GENERIC METHOD FOR PROPAGATION OF SHRIMP VIRUSES IN SF-9 INSECT CELLS

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ABSTRACT

Experiments with crustacean viruses are hampered by lack of susceptible continuous cell lines. To overcome this problem, lepidopteran cell lines were challenged with a shrimp DNA virus (white spot syndrome virus: WSSV) and RNA virus (yellow head virus: YHV) followed by serial, split-passage with immunohistochemical monitoring by confocal laser microscopy using labeled monoclonal antibodies to shrimp viral antigens. Stable, immortal cultures with 100% of the cells expressing shrimp-virus antigens were obtained, although the infected cells appeared grossly normal by phase contrast microscopy. Nor did they show any ultrastructural modifications characteristic of the challenge viruses. These persistently-expressing insect cell cultures were stable and could be continuously passaged, stored and revived as required. Since both DNA and RNA viruses were used, this appears to be a generic process that may be applicable to other shrimp viruses as well.

KEY WORDS: SHRIMP / VIRUS / INSECT CELL LINE / WSSV / YHV

60 pages

วิธีพื้นฐานสำหรับการเพิ่มจำนวนของไวรัสกุ้งในเซลล์แมลง Sf-9

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บทคัดย่อ

การศึกษาเกี่ยวกับไวรัสของสัตว์ในกลุ่มครัสเตเชียนมีข้อจำกัดเนื่องจากขาดเซลล์เพาะเลี้ยงทีมีความไวต่อเชื้อไวรัส การแก้ปัญหานี้ทำได้โดยการใช้เซลล์เพาะเลี้ยงของแมลงมาเหนี่ยวนำให้ติดเชื้อด้วยไวรัสตัวแดงดวงขาวซึ่งเป็นดีเอ็นเอไวรัสและไวรัสหัวเหลืองซึ่งเป็นอาร์เอ็นเอไวรัสของกุ้ง หลังจากนั้นทำการขยายเซลล์ในรุ่นถัดไปโดยใช้เซลล์ติดเชื้อจากรุ่นก่อนหน้ามาผสมกับเซลล์ปกติ และตรวจสอบการติดเชื้อโดยใช้วิธีติดตามเชื้อไวรัสในเซลล์ด้วยแอนติบอดีต่อเชื้อไวรัสนั้นและนำไปตรวจดูด้วยเทคนิค confocal microscopy ผลการทดลองพบว่ามีเซลล์เพาะเลี้ยงของแมลงติดเชื้อไวรัสทั้งหมด (100%) อย่างไรก็ดี เซลล์เพาะเลี้ยงที่ติดเชื้อเหล่านี้ไม่แสดงลักษณะภายนอกและลักษณะภายในที่เปลี่ยนแปลงหรือผิดปกติไปจากเซลล์เพาะเลี้ยงที่ไม่ติดเชื้อ เซลล์เพาะเลี้ยงที่ติดเชื้อไวรัสแบบเรื้อรังนี้มีความคงที่และสามารถถ่ายทอดไปยังรุ่นต่อๆไปได้ สามารถที่จะเก็บและเอามาใช้ได้เมื่อต้องการ การที่ทั้งไวรัสชนิดดีเอ็นเอและอาร์เอ็นเอสามารถเจริญได้ในเซลล์เพาะเลี้ยงของแมลง อาจจะถือว่าเป็นวิธีพื้นฐานที่สามารถประยุกต์ใช้กับไวรัสของกุ้งตัวอื่นๆได้

60 หน้า

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CHAPTER I
INTRODUCTION

Asia is the world leader in production of cultivated shrimp with a market value of several billions of US dollars per year. Thailand has been the world's leading producer since 1992 with export earnings up to 2 billion US dollars annually. Unfortunately, in 1995 Thai production dropped by 5000 metric tons due mainly to outbreaks of disease caused by yellow head virus (YHV). Again in 1996 and 1997, another disaster occurred due to disease outbreaks caused by white spot syndrome virus (WSSV). In that time, the estimated loss in export revenue was 1 billion US dollars. After 1997, Thai production recovered and reached 308,550 metric tons by the year 2000. In the succeeding years, more losses occurred due to monodon slow growth syndrome (MSGS) with production dropping to 263,428 metric tons in 2001. All of this production was based primarily on cultivation of the native black tiger or giant tiger shrimp *Penaeus monodon*. After that, domesticated, specific pathogen free (SPF) and genetically selected stocks of the exotic species *Penaeus vannamei* were widely adopted and production again rose, reaching 501,000 metric tons in 2007. During the period beginning around 1993, the rest of Asia did not fare so well. For example, WSSV outbreaks in China began in 1993, reducing export production from 115,000 metric tons to 35,000 metric tons in one year and recovery thereafter was relatively slow, until the introduction and widespread adoption of *P. vannamei* that has resulted in the highest production of cultivated shrimp ever recorded anywhere (more that 1 million metric tons in 2007) (1).

White spot syndrome virus (WSSV) is the most serious of the penaeid shrimp viruses in terms of global production losses. It has a wide host range including all cultured shrimp species as well as other crustaceans such as crabs and crayfish (2). The disease is highly lethal and contagious. Outbreaks of this disease can wipe out the entire population in a shrimp pond within a few days. For example, it was estimated in 1993 that more than 1 million tons of production was lost in all Asian countries. Later

world losses, including those from the USA, Central America and South America was much higher (3).

How the viruses spread among countries is unknown but it is suspected that moving grossly normal broodstock and post larvae freely amongst countries was responsible for the most rapid and effective means of spread. Almost certainly WSSV was spread from Thailand to Malaysia and India in this manner. In addition, WSSV was not reported from the Philippines until 2000 (4), probably because of an effective Philippine government ban on importation of broodstock and post larvae (5).

Another major shrimp virus is yellow head virus (YHV) that causes fatal disease in the giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*P. vannamei*) farmed in Thailand. Like outbreaks caused by WSSV, entire shrimp crops can be lost within a few days. The disease is called “hua leung” in Thai (6).

The most serious disease losses in the shrimp farming industry in the past decade have been caused by viral infections. Among the causative viruses, yellow head virus (YHV) and white spot syndrome virus (WSSV) are the two most serious causes of major production losses in Thailand (3,7,8). Reliable techniques for detection of these two viruses have been established using nucleic acid probes and PCR based methods (9,10) and the genomes of both have been completely sequenced. However, the function of most of the WSSV genes and some of the YHV genes is still unknown.

Cell culture is a basic tool for the study of pathogenic infections, especially for those pathogens such as viruses that replicate intracellularly. Experimental work on shrimp and other crustacean viruses is hampered by the lack of any continuous cell line (11). This is important because viral disease outbreaks are a constant threat to the shrimp culture industry that provides important export income for many developing countries. Although control of these diseases is vital, knowledge of the pathogens and their host defense responses is relatively poor but could be advanced more rapidly by the availability of infected cell lines. Therefore, in the present work, we performed experiments to determine whether the Sf-9 insect cell line could accommodate WSSV and YHV.

CHAPTER II
OBJECTIVES

Although numerous studies have been carried out on morphology, histopathology and genome sequence of shrimp viruses, the mechanism of virus infection is still unclear, largely due to the difficulty of establishing a continuous shrimp cell line. Since shrimp belong to the Phylum Arthropoda, we proposed to test an insect cell line as a possible host for the two shrimp viruses WSSV and YHV. Experiments were carried out to reach the following objectives

1. To investigate whether Sf-9 insect cells can accommodate WSSV and YHV.
2. To characterize the viruses produced from Sf-9 cells.
3. To find a method for easy storage, maintenance and production of shrimp viruses in the laboratory.

CHAPTER III
LITERATURE REVIEW

3.1 White spot syndrome virus (WSSV)

3.1.1 Structure and Morphology

White spot syndrome virus is a bacilliform, nonoccluded enveloped virus (12,13,14). Intact enveloped virions range between 210 and 380 nm in length and 70–167 nm in width (15,16,17,18). A tail-like appendage at one end of the WSSV virion is sometimes observed in negatively stained electron micrographs (14,19)

The viral envelope is 6–7 nm thick and is a lipidic, trilaminar membranous structure with two electron-transparent layers divided by an electronopaque layer (14, 20, 21). The nucleocapsid is located inside the envelope and is a stacked ring structure composed of globular protein subunits of 10 nm in diameter arranged in 14–15 vertical striations located every 22 nm along the long axis, giving it a cross-hatched appearance (20,21). When released from the envelope, the nucleocapsid increases in length indicating that it is tightly packed within the virion. The size of the nucleocapsid varies from isolate to isolate and ranges between 180 and 420 nm in length and 54–85 nm in width, with a 6 nm thick external wall (22,23,18).

