three hemocyte cell types. Immunoreactive signals for the protein were found to be strongest in large granular (LG) cells. This was followed by small granular (SG) cells and hyaline (H) cells which were found to possess the weakest signals for the protein. Within these cells, the immuno-reactive signals were found primarily (but not limited to) in the cell cytoplasm. Some positive reactions were also observed in the nucleus.

Among the three hemocyte cell types, large granular cells were found to possess the strongest immunoreactions for WSSV Vp28 envelope protein, whereas hyaline (H) cells were found to have the least intense immunoreactions. Although signals for the protein were observed in both cytoplasm and in the nucleus of the infected cultured cells, as with those of Pm Rab 7, the two proteins did not co-locate (See Fig. 8).

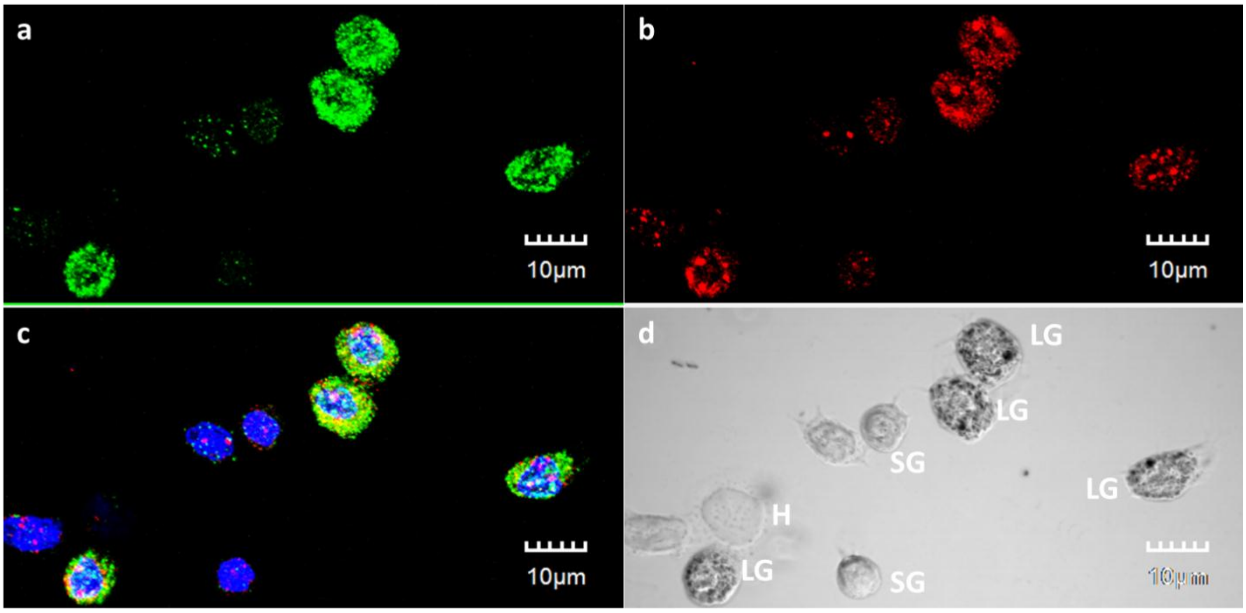

Fig. 8. Laser confocal micrographs of WSSV infected (2 h p.i.) cultured hemocyte cells of *Penaeus monodon* immunostained with specific antibodies for WSSV envelope protein Vp28 and host protein Pm Rab 7. (a) WSSV envelope protein Vp28 (green) immuno-stained with a specific mouse MAb and with Alexa Fluor 488 labeled goat anti-mouse antibody. (b) Host Pm Rab 7 protein (red) stained with a specific polyclonal antibody and with Alexa Fluor 546 labeled goat anti-rabbit antibody. (c) Merged image of a and b together with cell nuclei (blue) stained with TOPO 3. (d) Bright field image of the same field as in a and b showing hemocye types: large granular (LG) cells, semi granular cells (SG) and hyaline cells (H). The strongest positive immunoreactions for both proteins were observed LG and decreased in intensity for SG and H, with lowest signals in H.

6. Susceptibility of cultured cells to YHV

6.1 Time course infection of YHV in cultured hemocytes

From our time course YHV infection experiment, it was found that immunopositive signals for the virus nucleocapsid protein could be detected in the cytoplasm of cultured cells as early as 5 min post infection (P.I.). The first hemocyte cell type found to be positive with the test was the large granular (LG) cells. At 1 h P.I., most large granular cells and some small granular cells were found to be positive with the antibody whereas, most hyaline cells remained negative (Fig. 9). More cells of all types were found to be positive at later times after infection. Among the three hemocyte cell types, the strongest immunoreactions were observed in LG and especially in area where there were vacuoles/vesicles which could be the area where the virus particles/protein are packaged/formed (i.e., virus factories). These vacuoles were also found to be heavily stained with phalloidin-FITC, the fungal toxin which binds specifically to actin filaments. It is interesting to note that in infected granular cell more stress fibers (more polymerized actin) were observed in the cytoplasm when compared with the same areas in uninfected cells of the same cell type. By 15 h P.I. all cell types were found to be positive with the antibody. At this time, it was difficult to differentiate cell types, and especially between hyaline and small granular cells as both appeared to have cytoplasmic contents that were very similar to granules.

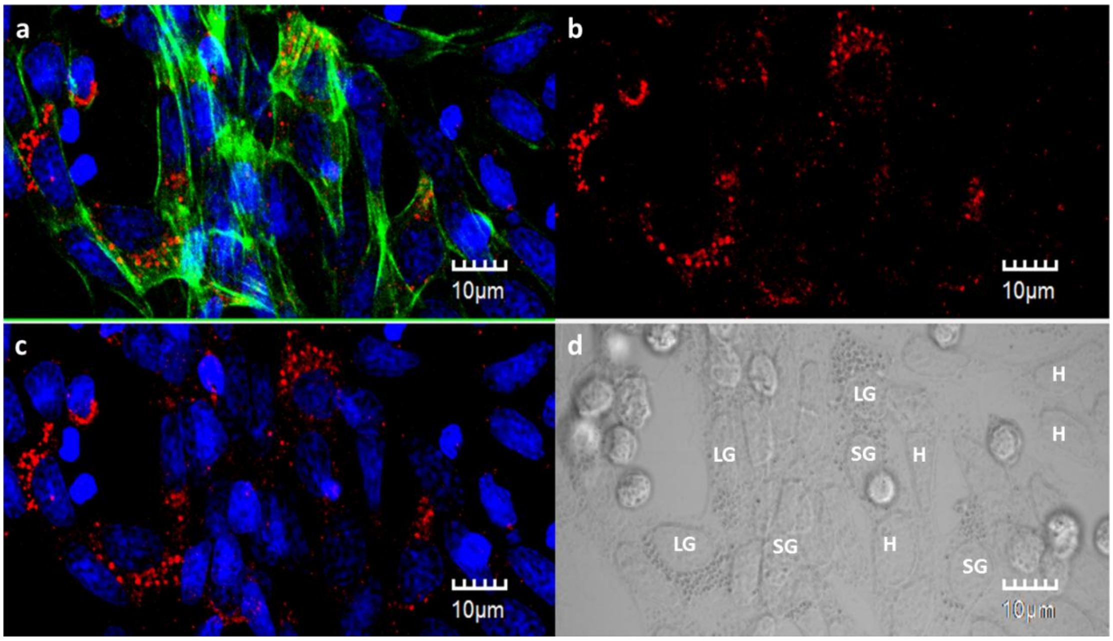

Fig. 9. Laser confocal micrographs of cultured *Penaeus monodon* hemocytes exposed (5 min) to yellow head virus (YHV) and then treated with antibodies specific to YHV nucleocapsid protein, and host actin. (a) Merged images showing YHV nucleocapsid protein (red) stained with specific antibody Y-19 and goat anti-mouse antibody labeled with Alexa fluor 633, host actin (green) stained with FITC labeled phalloidin and host nuclei (blue) stained with DAPI. (b) Image of the same field as in a but showing fluorescence from YHV nucleocapsid protein only. (c) Merged image of the same field as in (a) but showing fluorescence of only YHV nucleocapsid protein (red) and nuclei (blue). (d) Phase contrast of the same field as in a indicating different hemocyte types showing that large granular (LG) cells are the most intensely stained cells followed by small granular (SG) cells and hyaline (H) cells which are the least stained.