More than 40 WSSV proteins have been characterized. Some non-structural proteins are probably involved in transcriptional regulation (VP9) (24), virus proliferation (WSV021) (25) and/or regulation of DNA replication (WSV477) (26). At least 38 structural proteins have been located in the WSSV virion. Of these, 21 have been found in the envelope, 10 in the nucleocapsid and five in the tegument (a putative structure located between the envelope and nucleocapsid).

A cell attachment motif that suggests a role in viral entry has been found in the envelope proteins VP31, VP110 and VP281 (27,28,29,30), the tegument protein VP36A and the nucleocapsid proteins VP664 (28,31) and VP136A (28,30). Other proteins such as VP28, VP39B, VP41A, VP41B, VP51A, VP51B, VP68, VP124,

VP150, VP187, VP281, VP292 and a collagen-like protein (32) have been located in the envelope (27,30,33,34,35,36,37,38,39,40) whereas the proteins VP35 (41), VP466 (42), VP15 (35), VP51, VP76 (43) and others (44) have been located in the nucleocapsid and may have different putative functions (34,45).

The role of different WSSV proteins in infection has recently been studied by RNA interference. In *Penaeus (Litopenaeus) vannamei*, long doublestranded (ds) RNA corresponding to VP19 induced a specific antiviral response that inhibited WSSV infection and significantly reduced mortality (46). In *Penaeus (Fenneropenaeus chinensis)*, long dsRNA corresponding to VP28, VP281, WSSV protein kinases (PK) and an unrelated dsRNA from the green fluorescence protein (GFP) induced higher survival of WSSV-challenged shrimp. The highest survival rates were found in shrimp treated with dsRNA from VP28 and PK (47). A complete inhibition of a WSSV infection in shrimp was achieved by three consecutive injections of small short interfering RNA (siRNA) against VP28 in *Penaeus (Marsupenaeus) japonicus* (48).

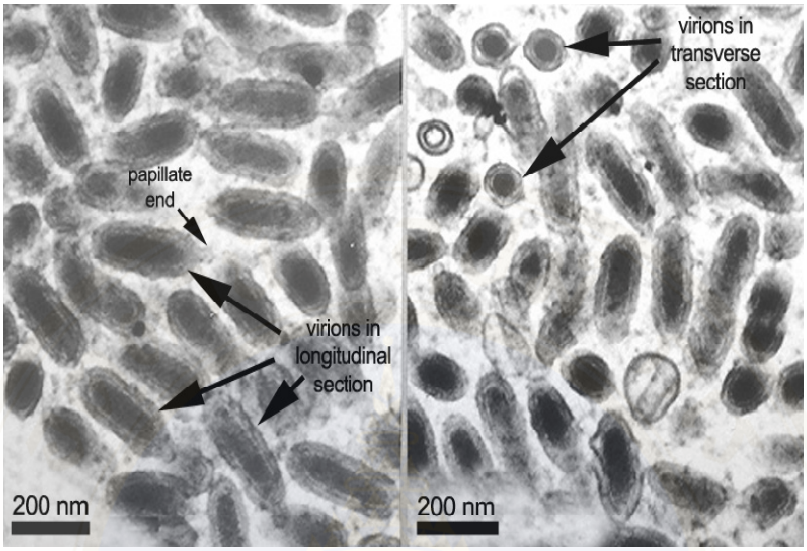

Figure.3.1 High magnification TEM . Thin section of WSSV in a cell nucleus.

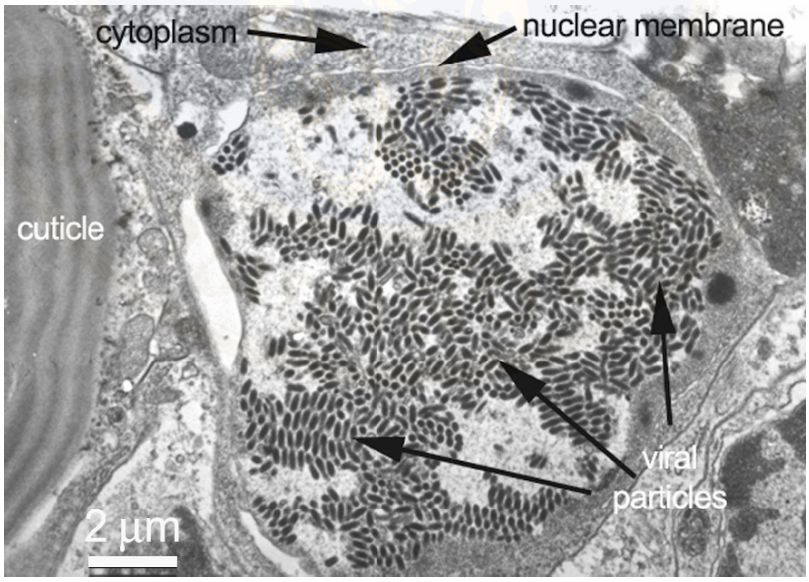

Figure.3.2 Low magnification TEM of WSSV infected gill tissue showing large numbers of rod shaped virions in the nucleus of a subcuticular epithelial cell (Wongteerasupaya et al., 1995b).

3.1.2 Genome and classification

The WSSV genome is a circular, dsDNA molecule with an A+T content of 59% (homogeneously distributed). The genome size varies according to the viral isolate. For example, the genome size of one Thai isolate is 293 kbp while those from China and Taiwan contain 305 kbp and 307 kbp, respectively. (34, 45, 49).

Sequence analysis shows that the WSSV genome contains between 531 and 684 open reading frames (ORFs) with an ATG initiation codon. Of these, 181–184 ORFs are likely to encode functional proteins with sizes between 51 and 6077 amino acids, which represent 92% of the genetic information contained in the genome (33,45). Only 21–29% of such ORFs have been shown to encode proteins that share identity with other known proteins. These proteins include enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase (49).

WSSV was originally classified as an unassigned member of the *Baculoviridae* because of its bacilliform, enveloped, rod-shaped morphology (50). However, it was later listed as unclassified due to the lack of adequate molecular information (51). In 2001, the WSSV genome was completely sequence and found to show no similarity to any existing virus family (34,45). Based on this information, WSSV was allocated to a new virus family *Nimaviridae* and genus *Whispovirus* (52,53)

3.1.3 Morphogenesis

The stages of WSSV morphogenesis have been characterized and are directly related to the development of cellular lesions (20,54,55)

Stage 1: the early stage of cell infection. Infected cells show slightly hypertrophied nuclei. A viral nucleosome appears before the formation of viral particles. It is composed of viral proteins organized in fibrillar fragments. In the cytoplasm, the endoplasmatic reticulum (ER) becomes enlarged with abundant free ribosomes.

Stage 2: in the nucleus, the fibrillar material induces the formation of circular membranes that are soon filled with viral core material starting viral assembly. At this stage, Cowdry-A type inclusions appear as a translucent zone between the

virogenic stroma and the very electron-dense marginated chromatin. The nuclei become hypertrophied and rounded.

Stage 3: in the nucleus, the nucleocapsids appear with low electron density and gradually grow from one end towards the other. The central intranuclear inclusion appears smaller than in cells in stage 2 and is more electron dense because of the presence of abundant viral particles. When the marginated chromatin disappears, the nuclear membrane is disrupted and the marginal transparent zone is fused with the lucent cytoplasm. Most organelles are abnormal, disintegrated or form membranous structures.

Stage 4: in the nucleus, the nucleocapsid is completed with 12–14 rings of globular protein units arranged in a stacked series. Each nucleocapsid has one round and one square end. The nucleocapsid becomes completely enclosed by the envelope.

Stage 5: the late stage of viral morphogenesis. The viral particles become ovoid in shape and a long tail-like projection derived from the envelope is observed. The inner material of the tail is separated from the nucleocapsid. Afterwards, the nucleocapsids become shorter, thicker and more electron dense because of the packing of the viral DNA-VP15 complex.

i) *Stage 6: the final phase of morphogenesis*. The mature virions are elliptical with complete smooth envelopes enclosing an electron-dense nucleocapsid and with a tail-like projection at the last enclosed end. Sometimes assembly of nucleocapsids occurs completely separated from the envelopes and later they are wrapped by the envelopes. At this final stage, infected cells are severely damaged and disrupted. Void spaces are observed in tissues as cells disintegrate.

Replication cycle

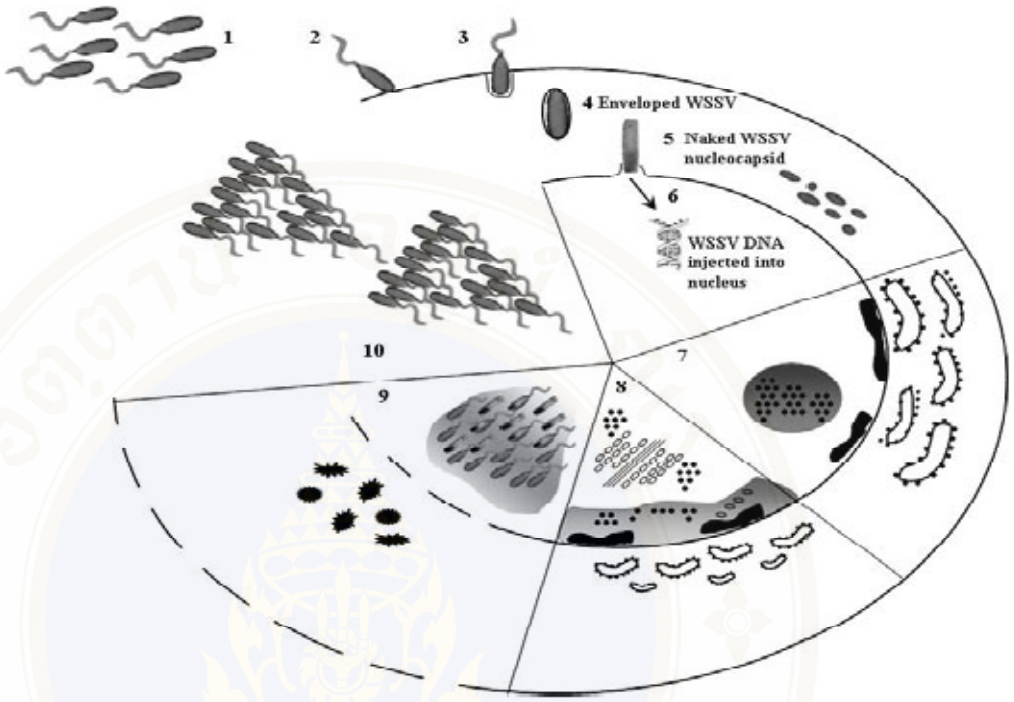

Figure.3.3 A proposed model of the morphogenesis of white spot syndrome virus (WSSV). (1) Infectious WSSV particles. (2) An infectious WSSV virion attaches to a susceptible cell using envelope proteins with a cell attachment motif. (3) WSSV enters the cell. (4) The envelope of the WSSV virion probably fuses with the endosome and the naked nucleocapsid is transported to the nucleus, in a similar way as in baculoviruses (see Lua & Reid 2000). (5) The naked WSSV nucleocapsid attaches to the nuclear membrane and the WSSV genome is released into the nucleus. (6) The WSSV genome replication starts. In the cytoplasm, the mitochondria start degenerating (Wang et al. 1999a). (7) In the nucleus the early virogenic stroma appears composed of loose granular material. Cellular chromatin accumulates near the nuclear membrane and the rough endoplasmic reticulum (RER) becomes enlarged and active. (8) The marginated chromatin is transformed in a dense ring zone (shaded area). The virogenic stroma is less dense and starts forming vesicles that will form the viral envelope. The vesicles are probably formed with membranous material found in the ring zone, as in baculoviruses (see Lua & Reid 2000). A viral nucleosome is also observed as a filamentous structure in the virogenic stroma. This structure contains proteins that will form the nucleocapsid. (9) New WSSV particles are assembled in the nucleus within an electron-dense inclusion. The empty envelopes are filled with a nucleocapsid. In cytoplasm, organelles become disintegrated and the cellular and nuclear membranes are disrupted (Wang et al. 1999a). (10) WSSV virions are completely formed and ready to be released from the disrupted cell to begin the cycle in other susceptible cells. (C M Escobedo-Bonilla et al. 2008).

3.1.4 clinical signs and Pathology

Clinical signs of disease caused by WSSV include a sudden reduction in food consumption, lethargy, loose cuticle and often reddish body discoloration, and the presence of white spots of 0.5 to 2.0 mm in diameter within the cuticle of the carapace, appendages and abdominal segments (56). White spots on the shell of infected shrimp under scanning electron microscope appear as large dome shaped spots on the carapace measuring 0.3 to 3 mm in diameter. Smaller white spots of 0.02 to 0.1 mm appear as linked spheres on the cuticle surface. Chemical composition of the spots is similar to the carapace with calcium being the major component and it is suggested to be derived from abnormalities of the cuticular epidermis (57).

By histopathology, WSSV infection is characterized by cells with hypertrophied nuclei showing amphophilic intranuclear inclusions and marginated chromatin (20,58). These intranuclear inclusions are markedly distinct and bigger than the Cowdry A-type inclusions characteristic of the infectious hypodermal and haematopoietic necrosis virus (14). Infected nuclei become progressively more basophilic and enlarged (15; 19, 20; 59; 60; 61; 62; 63). In the late stages of infection, karyorrhexis and cellular disintegration may occur, leading to the formation of necrotic areas characterized by vacuolization (22,54,64).

3.1.5 Host range

White spot syndrome virus has a broad host range, including penaeid shrimp (10,20,65,66,67), crayfish (61,68,69), crabs, freshwater crabs (70,71,72), lobsters (18,73) and non-penaeid shrimp (74). Some aquatic insect larvae (59,60) and polychaete worms have tested positive for WSSV by PCR assay, but they are probably mechanical carriers (74,75). Although many of these species have been confirmed to support WSSV replication under experimental conditions, some other species collected from the wild have only been found WSSV positive by PCR. This indicates that many such species are not necessarily WSSV natural hosts, but may only be mechanical carriers.

3.1.6 Transmission mechanisms

The infection can be transmitted vertically (trans-ovum), horizontally by consumption of infected tissue (e.g. cannibalism, predation, etc.), and by water-borne routes. Transmission can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can also be a source of disease transmission (76).

3.1.7 Control and Prevention

Although the underlying mechanism remains unknown, laboratory experiments have shown that 'vaccinated' shrimp and crayfish have better survival rates after WSSV challenge. It was first shown that *Penaeus japonicus* shrimp that survived natural and experimental WSSV infections displayed resistance to subsequent challenge with WSSV (77). Later studies showed that intramuscular injection of inactivated WSSV virions or recombinant structural protein, (VP28), provided shrimp with some protection against experimental WSSV infection. Furthermore, shrimp fed with food pellets coated with inactivated bacteria over expressing VP28 showed better survival rates after WSSV challenge (78). However, although these results seemed promising, the protection was effective only when the shrimp were infected with a low dosage of WSSV. Also, the effect usually lasted for only a few days, or in the case of crayfish, for about 20 days.

Another potential means of protecting shrimp against WSSV infection is to use RNA interference (RNAi). WSSV gene-specific double-stranded (ds) RNAs produced strong anti-WSSV activity, protecting the shrimp against WSSV infection, but the same study showed that long dsRNA induced both sequence-dependent and independent anti-viral responses in shrimp (79). A more recent study even showed that oral administration of bacterially expressed VP28 dsRNA could protect shrimp against WSSV infection (80). To date, however, there are still no field trial data for either the vaccination or the RNAi approach.

There are no available treatments for WSSV, although a large number of disinfectants are widely used in shrimp farms and hatcheries to prevent disease outbreaks. Stocking of uninfected shrimp seeds and rearing them away from environmental stressors with extreme care to prevent contamination are useful management measures (81).

3.2 Yellow head virus (YHV)

3.2.1 Structure and Morphology

YHV virions are rod-shaped, enveloped particles (50nm x 175nm) with prominent diffuse spikes (8nm x 11nm) projecting from the surface. Internal helical nucleocapsids are approximately 25 nm in diameter and have a periodicity of 5-7 nm. Filamentous nucleocapsid precursors, approximately 15 nm diameter and of variable length (80nm-450nm) are observed in the cytoplasm, sometimes densely packed in paracrystalline arrays. Nucleocapsids acquire trilamellar lipid envelopes by budding through membranes into intracytoplasmic vesicles or at the cell surface. It has been reported that long nucleocapsid precursors generate elongated, enveloped structures that subsequently fragment into mature virions.

YHV virions contain a polyadenylated 26.6 kDa (+) ssRNA genome and three structural proteins. The nucleoprotein (p20) is a highly hydrophilic, basic protein that complexes with the genomic RNA in nucleocapsids. Transmembrane glycoproteins gp64 and gp116 are components of the envelope that from visible projections on the virion surface.