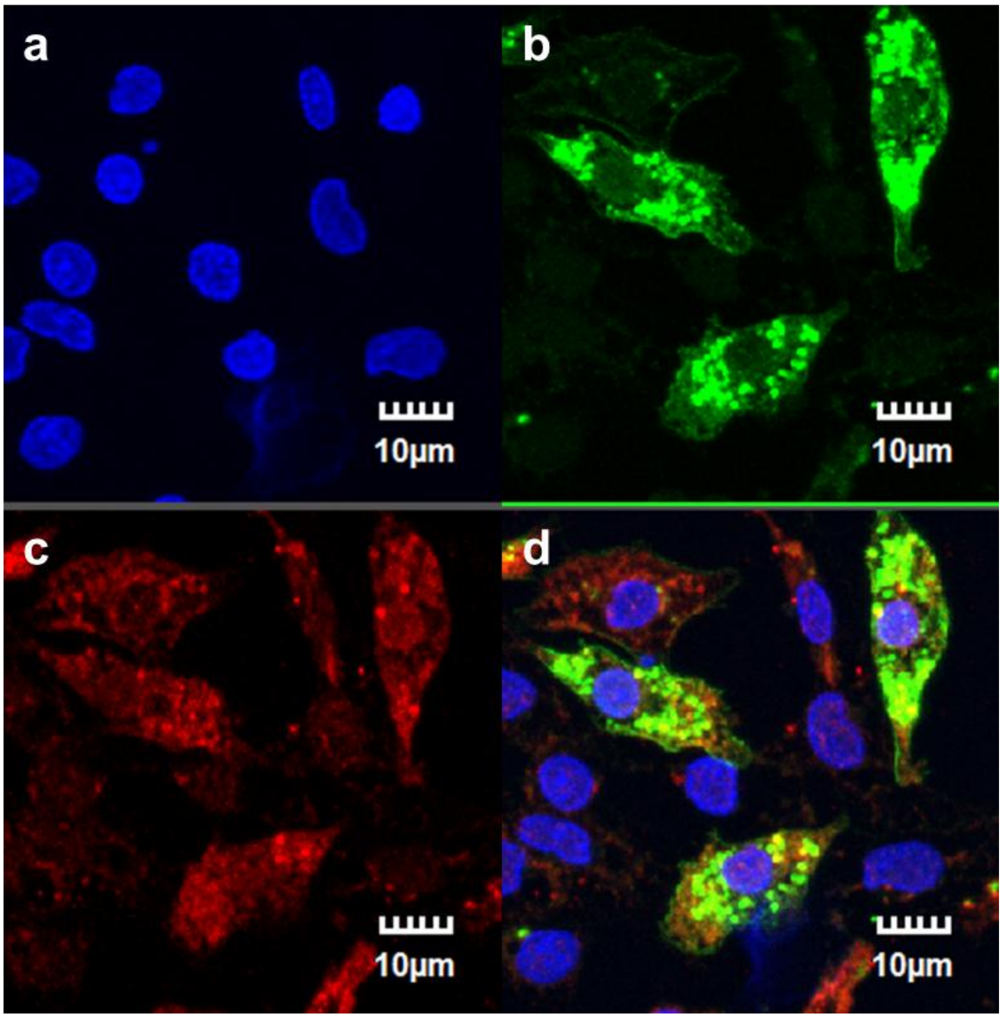

Fig.10. Multilayer, laser confocal micrographs of *Penaeus monodon* cultured hemocytes infected with YHV for 15 hours and stained with specific antibodies to YHV nucleocapsid protein and host actin. (a) Photomicrograph of host nuclei (blue) stained with DAPI. (b) Same field as in (a) showing host actin (green) stained with FITC labeled phalloidin. (c) Same field showing YHV nucleocapsid protein (red) stained with specific monoclonal antibody Y-19 and goat anti- mouse antibody labeled with Alexa fluor 633. (d) Merged images (a) to (c).

6.2 Association of actin polymerization with the YHV replication cycle

In this study the role of actin filaments or actin polymerization in YHV replication was also investigated. YHV infected culture cells, pre-treated or not with cytochalasin D, were probed with monoclonal antibodies against all three structural proteins of YHV and with FITC labeled phalloidin to detect polymerized host actin filaments. The cells were also stained with DAPI to detect host nuclei. Some of the infected cell nuclei appeared shrunken or condensed, perhaps indicating initial stages in apoptosis. As expected, it was found that actin in treated cells was less polymerized than actin in untreated cells. However, for the 3 YHV virion proteins, some differences were noted in cells either treated or not with cytochalasin D. specifically, there were fewer positive cells located in focal areas for the treated wells but generalized positive cells in the un treated wells. The cells either treated or not were found to be positive with all three antibodies. These results suggested that YHV infection could induce actin polymerization, at least in granular cells (Fig. 11). It was also interesting to note that some of the polymerized actin was co-localized with YHV envelope protein gp64. These areas of co-localization are in vacuoles/vesicles located in the cytoplasm of the infected cells. The significance of this interaction is still not known, but it suggests that YHV may use actin filaments to transport its proteins or virus particles (or the vesicles containing virus particles) along the viral maturation path.

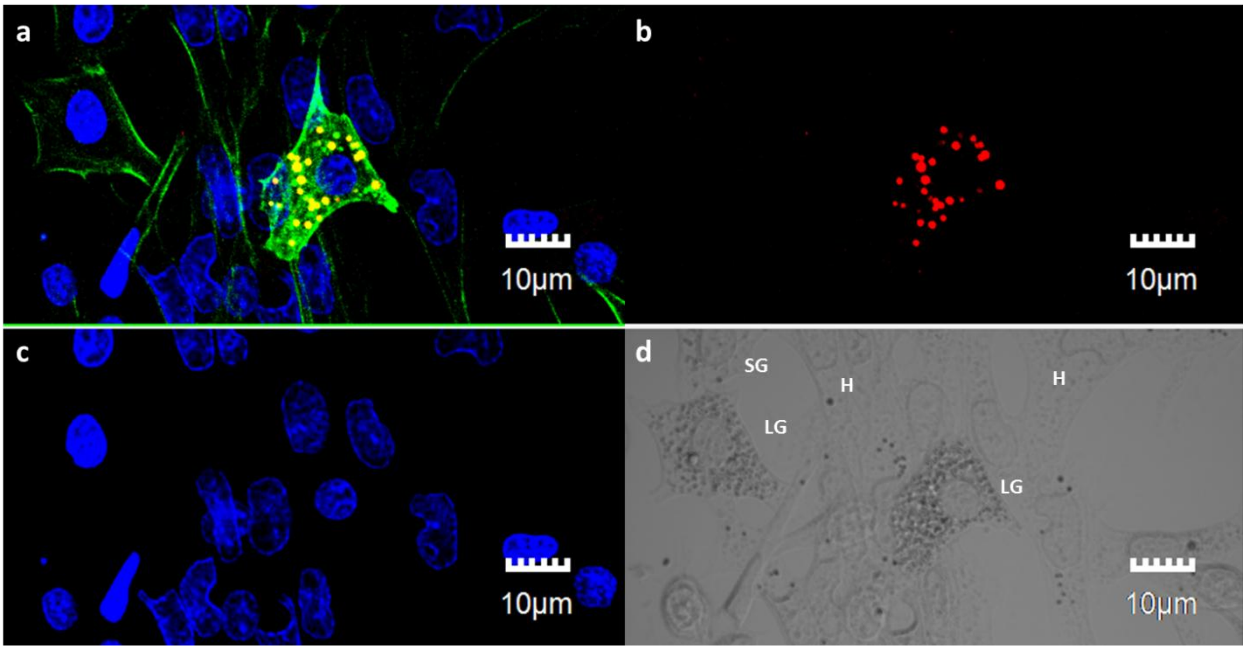

Fig. 11 Laser confocal micrographs of cultured *Penaeus monodon* hemocytes exposed (1 h) to YHV and then treated with specific monoclonal antibody and FITC labeled phalloidin for YHV envelope protein gp64 and for host actin respectively. (a) Merged image showing YHV envelope protein gp64 (red) stained with specific antibody Y-18 and goat anti-mouse antibody labeled with Alexa fluor 633, host actin (green) and host nuclei (blue) stained with DAPI. (b) Same field as in (a), but showing only fluorescence of YHV envelope protein gp64 (red) (c) Same field as in (a) and (b) showing only host nuclei. (d) Phase contrast of the same field indicating different hemocyte types. More intense staining of actin filaments was observed in cytoplasm of infected large granular (LG) especially in the vacuoles when compared to that in un-infected cell of the same cell type. The two proteins appear to collocate primarily in the vacuoles locating in the cell cytoplasm as shown in the merged picture (a) as yellow color (i.e., red plus green).

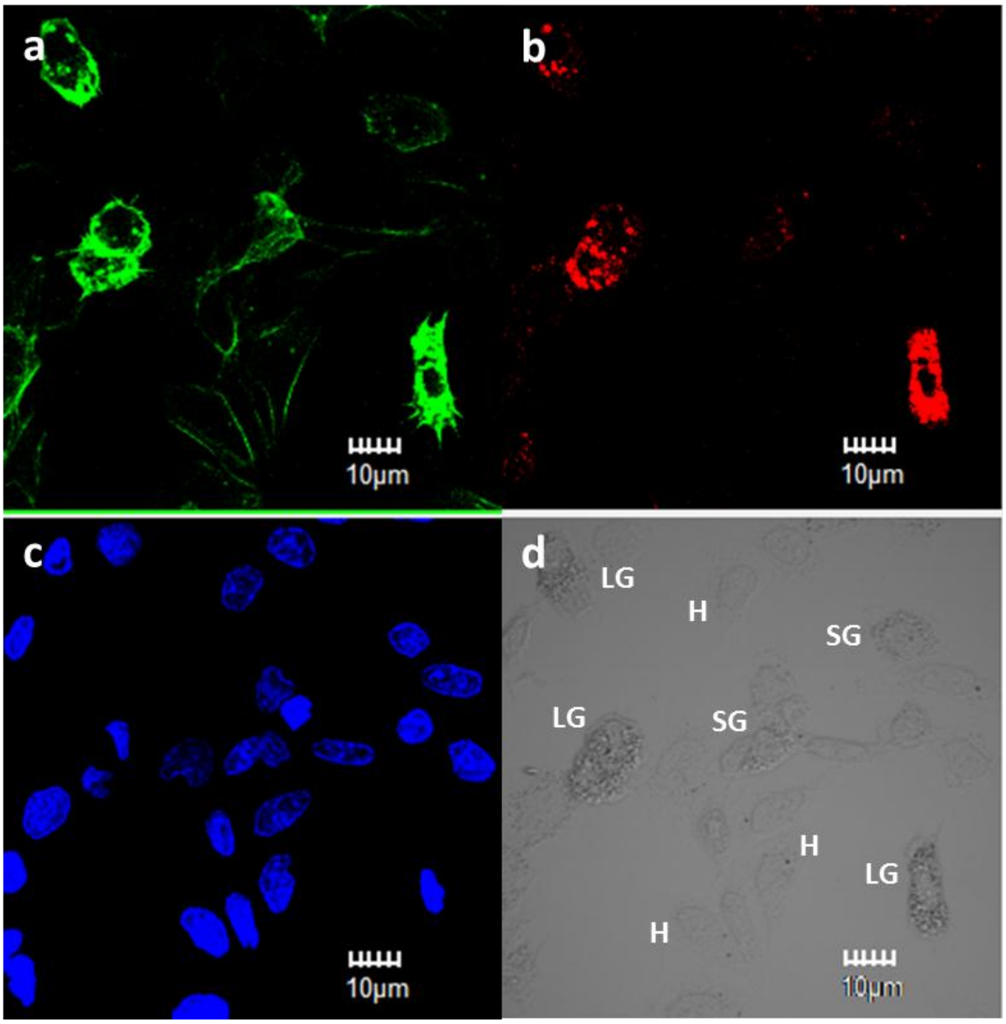

Fig. 12 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15 exposed to YHV (1 h) then treated with a mouse monoclonal antibody for YHV envelope protein gp64 and for host actin. (a) Photomicrograph of host actin (green) stained with FITC-phalloidin. (b) Same field as in (a) showing YHV gp64 (red) stained with Alexa Fluor 633. (c) Same field showing DAPI stained host nuclei (blue). (d) Phase contrast of the same showing that large granular (LG) cells are the most intensely stained cells followed by small granular (SG) cells and hyaline (H) cells which are the least stained.