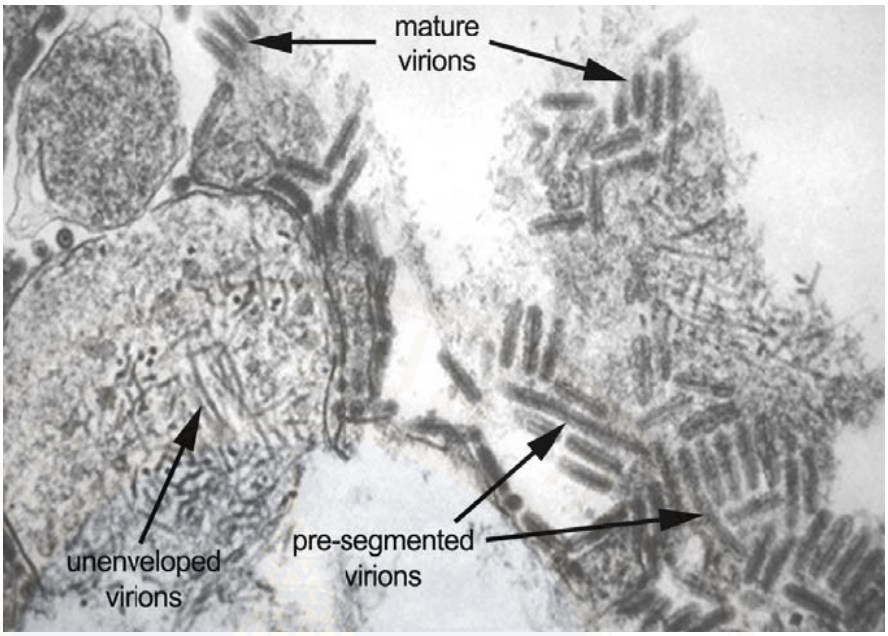

Figure.3.4 Transmission electron micrograph of a YHV-infected shrimp tissue section showing the unusual filamentous nucleocapsid precursors (on the left) and mature, rod-shaped, enveloped virions (on the right). Flegel TW.2006. Aquaculture 258:1-33

3.2.2 Genome and classification

Yellow head virus was originally classified as a coronavirus or rhabdovirus based upon virus morphology and the presence of a single-stranded RNA genome (82). It was subsequently shown to have a positive-sense RNA genome (83). Based on sequence identity, genome organization, and gene expression, it was deemed to be closely related to gill-associated virus from Australia (84) and both have been placed in a new genus *Okavirus* within the family *Roniviridae* in the order *Nidovirales* (85).

Analysis of the structural proteins of the virus by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) revealed three major structural proteins of molecular masses 116 kDa (gp116), 64 kDa (gp64), and 20 kDa (p20). Structural proteins gp116 and gp64 are glycosylated (86,87) and are believed to be the proteolytic products of a predicted membrane polyprotein containing six hydrophobic transmembrane domains encoded by open reading frame 3 (ORF3) (86).

3.2.3 Replication cycle

The YHV nucleocapsid has helical symmetry and comprises a coiled filament of diameter 16-30 nm with periodicity of 5-7 nm (87,88,89). Filamentous nucleocapsid precursors approximately 15 nm in diameter and of variable length (80-450 nm) occur abundantly in the cytoplasm of infected cells. Nucleocapsids acquire envelopes by intracytoplasmic budding at the membranes of the endoplasmic reticulum from which it is presumed the trilaminar lipid envelope of virions is derived. The long nucleocapsid precursors appear to generate elongated enveloped virion precursors that subsequently fragment into discrete rod-shaped virions (89). Purified YHV virions pair end to end with an appearance suggesting they may have arisen by fragmentation of longer virions (82). Nucleocapsid precursors and mature enveloped virions are characteristically observed throughout the cytoplasm of infected cells and often near membranous vesicles in which budded virions often align in paracrystalline arrays (88, 89). Virions have also been observed near or between the outer and inner nuclear membranes (87,89) in proximity to cytoplasmic nucleocapsid filaments, suggesting that virion maturation can sometimes occur at these membranes. Virions have also been observed budding from cytoplasmic membranes (88).

3.2.4 clinical signs and Pathology

Gross clinical signs of YHV infection including yellowing of the hepatopancreatic tissue underlying the cuticle of the cephalothorax and erratic swimming behavior are not observed consistently and are not sufficiently pathognomonic to be useful for disease diagnosis. Histologically, moderate to large numbers of basophilic spherical, cytoplasmic inclusions in tissues of ectodermal and mesodermal origin are indications of YHV infection and can be used for presumptive diagnosis.

Shrimp are susceptible to YHV infection from late postlarval stages but mass mortality in ponds usually occurs in early-to-late juvenile stages. Disease and mortalities usually occur within 2-4 days of a period of exceptionally high feeding activity followed by an abrupt cessation of feeding. Moribund shrimp congregate at pond edges near the surface and may exhibit a bleached overall appearance and

discoloration of the cephalothorax caused by yellowing of the underlying hepatopancreas.

YHV infects tissues of ectodermal and mesodermal origin, including lymphoid organ, hemocytes, hematopoietic tissue, gill lamellae, and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts, and ganglia. In severe infections, there is a generalized cell degeneration with prominent nuclear condensation, pyknosis and karyorrhexis, and basophilic, perinuclear cytoplasmic inclusions in affected tissues. There is evidence of apoptosis, including chromatin condensation and DNA fragmentation, in hemocytes, lymphoid organ, and gill tissues and it has been suggested that widespread apoptosis rather than necrosis is the cause of disease and mortalities. (90)

YHV, GAV, and other viruses in the yellow head complex can also occur as low-level chronic infections in apparently healthy shrimp. Chronic infections have been observed in shrimp of all life stages collected from hatcheries and farms, and in the survivors of experimental infection. For YHV the onset of disease has been associated with the stress of molting. During chronic infections, there is little histopathology other than the accumulation of partitioned foci of cells with hypertrophic nuclei (spheroid bodies) in the lymphoid organ. Spheroid bodies appear to form in shrimp as part of a nonspecific defense mechanism for clearance of infectious agents and other foreign bodies.

3.2.5 Host range

Yellow head disease has been reported in farmed tiger shrimp from Thailand, Taiwan, China, the Philippines, Vietnam, Malaysia, Indonesia, India, Sri Lanka, and Madagascar. Although natural infection and disease have been reported only in black tiger shrimp and kuruma shrimp (*Marsupenaeus japonicus*), YHV can cause high rates of mortality following experimental infection of most other farmed marine shrimp species, including Pacific white shrimp (*Penaeus (Litopenaeus) vannamei*), Pacific blue shrimp (*Penaeus (Litopenaeus) stylirostris*), brown tiger shrimp (*Penaeus esculentus*), white banana shrimp (*Penaeus (Fenneropenaeus) merguiensis*), white shrimp (*Penaeus (Litopenaeus) setiferus*), brown shrimp (*Penaeus*

(*Farfantepenaeus) duorarum*), red endeavour prawn (*Metapenaeus ensis*), and Jungas shrimp (*Metapenaeus affinis*). Some species of palemonid shrimp and krill (*Acetes* sp.) are also susceptible to experimental infection. Crabs appear to be refractory to YHV infection and disease. (90)

3.2.6 Host Response to Infection

As invertebrates, shrimp lack antibodies, cytokines, T-lymphocytes, and other powerful components of the vertebrate immune system that allow a specific adaptive response to viral infection, clearance of virus and infected cells, and long-term immunological memory. There is also no evidence in shrimp of interferon, natural killer (NK) cells, or other key components of the vertebrate natural immune system that allow an immediate nonspecific defense against viruses. Nevertheless, shrimp do appear to have a capacity to response to viral infection and highly pathogenic viruses are commonly present as low-level chronic infections in apparently healthy shrimp. For YHV, there is no evidence of an inflammatory response at the primary sites of infection. However, YHV accumulates in spheroid bodies in the lymphoid organ during chronic persistent infections, and it is thought that the lymphoid organ has an important role in filtering granulated hemocytes and the clearance of viruses from infected shrimp. It has been reported that cells within lymphoid organ spheroids become apoptotic during infection and may be cleared during molting. Apoptotic cells have been observed in lymphoid organ, hemocytes, and gills during acute YHV infections in what appear to be a fundamental host defense reaction. It has also been reported that double-stranded RNA (dsRNA) corresponding to sequences in the viral replicase and glycoprotein genes specifically inhibits YHV infection *in vitro* and *in vivo*, suggesting that RNA interference may play a role in the host response to infection.

3.2.7 Transmission mechanisms

YHV can be transmitted horizontally by injection, ingestion of infected tissue, immersion in membrane-filtered tissue extracts, or by co-habitation with infected shrimp (91,92). Transmission has also been demonstrated by injection of

extracts of paste prawns (*Acetes* sp.) collected from infected ponds (93). For GAV, vertical transmission has been shown to occur from both male and female parents, probably by surface contamination or infection of tissue surrounding the fertilised egg (94). The dynamics of YHV infection in ponds have not been studied, but the rapid accumulation of mortalities during disease outbreaks suggests very effective horizontal transmission, probably by cannibalism.