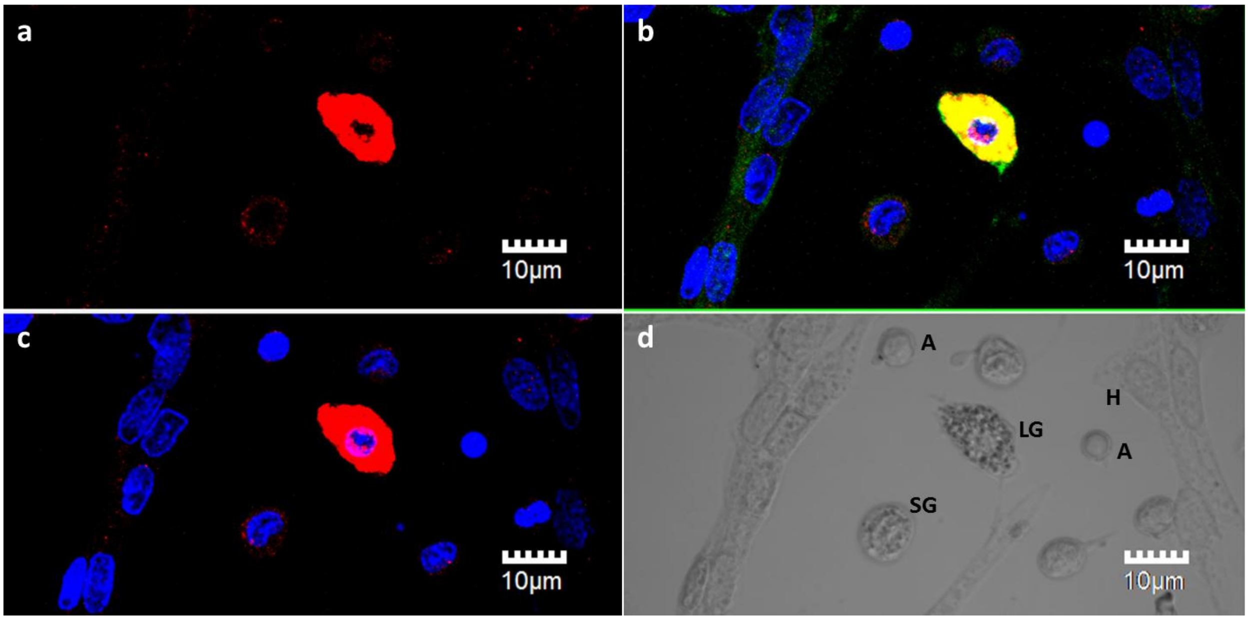

Fig. 13 Laser confocal micrographs of cultured hemocyte cells pretreated with cytochalasin D before being exposed to YHV (1 h) and treated with a mouse monoclonal antibody for YHV envelope protein gp64 and FITC-phalloidin for host actin filaments. (a) Photomicrograph of YHV gp64 (red) stained with Alexa Fluor 633. (b) Same field as in (a) showing YHV gp64, host actin (green) and the cell nuclei stained with DAPI (blue). (c) Same field showing DAPI stained host nuclei (blue) and YH gp64 (red). (d) Phase contrast of the same showing that large granular (LG) cell is the most intensely stained cells followed by small granular (SG) cells and hyaline (H) cells which are the least stained.

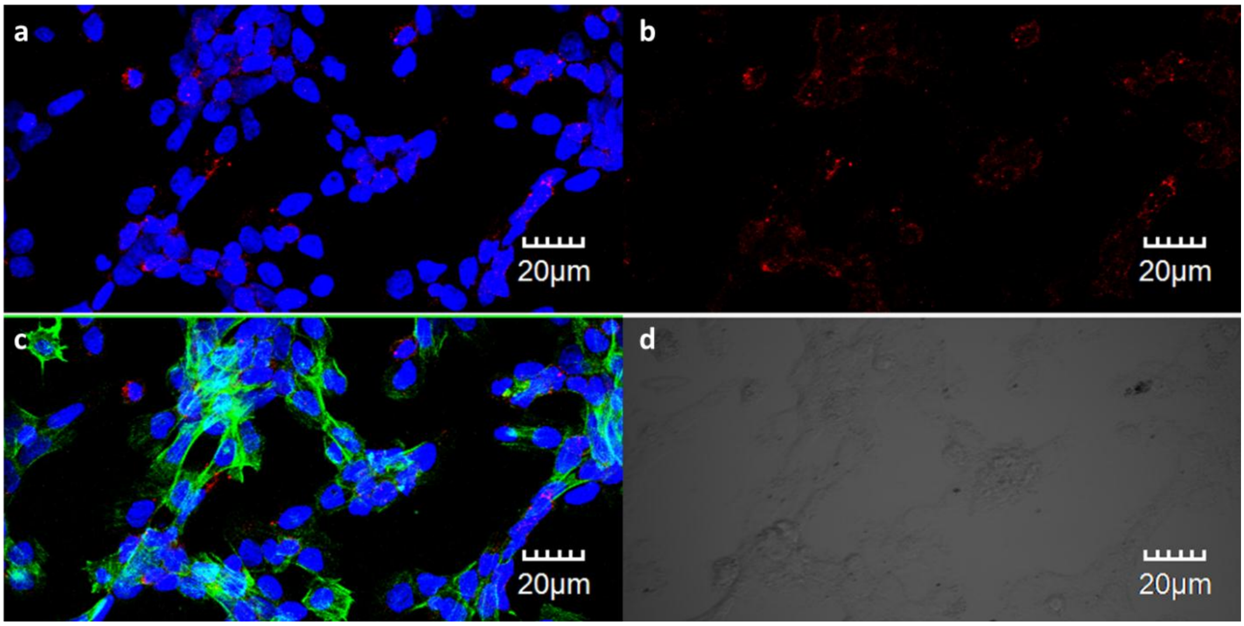

Fig. 14 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15 exposed to YHV (15 h P.I) treated with an antibody for YHV gp64 and FITC-phalloidin for host actin filaments. (a) Photomicrograph of YHV gp64 (red) stained with Alexa Fluor 633 and host nuclei (blue) stained with DAPI. (b) Same field as in (a) showing only YHV gp64 (red). (c) Same field showing host nuclei, host actin filaments (green) and the YHV gp64 (red). (d) Phase contrast of the same.

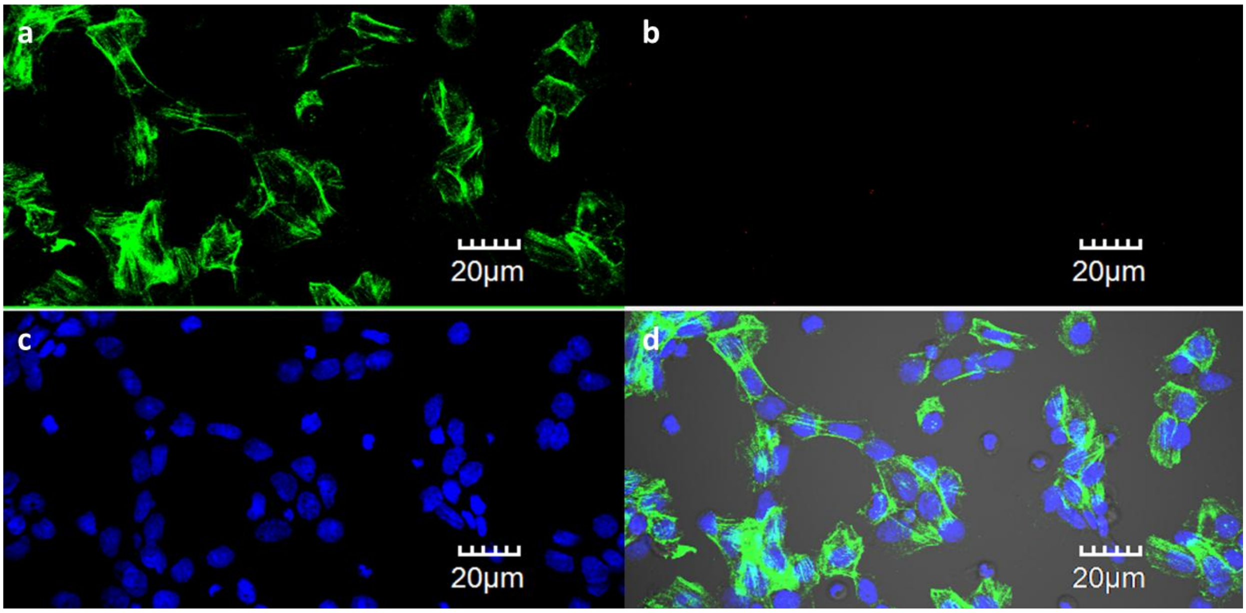

Fig. 15 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15 un-treated with cytochalasin D and un-infected with YHV. These cells were treated with monoclonal antibody specific for YHV envelope protein gp64 and FITC-phalloidin for host actin. (a) Photomicrograph of host actin filaments (green). (b) Photomicrograph of YHV gp64 stained with Alexa Fluor 633(red), no positive signal for the protein was observed. (c) Host nuclei (blue) stained with DAPI. (d) Merged images a to c.