3.2.8 Control and Prevention

No effective vaccines or therapeutics are currently available for the control of YHV and no genetically resistant shrimp stocks have been reported yet. Disease management is primarily through pathogen exclusion by PCR screening of broodstock and /or seed, the application of on-farm biosecurity and sanitary measures, and stress reduction by careful management of water quality during grow-out. (95)

3.3 Cultivation of shrimp viruses

3.3.1 Conventional methods for shrimp virus propagation.

Currently, a small amount of viral stock of a shrimp virus is subjected to amplification by passage through healthy shrimp. The shrimp are pre-screened as negative for both YHV (RT-PCR) and WSSV (PCR). The viral stock in lobster hemolymph media buffer (LHM) is injected into the abdominal muscle of healthy shrimp at 0.1 % body weight. When the shrimp became moribund, hemolymph was drawn in LHM buffer in the ratio 1:10. The supernatant is filtered through 0.2 μm (pore size) membrane and stored in aliquots at -80 ºc.

However, to study its infectivity, one has to rely on *in vivo* bioassay which requires large numbers of animals. Although much new information is available for this virus, many issues remain open, especially those related to interaction between the virus and its host. These studies are difficult due to the lack of shrimp cell lines for production of the virus and for *in vitro* studies.

3.3.2 Primary shrimp cell cultures

A complete understanding of shrimp viruses is dependent upon the development of laboratory techniques for the maintenance and culturing of these viruses and their host cells (96). Attempts were made by various researchers to establish primary cell cultures and continuous cell lines from different organ sources of shrimp (97,98,99) such as the lymphoid (Oka) organ (96,100,101,102,103,104), the heart (103), nerve cord (100,103), gut (100), hepatopancreas (103) and gonads (97, 100, 103, 105). The success of such approaches was limited by two steps which were the medium and the preparation of the cells.

With respect to medium, it can be concluded that Leibovitz's L-15 medium has been the most popular choice for prawn cell culture. M199 or variants of it have also been shown to support prawn cell growth (102,106,107,108). However, Toullec et al. (109) found that M199 was best for the culture of ovarian tissues while Grace's Insect Medium was best for the culture of limb bud regeneration tissue.

Concerning the treatment of the tissues, several techniques are available but all of them appear not to be adapted to every different tissue (110). Among the tissues tested, the lymphoid organ and heart gave the best results which is in agreement with Nadala et al. (100) who reported that the Oka (lymphoid) organ was the best for primary culture. However, Chen et al. and Luedeman and Lightner (97) reported that the ovary was the best tissue source for cell culture. This disagreement might be attributable to the different media and species and age of shrimp used.

3.4 Insect cell cultures

The first insect cell culture was established from pupal ovarian tissue. Since then, over 500 insect cell lines have been successfully established from many insect species representing numerous insect orders and from several different tissue sources. The utility of insect cell lines for protein production has grown from laboratory-scale experimental work to industrial applications (111). These cell lines are used as research tools in virology, in studies of signaling mechanisms to study insect immunity, hemocyte migration, to test hypotheses about gene expression and in screening programs designed to discover new insecticide chemistries. Virology

research is revealing fundamentally new information on virus/host cell interactions (112,113,114,115). Studies in gene expression are uncovering signal transduction pathways that are new to insect science. Research is leading to the development of high-speed screening technologies that are essential in the search for new insect pest management tools. A few insect cell lines are, in routine industrial processes, designed to produce proteins of biomedical significance. Both primary cell cultures and established lines are used in basic biological studies to reveal how insect cells work. In recent years, there is renewed interest in developing new lepidopteran cell lines due to their potential application in biotechnology. The advances made in the genetic engineering technology in the early eighties have helped in the production of new recombinant proteins and genes useful in medicine and agriculture (116,117).

Because of the long generation time of shrimp and the lack of continuous cell lines, work was begun to test the viral accommodation concept using mosquitoes and mosquito cell lines as models (118,119). As with previous studies, it was shown that the densovirus *Aal*DNV could be used to infect and establish persistent infections without CPEs in a subclone of *Ae*. Albopictus C6/36 cells (120). The percentage of cells infected declined with increasing passage number (from an initial 75% to a final steady state of about 20%) as monitored by antibody to the viral coat protein, while the production of *Aal*DNV virions fluctuated cyclically, apparently due to the dynamics of defective interfering vial particle (DIP) production. DIP have long been suggested to interfere with viral production or to compete with infective particles for cell surface receptors (121). The authors suggested (118) that whatever the mechanism, serial passage resulted in a persistent infection that was less severe than the initial acute infection. In addition, the percentage of cells infected could not be increased by superchallenge of the persistently infected culture. These phenomena in cell cultures resembled those for persistent, innocuous viral infections in shrimp populations.

With a mosquito population, it was also possible to mimic results from shrimp in that viral challenge of four successive generations of mosquito survivors resulted in a stepwise increase in survival from 15% to 58% (118,119). Also like shrimp, many of the surviving mosquitoes were infected with the virus but showed no signs of disease and were capable of successfully producing offspring to which they could transmit the virus vertically. An unexpected result of the study was an indication

that the number of defective viral genomes in the surviving mosquito population was considerably higher than that in the viral stock used for the successive generation challenges.

3.4.1 Lepidopteran cell lines

Cell lines are used as tools to grow many entomopathogenic viruses that have potential for use as as biopesticides. These viruses are highly virulent to susceptible insect hosts and are ecofriendly. Many types of viruses infect insects, with the most common belonging to the family Baculoviridae. The most popular invertebrate expression vector system is based on the *Autographa californica* nuclear polyhedrosis virus (AcNPV), an insect baculovirus isolated from the Alfalfa looper that replicates in the nucleus of over 30 lepidopteran insect cell lines. Members of the family baculoviridae, in particular, have been projected as bio-pesticides of the future and are being used in many countries for pest control (122). Baculoviruses have been isolated from the members of Phylum Arthropoda (insects and crustaceans) and no isolations have been reported from vertebrates so far (123). Baculoviruses are extremely virulent to their susceptible hosts and are safe for humans.

Lepidopteran cell lines were primarily being established to propagate insect viruses as a biopesticide for the control of insect pests. Lately, the baculovirus expression vector system combined with insect cell cultures has become more attractive for the expression of many heterologous proteins than other systems *viz*., bacterial, yeast, vertebrate viruses, *etc*., due to their unique characteristics. This technology is also being used in the construction of recombinant baculoviruses for use as biopesticides that offer comparatively faster killing of insect pests than by wild type baculoviruses (124,125). The most widely used lepidopteran cells for the baculovirus expression vector system (BEVS) are Sf9 and Sf21 cell lines isolated from ovarian tissue of the fall army worm, *Spodoptera frugiperda*, and the High Five cell line, designated BTITn-5B1-4, originally established from *Trichoplusia ni* embryonic tissue. Sf9 cells are a sub-clone of Sf21 cells and were selected for their faster growth rate and higher cell densities than Sf21 cells. *Spodoptera frugiperda* cells, either Sf9 or Sf21 are preferred for virus expansion. Sf21 cells can compare favorably, in terms of

heterologous protein expression, to both High Fives and the Sf9 cell lines in certain situations (126).

CHAPTER IV
MATERIALS AND METHODS

4.1 Source of viruses

The virus stocks used in this study were prepared from hemolymph of infected shrimp and kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Co. Ltd. The WSSV and YHV stocks contained $2.0x10^7$ and $5.0x10^7$ viral genome copies/µl, respectively. The virulence of the stocks was confirmed by challenging healthy *P. vannamei* juvenile shrimp (approximately 7-10 g). The mortality of shrimp began 2 days post challenge. The moribund shrimp were tested for YHV or WSSV by RT-PCR and PCR assay, respectively. The stocks were kept at -80 ºC. The working seed for WSSV and YHV were diluted with Sf-900II medium.

4.2 Insect cells

Sf9 cells are a clonal isolate, derived from *Spodoptera frugiperda* IPLB-Sf21-AE (Fall Armyworm) (Gibco Invitrogen). The cells were provided by Centex Shrimp, Faculty of science, Mahidol University. The Sf9 cells were cultured and maintained in 1X Sf-900 II SFM, serum free medium (Gibco Invitrogen) containing 1% of 100X antibiotic-antimycotic (Gibco Invitrogen). The Sf9 cells were sub-cultured every 3 days at 1:3 split ratio.

4.3 Experimental animals

Healthy *P. vannamei* shrimp used in this study were collected from shrimp farms of Charoen Pokphand Co.Ltd. Shrimp of 10-15 g body weight were maintained in 200 litre fiberglass tanks with air-lift biological filters at room temperature (27–30 ºC) with salinity between 15 and 20 ppt. These shrimp were fed with artificial pelleted

feed (CP feed, Thailand) and were kept in tank for 3 days for acclimatization before tests were carried out.

4.4 Persistent infections

The Sf9 Cells were maintained in 1X Sf-900 II SFM, serum free medium, (Gibco Invitrogen) containing 1% of 100X antibiotic-antimycotic (Gibco Invitrogen). Monolayers were prepared within 24 hours before being inoculated with YHV or WSSV. The WSSV and YHV stocks at 2.0 x 10^7 and 5.0x10^7 viral genome copies/µl (as determined by real-time PCR) were diluted 1000 times as working solutions. The WSSV working stock was used to challenge monolayers of Sf9 cells (approximately 1 x 10^6 cells) at an approximate multiplicity of infection (MOI) of 1 in a 6-well plate (Costar, Corning) by incubating on a shaker at 28 ºC for 2 hours. The virus solution was removed by washing 3 times with PBS before 2 ml fresh, sterile medium was added followed by incubation at 28 ºC. The cells were sub-cultured every 2–3 days by split-passage.