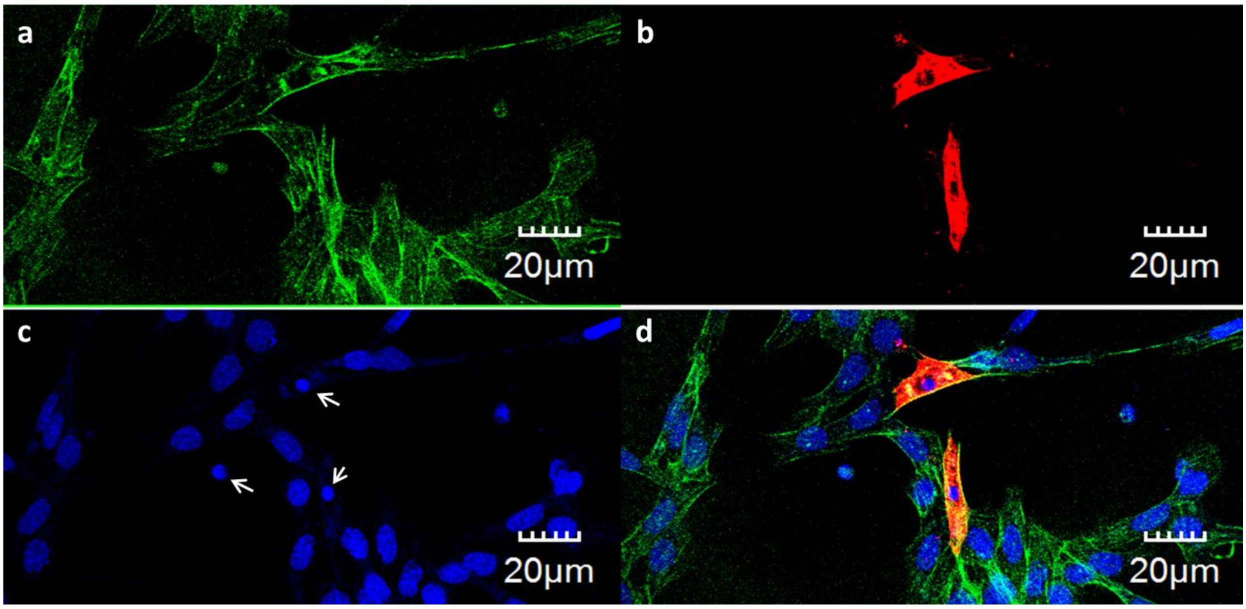

Fig. 16 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15. The cells were pretreated with cytochalasin D for 1 hour before infection with YHV for 15 h. These cells were stained with a mouse monoclonal antibody specific for YHV nucleocapsid protein (p20) and FITC-phalloidin for host actin filaments. (a) Photomicrograph of host actin filaments (green). (b) YHV gp64 stained with Alexa Fluor 633(red) showing two positive cells. (c) Photomicrograph of host nuclei (blue) stained with DAPI showing condensed or shrunken nuclei (Arrow heads). (d) Merged images a to c showing one focal area where two infected cells were found to contain shrunken nuclei or the cells were undergoing apoptosis.

6.3 Effect of tunicamycin treatment on YHV infection in cultured cells

To study the role of *N*-linked glycosylation in the YHV replication cycle, the *N*-linked glycosylation inhibitor tunicamycin was used. YHV infected, cultured cells treated or not with tunicamycin for different times were immuno-stained with monoclonal antibodies against YHV nucleocapsid and envelope glycoproteins (gp116 and gp64) and reactions were detected with goat anti mouse antibody labeled with Alexa-Fluor 966. Actin filaments and nuclei were stained with phalloidin-FITC and DAPI respectively. It was found under the conditions used, that positive immunoreactions were obtained for all three structural proteins in both the tunicamycin treated and untreated cells. However, the signals for all three antibodies were generally stronger in untreated cells than in treated cells. These results suggested that under the conditions used the virus could still replicate and that virus particles could still be formed.

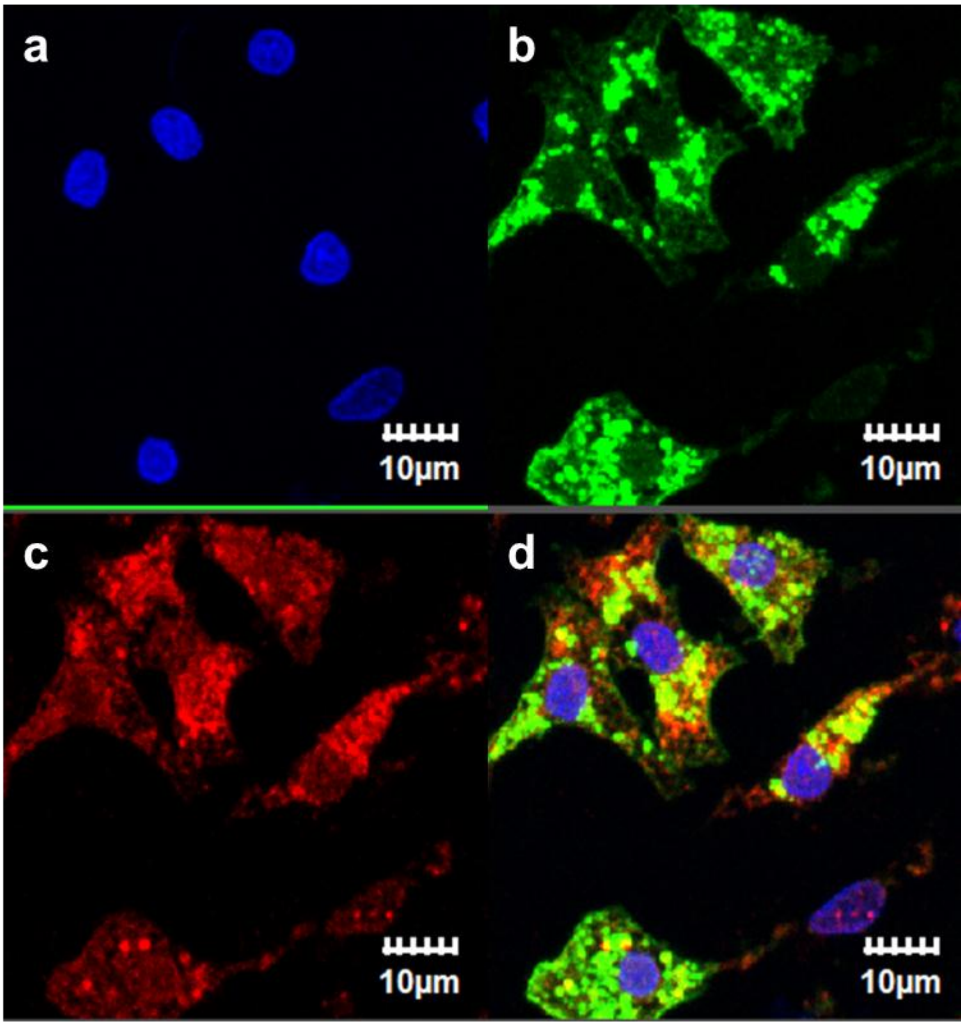

Fig. 17 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15 pre-exposed cytochalasin D for 1 h before exposure to YHV (15 h). The cells were treated with a mouse monoclonal antibody specific for YHV nucleocapsid protein (p20) and FITC-phalloidin for host actin filaments. (a) Host cell nuclei (blue) stained with DAPI. (b) Photomicrograph of host actin filaments (green). (c) YHV p20 stained with Alexa Fluor 633(red) showing positive reaction in all cells. (d) Merged images a to c.