4.5 Viral genome copy numbers

Sf-9 cells were mixed with 500 µl of lysis solution followed by DNA extraction using the protocol of a genomic DNA purification kit. DNA pellets were dissolved in 50 µl of sterile deionized water by gentle vortexing. DNA was measured by spectrophotometry. Diluted DNA solutions (about 200 ng/µl) were used for PCR reactions. The sequences of PCR primers and the TaqMan probe used for the detection of WSSV were selected from the 1,447 STS target region in the WSSV genomic sequence in GenBank AF440570 (Yang et al., 2001). The primers (WSSV1; nt259851–259867 and WSSV2; nt 259906–259927) generated a 77 bp-amplicon. The TaqMan probe starting from nt 259870 and running to nt 259889 was synthesized and labeled with the fluorescent dyes 5-carboxyfluoroscein (FAM) at the 5′ end and N,N,N′,N′-tetramethy-6-carboxyrhodamine (TAMRA) at the 3′end (PE Applied Biosystems, Foster City, CA,USA). The TaqMan assay was carried out using a Taq-Man Universal PCR Master Mix containing AmpliTaq Gold DNA polymerase,

AmpErase uracil-N-glycosylase (UNG), dNTPs with dUTP and optimized buffer components (PE Applied Biosystems, Foster City, CA, USA). Samples of diluted STS stock to give 10^6, 10^5, 10^4, 10^3, 10^2 STS copies were added to PCR mixtures containing 0.4 μM of each primer and 0.2 μM of TaqMan probe in a final volume of 20 μl. Two replicates for each concentration were run. Amplification was preformed for 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 50 °C and 1 min at 60 °C. After PCR amplification, a baseline and threshold were defined using the sequencer computer program (ABI Prism 7000 Sequence detection software, AB Applied Biosystems, Foster City, CA, USA).

4.6 Immunohistochemistry and confocal microscopy

Infected cells were seeded on cover glasses and left to attach for 2 hours before fixation with 4% paraformaldehyde in PBS for 15 min and washing twice with PBS. They were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 10% normal bovine serum. After incubation with primary antibody Y-19 against YHV nucleocapsid protein p20 (Sithigorngul et al., 2002) or VP-28 against WSSV envelope protein VP28 (Sritunyalucksanaet al., 2006) for 1 hour at 37ºc, cells were washed with PBS-T. The stained cells were incubated with GAM Alexa Flour 486 (Molecular Probes, Invitrogen Corp.) (1:500) for 30 min and washed with PBS-T for 5 min. This was followed by a second fixation in 4% formaldehyde in PBS for 15 min and counterstaining with TO-Pro 3 (Molecular Probes, Invitrogen Crop.) (1:500) nuclear stain for 1 hour before a final wash with PBS. The cells were then examined by confocal microscopy. The stained naïve SF-9 cells passaged in parallel with the infected cells were used as a negative control.

4.7 Shrimp challenge tests

Shrimp were challenged with viral preparations from either the supernates of persistently infected cell cultures or from homogenized persistently infected cells. For cell homogenization, whole cells were collected and washed with PBS before being homogenized using a sonicator. The homogenate was injected (100 μl) at the

average concentration of 1×10^6 Sf9 cells (for WSSV) per shrimp. For culture media challenge tests, 5 ml of culture medium from persistently infected cultures, day-2 of cultivation was filtered through a 5 kDa protein membrane (Amicon Ultra, Millipore) until only 500 µl remained (i.e., 10 x concentration). This filtrate was used to inject shrimp. Filtrate (100 µl) was injected intramuscularly into the 5th abdominal segment (*Penaeus vannamei* of approximately 10-15 g each). These infected shrimp were fed twice daily with a commercial shrimp feed and excess feed was removed daily. Shrimp used in this experiment were tested for the absence of WSSV using a commercial PCR detection kit for WSSV according to the manufacturer's instructions (IQ2000 detection system, Farming Intelligene, Taiwan).

4.8 Histological analysis of inoculated shrimp

Hemolymph from inoculated shrimp was collected in anticoagulant at day 2 post challenge. Cells were pelleted by centrifugation at 1500 rpm for 5 min followed by washing with medium. Hemocytes were cultured in fresh Sf-900 II SFM serum free medium at a ratio of 1:1. After one day of cultivation, cells were fixed with 4% paraformaldehyde and subjected to appropriate immuno-staining as described above. In addition to shrimp cells, whole moribund shrimp from the challenge tests were fixed with Davidson's fixative and subjected for histological examination with H&E staining as described by Bell and Lightner (1988). Tissues were observed for typical signs of WSSV and YHV infection (Lightner 1996).

4.9 Electron microscopy

Infected insect cells were fixed with 4% paraformaldehyde, washed twice with PBS and collected in a microtube by centrifugation at 1,500 rpm for 5 min. Cells were postfixed in 1% OsO_4 in 0.1 M PBS for 1 h before being subjected to centrifugation in order to remove fixative and washed with 0.1 M PBS. After discarding the supernatant solution, cells were transferred to filter paper to remove excess water before being embedded into 2% melted agar. The excess solidified agar was removed. The cube of cells was dehydrated in a graded ethanol series from 50%

to absolute ethanol with 15 min for each step. This was followed by two 15 minute transfers to propylene oxide and then 50 and 75% Epon (Embed-812, EMS) in propylene oxide each for 60 min. Finally the block was transferred to 100% Epon in an embedding capsule (70000, EMS) and allowed to polymerize at 70ºc for a few days. Cells were cut at 1 μm thickness and stained with toluidene blue or H&E for light microscopy and sections at 30 nm thickness were stained with uranyl acetate and lead citrate for transmission electron microscopy.

CHAPTER V
RESULTS

5.1 Replication pattern of shrimp viruses in Sf-9 cells

To investigate whether or not shrimp viruses could replicate in Sf-9 cells, Sf-9 cell cultures were exposed to WSSV at a multiplicity of infection (MOI) of 1 and 5. The supernatant solution and exposed cells were harvested on days 2, 4 and 6 post-exposure.

No viral genomic material was detected in the supernatant solution at either MOI. In contras (Fig 5.1), PCR testing of extracts from whole cells revealed that intracellular viral genome synthesis was prominent within 2 days post exposure. The genome synthesis peaked on day 4 at 3.74 x 10^6copies/µl for MOI 1 and at 2.08 x 10^7copies/µl for MOI 5 (Fig. 5.1).

Despite the high replication level of WSSV, the infected Sf-9cells showed no cytopathic effects or other visible signs of viral infection. The data indicated that WSSV genomic material could replicate in Sf-9 cells. Therefore, accommodation of YHV and WSSV by Sf-9 cells was investigated further.

5.2 Establishment of YHV/WSSV persistent infections

In order to answer the question whether or not Sf-9 cells could serve as a hosts for continuous maintenance of YHV and WSSV in passaged laboratory cultures, Sf-9 cells were inoculated with YHV or WSSV at the MOI of 1 and then sub-cultured every 2-3 days by split-passage at the ratio of 1:3. These cultures were sub-passaged up to 150 times. The state of infection, cell morphology and biological characteristics of the cells from these cultures were characterized using confocal microscopy for normal phase contrast viewing and for immunocytochemistry with antibodies against antigens of the respective viruses.