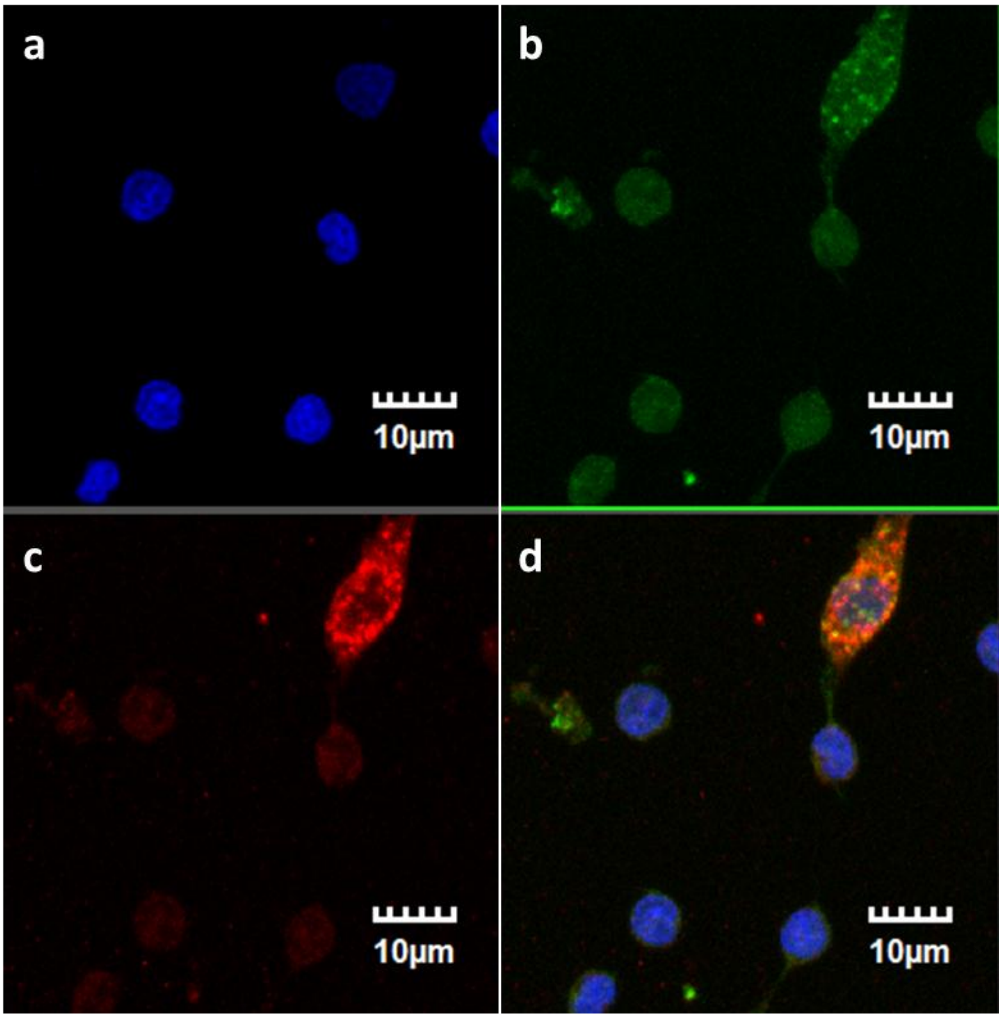

Fig. 18 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15 exposed to tunicamycin and YHV (15 h P.I.) treated with a mouse monoclonal antibody specific for YHV nucleocapsid protein (p20) and FITC-phalloidin for host actin filaments. (a) Host cell nuclei (blue) stained with DAPI. (b) Photomicrograph of host actin filaments (green) showing very faint actin staining. (c) YHV p20 stained with Alexa Fluor 594(red) showing positive reaction in all cells. (d) Merged images a to c. The morphology of cells in tunicamycin containing culture medium were not as good as those in normal medium, in that many of them appeared to have very little cytoplasm.

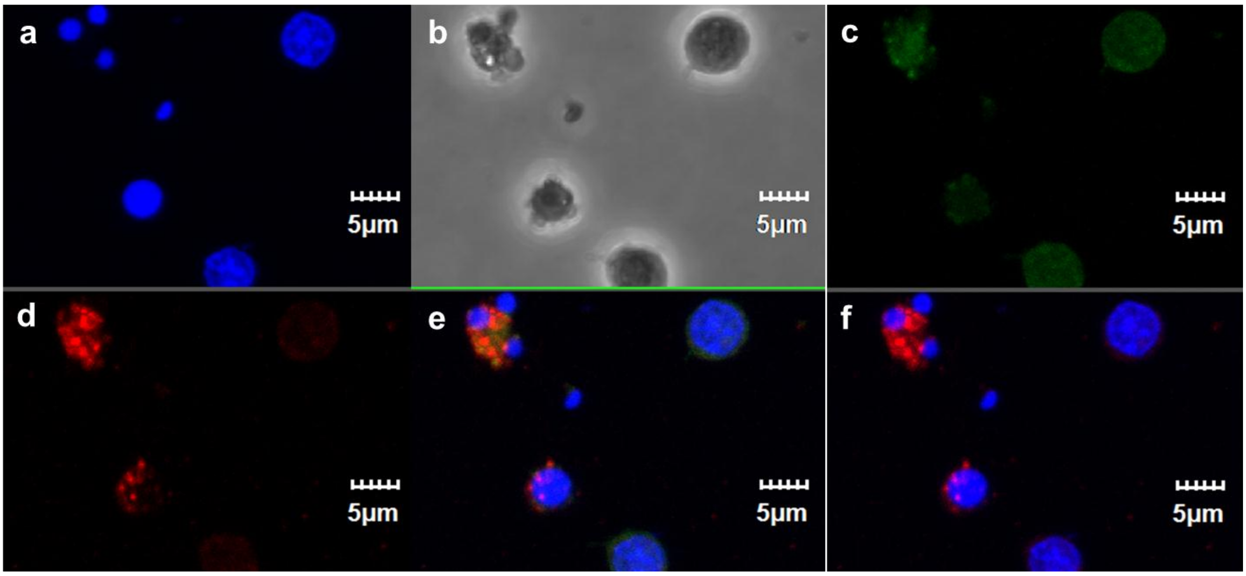

Fig. 19 Laser confocal micrographs of *Penaeus monodon* cultured hemocyte cells in 2x L15 exposed to tunicamycin and YHV (15 h P.I.) treated with a mouse monoclonal antibody specific for YHV envelope glycoprotein protein (gp116) and FITC-phalloidin for host actin filaments. (a) Host cell nuclei (blue) stained with DAPI showing some fragmented nuclei (apoptosis). (b) Photomicrograph of phase contrast of the same. (c) Host actin filaments (green). (d) YHV gp116 stained with Alexa Fluor 633(red) showing positive reactions in some cells. (e) Merged images a, c and d. (f) Merged images of (a) and (d) showing two YHV positive cells undergoing apoptosis.