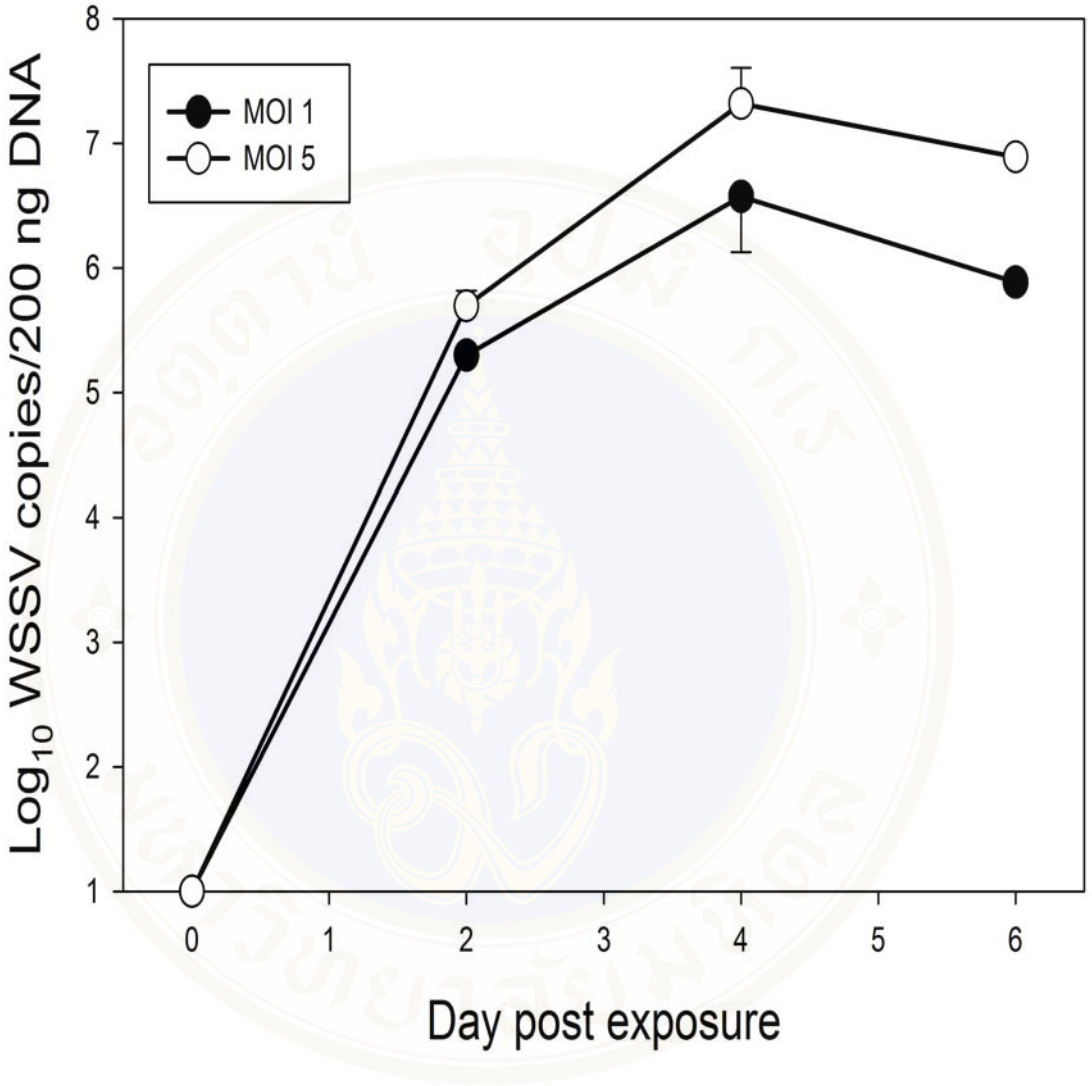

Figure.5.1. Detection of WSSV genome synthesis in infected Sf-9 cells exposed to WSSV at MOI 1 and 5. On days 2, 4 and 6 post-challenge, infected cells were harvested and numbers of viral DNA copies were determined by quantitative PCR.

5.3 Persistent maintenance of WSSV in Sf9 cells: morphological characterization

To determine the distribution of WSSV in the exposed cells and to investigate the impact of persistent WSSV infection on Sf-9 morphology, cultures from passage 1 to passage 150 were stained with antibody against VP28 and were then examined by confocal microscopy. Immunocytochemical analysis using antibodies against envelope protein (VP28) revealed that WSSV-exposed Sf-9 cultures became 100% positive for the WSSV VP28 antigen from the first passage onward (Fig. 5.2a–c), even though the cells showed no visible signs of viral infection by phase contrast microscopy (Fig.5.3a–c). Numbers of positive cells and fluorescence intensity were somewhat lower at initial challenge but increased by passage 2 and remained intense thereafter, suggesting that the cells produced the antigens in an adaptive manner. At the end of the thesis work, the cells had been passaged more than 150 times with undiminished fluorescence and with all cells positive. Despite the intense fluorescence in late passage cultures, morphology of Sf9 cells (Fig.5.3a–c) by phase contrast microscopy did not differ greatly from that of the respective naïve, unchallenged cells. Nor were any WSSV virions or recognizable pre-virion parts seen in the nuclei of the immunopositive Sf9 cells (Fig. 5.4) by transmission electron microscopy (TEM), although numerous cytoplasmic vesicles and inclusions were present that were absent from naïve cells.

5.4 Persistent maintenance of YHV in Sf9 cells: morphological characterization

Similar investigations were performed with Sf-9 cells exposed to YHV. With the YHV-exposed Sf9 cells, immunohistochemical analysis by confocal microscopy using antibodies against nucleocapsid protein (p20) revealed 100% expression of the relevant viral antigen from the first passage (Fig. 5.5a). The fluorescence occurred as discrete spots in the cell cytoplasm. As with WSSV above, numbers of positive cells and fluorescence intensity were high from early exposure and remained high thereafter (Fig. 5.5 b, c). Despite the intense fluorescence, morphology of cells by phase contrast microscopy did not differ from that of naïve,

unchallenged cells (Figs.5.3). By TEM, no typical YHV virions or nucleocapsids were seen in the immunopositive the Sf9 cells (Fig. 5.6) although they differed from naïve cells in showing numerous unusual cytoplasmic inclusions and vesicles absent from the latter.

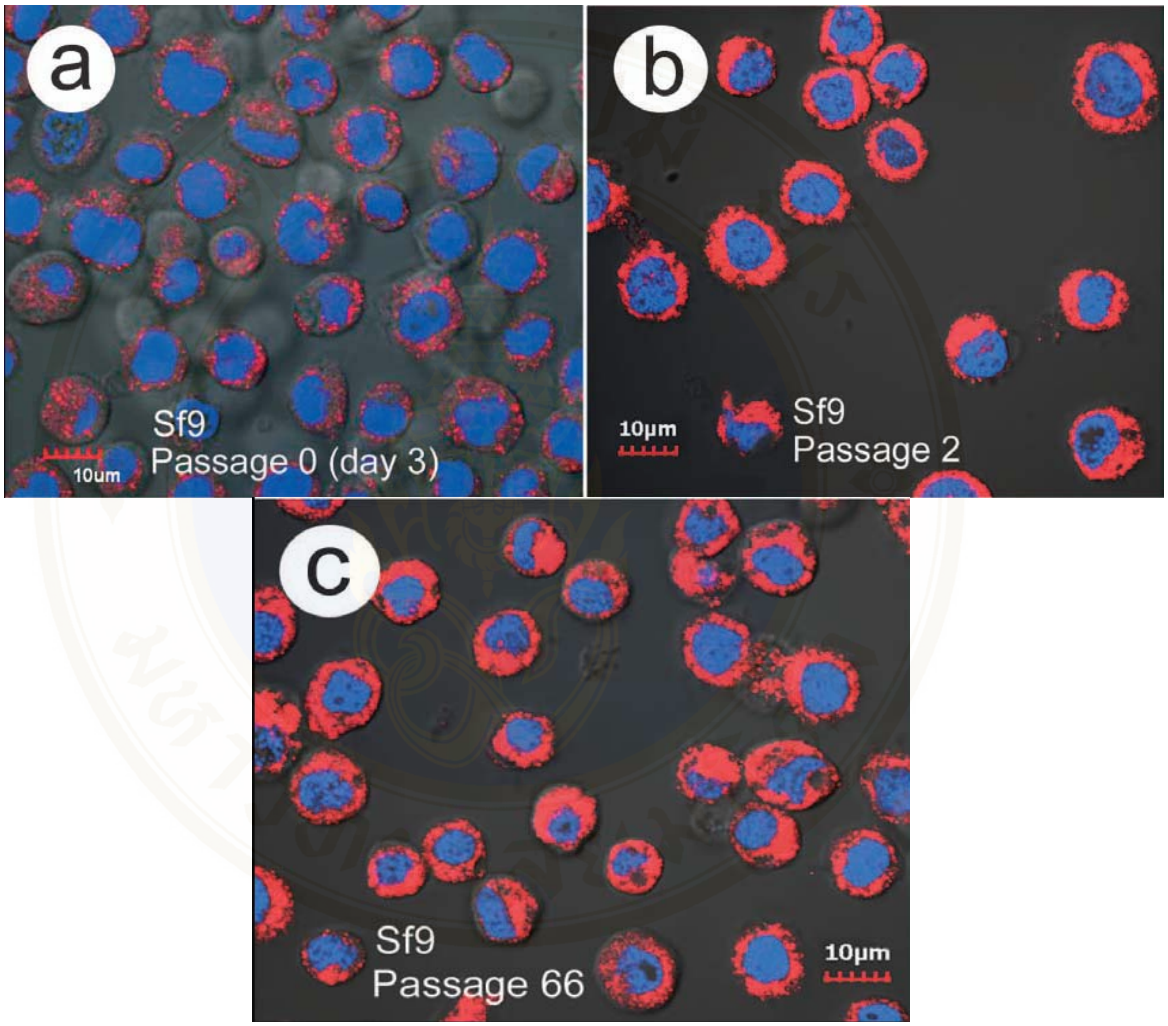

Fig.5.2. Examples of confocal microscopy images of Sf9 cells challenged with WSSV and stained with TOPO-3 for the nuclei (blue) and anti-VP28 of WSSV (red). (a) Initial challenge culture at day 3 post challenge showing positive immunofluorescence as discrete spots in the cell cytoplasm. (b) Cells from passage 2 showing more uniform immunopositive cytoplasm. (c) Cells from passage 66 that resemble those from passage 2.