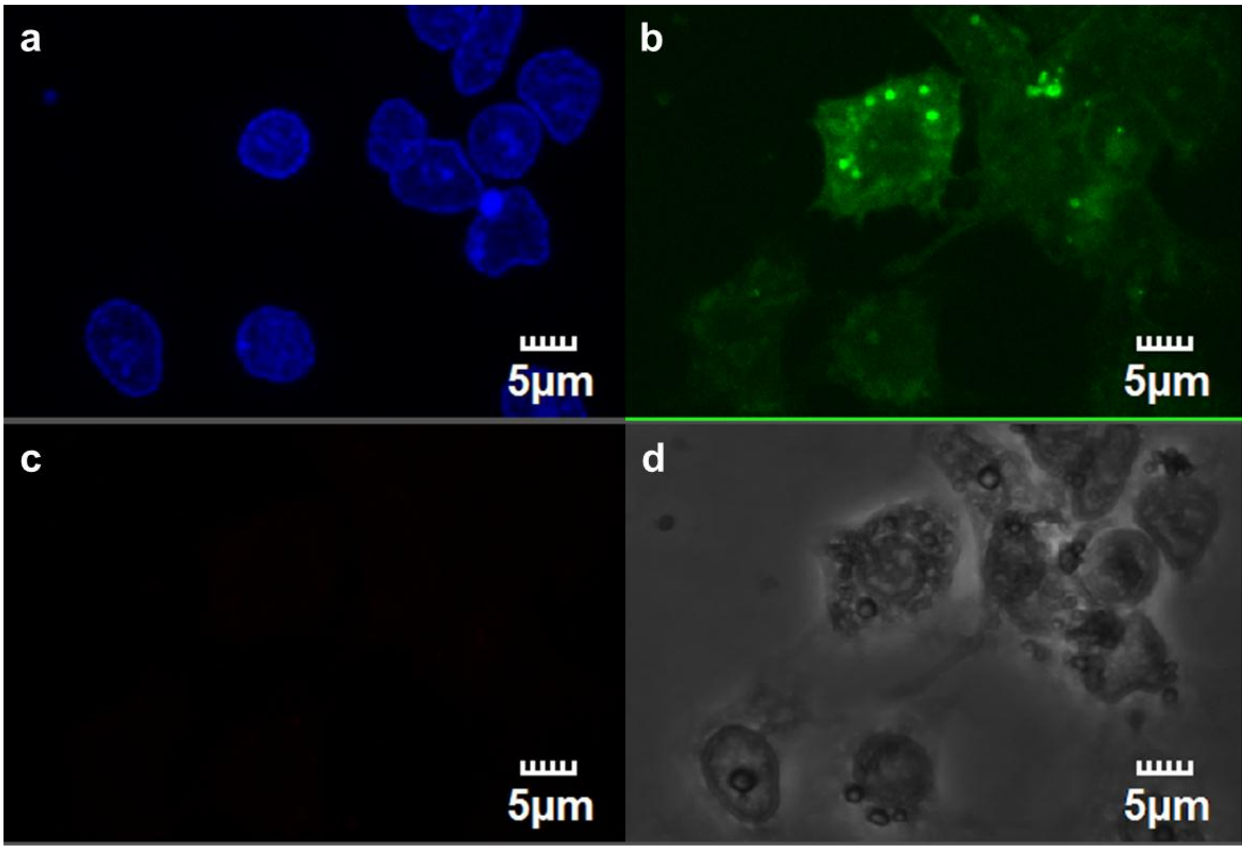

Fig. 20 Control uninfected cells treated with tunicamycin (15 h post treatment) The cells were immuno-stained with a monoclonal antibody against YHV nucleocapsid protein (clone Y19) detected with goat anti-mouse antibody labeled with Alexa Fluor 633 dye, red, c) and FITC labeled phalloidin to stain actin filaments (green, in b). The cell nuclei were stained with DAPI (blue, a). No immuno-positive signal for the virus protein was observed as expected.

Discussion

In this project, methods for routine primary shrimp cell culture were successfully established using double strength-L-15 or single strength Grace's insect medium and Sf900 insect medium as basal media with supplements including 15% fetal bovine serum, 0.5% NaCl and antibiotics including 2% penicillin and streptomycin (stock Penicillin and streptomycin, 5000 units and 5000 µg/ml). The cultured cells could be maintained at 30 ºC in an incubator for up to three weeks in modified 2x- L15 and up to 9 months in modified Grace's insect medium. Similar media formulas, especially with 2x-L15 have been used to culture different shrimp tissues with some success (Assavalapsakul et al., 2003; Kasornchandra et al., 1999; Lu et al., 1995). Grace's medium has also been used (Luedeman and Lightner, 1992). Since the cultured cells in Grace's insect medium were not susceptible to YHV infection, 2xL-15 was chosen as the basal culture medium for all our hemocyte cell culture and infection experiments.

To determine whether primary culture in 2x L15 was suitable for in vitro studies on shrimp viruses, susceptibility tests for three viruses including YHV, WSSV and TSV were carried out. It was found that the primary hemocyte cell cultures were susceptible to all three viruses tested. Many viral structural proteins were found to be expressed in these cells, including two envelope proteins (VP19 and VP28) of WSSV, a nucleocapsid protein (Vp1) of TSV and all three structural proteins of YHV. These results suggested that cultured shrimp cells, unlike insect cell lines (Sriton et al., 2009), could support virus replication and the formation of normal, enveloped particles. In other words, the cells were shown to be permissive to YHV and probably to TSV and WSSV as well.

In this study we found that all three hemocyte cell types were susceptible to YHV although they were not infected at the same time. These results contradicted results reported earlier by another research team who suggested that only granular hemocytes were susceptible to the virus (Taengchaiyaphum et al., 2013). It is possible that in their study of cultured cells was too short (only 1 hour after challenge) and fell within the period during which most of the hyaline cells remain negative for YHV by immuno-histochemical testing. It is possible that these cells might be infected by the virus at the same time as the large granular and semi granular cells but that the viral replication rate in these cells is slower than in granular cells.

In our study we also investigated the expression pattern of the receptor protein laminin. This protein is known to interact with the gp116 envelope protein of YHV and injection of recombinant laminin protein leads to protection of shrimp against the virus (Busayarat et al., 2011). It was found that all hemocyte cell types expressed this protein in their cytoplasm. If this protein is the sole receptor protein for YHV, then all those cells should have become positively immuno-labeled at the same time. Thus, it is likely that other factors or receptors are involved in the YHV infection and replication processes. In fact, another possible YHV receptor has been identified by a technique called viral overlay protein binding assay (VOPBA), dsRNA targeting the mRNA of this gene was found to inhibit virus infection completely (Assavalapsakul et al., 2006). Unfortunately we did not have access to the antibody against this protein.

In the WSSV infection experiment, we also found that the large granular cells were the first cell type to be infected with the virus. Again our results contradict another report on crayfish which concluded that small granular cells were most susceptible to the virus and that the viral replication rate in them was higher than that in large granular cells (Jiravanichpaisal et al., 2006). It is possible that differences among crustacean species will turn out to be major contributors to these discrepancies. As with the laminin receptor protein, the expression pattern of the putative WSSV receptor protein Pm Rab7 was also investigated. It was found that this protein was expressed by all hemocyte cell types, but in varying degrees. It was found to be highest in large granular cells, similar to envelope protein Vp28 of the virus. But since the protein was found in both cytoplasm and in the nucleus, it is possible that the protein may have more functions than originally expected, (i.e. more than just as receptor protein) (Sritunyalucksana et al., 2006).

Actin filaments have been known to play many different roles during virus replication. Some viruses incorporate actin filaments into their particles and many viruses use actin filaments to transport their own proteins or particles within cells (Cudmore et al., 1995; Cudmore et al., 1996; Cudmore et al., 1997). In this research project, cells infected with YHV treated and not treated with actin polymerization inhibitor, cytochalasin D, were detected with Mabs against all three virus proteins. It was found that the viral envelope glycoprotein gp64 co-located with actin filaments. The immuno reactive signals for both proteins were found to be very strong in vesicles in the cytoplasm of infected cells. It was also clear in virus infected granular cells that there was more intense actin filament staining than un-infected cells. This result suggested that virus infection induced or promoted actin polymerization. Similar observations have been reported in cells many other viruses such as vaccinia virus (Rietdorf et al., 2001) and Epstein-Barr virus (Melamed et al., 1994). These two viruses have been shown to use actin filaments to transport their particles and to assist with or enhance their cell to cell spread. It is possible that YHV employs actin filaments in a similar manner since the positive signals for YHV proteins in cytochalasin D-treated cells were in the same focal areas of the culture plate and not as widely spread as they were in untreated wells.

In our recent study, we reported that *N*-linked glycosylation is essential for virus replication and envelopement (Soowannayan et al., 2013). We showed that YHV-infected shrimp that received the *N*-linked glycosylation inhibitor tunicamycin formed no enveloped particles and that the viral RNA copy numbers in this shrimp were much less than in the untreated shrimp. In this study, when YHV infected cultured hemocytes were treated with the same reagent, it was found that immunoreactive signals for all three viral structural proteins were observed in both treated and untreated cells. Although the intensities of the positive reactions for all three proteins were generally less in the treated cells, the results suggested that the virus could still be formed. It is possible that the quantity of tunicamycin used may not have been optimal, since the morphology of the treated cells was not as normal as in untreated cells. These facts need to be investigated further.

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.

Assavalapsakul, W., Smith, D.R., Panyim, S., 2003. Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of *Penaeus monodon*. Dis Aquat Organ 55, 253-258.

Assavalapsakul, W., Smith, D.R., Panyim, S., 2006. Identification and characterization of a *Penaeus monodon* lymphoid cell-expressed receptor for the yellow head virus. J Virol 80, 262-269.

Busayarat, N., Senapin, S., Tonganunt, M., Phiwsaiya, K., Meemetta, W., Unajak, S., Jitrapakdee, S., Lo, C.-F., Phongdara, A., 2011. Shrimp laminin receptor binds with capsid proteins of two additional shrimp RNA viruses YHV and IMNV. Fish Shellfish Immun 31, 66-72.

Chen, S.N., Kou, G.H., 1989. Infection of cultured cells from the lymphoid organ of Penaeus monodon Fabricius by monodon-type baculovirus (MBV). J Fish Dis 12, 73-76.

Crane, M.J., Benzie, J.H., 1999. The Aquaculture CRC International Workshop on Invertebrate Cell Culture 2--4 November 1997, University of Technology, Sydney, Australia. Methods Cell Sci 21, 171-172.

Cudmore, S., Cossart, P., Griffiths, G., Way, M., 1995. Actin-based motility of vaccinia virus. Nature 378, 636-638.

Cudmore, S., Reckmann, I., Griffiths, G., Way, M., 1996. Virus movement: actin takes centre stage. Trends in Cell Biology 6, 458.

Cudmore, S., Reckmann, I., Way, M., 1997. Viral manipulations of the actin cytoskeleton. Trends Microbiol 5, 142-148.

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

Itami, T., Maeda, M., Kondo, M., Takahashi, Y., 1999. Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, Penaeus japonicus. Methods Cell Sci 21, 237-244.

Jiravanichpaisal, P., Sricharoen, S., Soderhall, I., Soderhall, K., 2006. White spot syndrome virus (WSSV) interaction with crayfish haemocytes. Fish Shellfish Immun 20, 718-727.

Kasornchandra, J., Khongpradit, R., Ekpanithanpong, U., Boonyaratpalin, S., 1999. Progress in the development of shrimp cell cultures in Thailand. Methods Cell Sci 21, 231-235.

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.

Luedeman, R., Lightner, D.V., 1992. Development of an in vitro primary cell culture system from the penaeid shrimp, Penaeus stylirostris and Penaeus vannamei. Aquaculture 101, 205-211.

Melamed, I., Stein, L., Roifman, C.M., 1994. Epstein-Barr virus induces actin polymerization in human B cells. J Immunol 153, 1998-2003.

Rietdorf, J., Ploubidou, A., Reckmann, I., Holmstrom, A., Frischknecht, F., Zettl, M., Zimmermann, T., Michael, W., 2001. Kinesin-dependent movement on microtubules precedes actin-based motility of vaccinia virus. Nat Cell Biol 3, 992-1000.

Senapin, S., Phongdara, A., 2006. Binding of shrimp cellular proteins to Taura syndrome viral capsid proteins VP1, VP2 and VP3. Virus Res 122, 69-77.

Soowannayan, C., Chanarpakorn, N., Phanthura, M., Deekhlai, N., Kunasol, C., Sriurairatana, S., 2013. N-Linked glycosylation is essential for the yellow head virus replication cycle. J Gen Virol 94, 2458-2468.

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, 86–93.

Sritunyalucksana, K., Wannapapho, W., Lo, C.F., Flegel, T.W., 2006. PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. J Virol 80, 10734-10742.

Taengchaiyaphum, S., Havanapan, P.-o., Roytrakul, S., Lo, C.-F., Sritunyalucksana, K., Krittanai, C., 2013. Phosphorylation is required for myosin regulatory light chain (PmMRLC) to control yellow head virus infection in shrimp hemocytes. Fish Shellfish Immun 34, 1042-1049.

Output from this project:

- A paper partially supported by this project was published in the Journal of General Virology.

  Soowannayan, C., Chanarpakorn, N., Phanthura, M., Deekhlai, N., Kunasol, C., Sriurairatana, S., 2013. *N*-Linked glycosylation is essential for the yellow head virus replication cycle. J Gen Virol 94, 2458-2468 (Attached)

- A poster entitled: Establishment of routine primary hemocyte cell cultures for shrimp virus research and their use in studying envelope formation by yellow head virus (YHV) was awarded “เกียรติบัตรเสนอผลงานวิจัยยอดเยี่ยมแบบโปสเตอร์” จากการประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. สำนักงานคณะกรรมการการอุดมศึกษา กระทรวงศึกษาธิการและสำนักงานกองทุนสนับสนุนการวิจัยแห่งชาติ ระหว่างวันที่ 16-18 ตุลาคม 2556

- During this period the author was invited to

1. review 2 papers, one for the Journal of Virology and one for Diseases of Aquatic Organisms
2. be a member of Nidovirus study group of the international committee on taxonomy of viruses (ICTV) and
3. be an observer in the executive committee of the Fish health Section in the Asian Fisheries Society