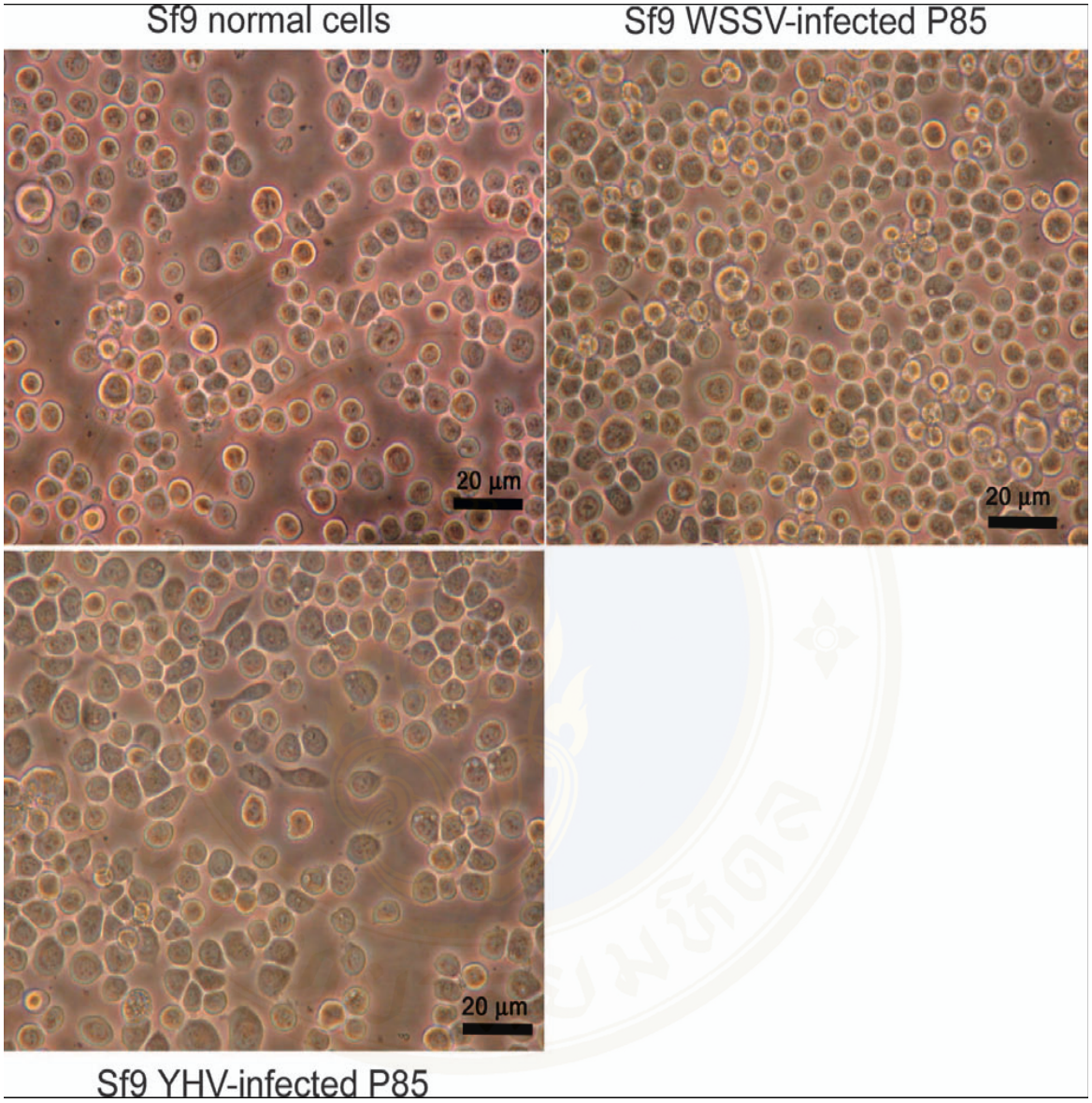

Fig.5.3. Phase contrast photomicrographs of Sf9 cells persistently infected with WSSV and YHV.

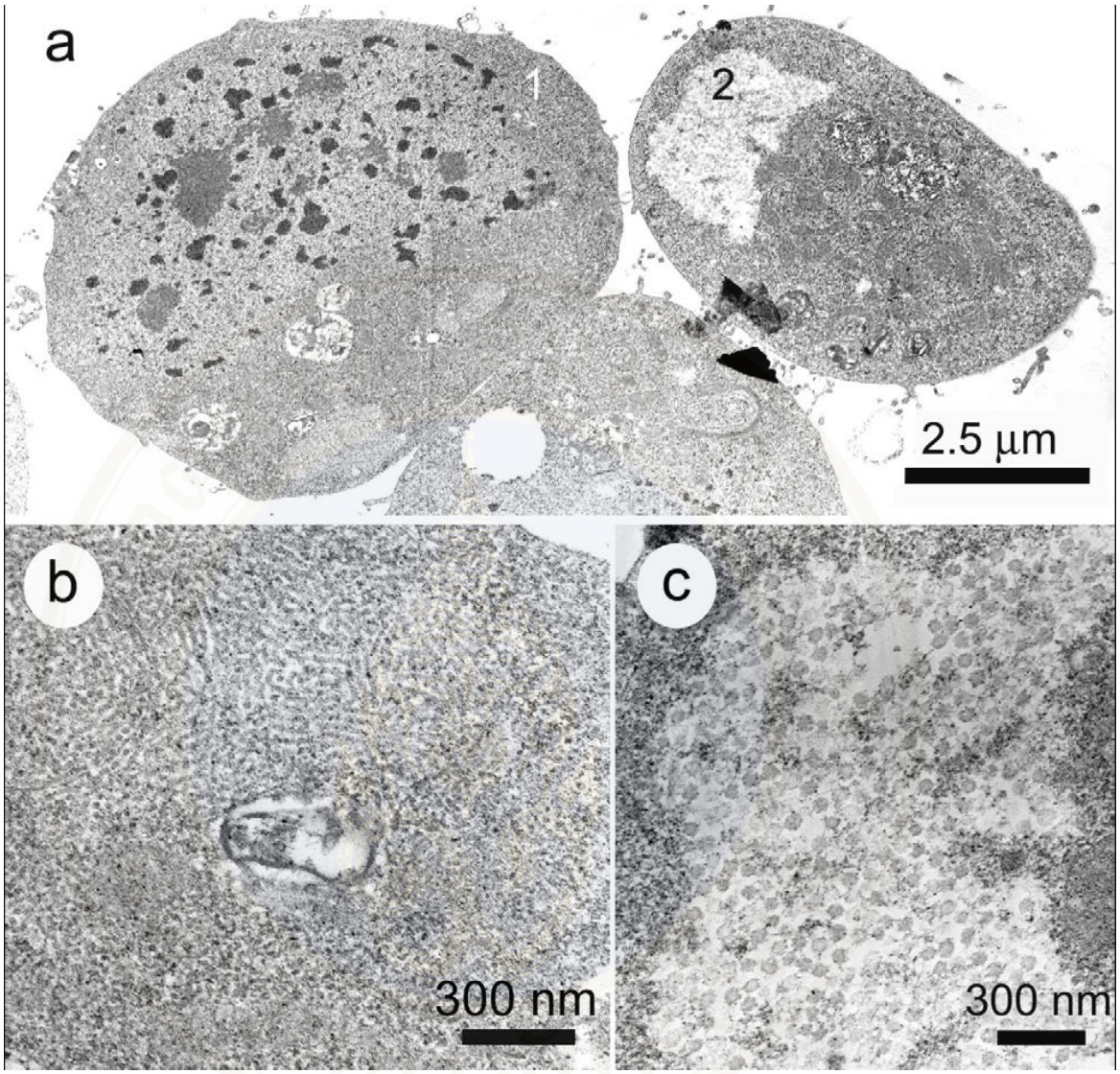

Fig.5.4. Transmssion electron microgroaphs of Sf9 cells immunopositive for WSSV.(a) Low magnification micrograph showing cells with unusual cytoplasmic inclusions (1, 2) but no evidence of WSSV virions in the nucleus. (b) High magnification micrograph of the area marked 1 in (a) and showing aggregation of unusual, short filamentous structures. (c) High magnification of the area marked 2 in (a) showing unusual round structures. The structures shown in b and c do not resemble virogenic material previously described for WSSV.

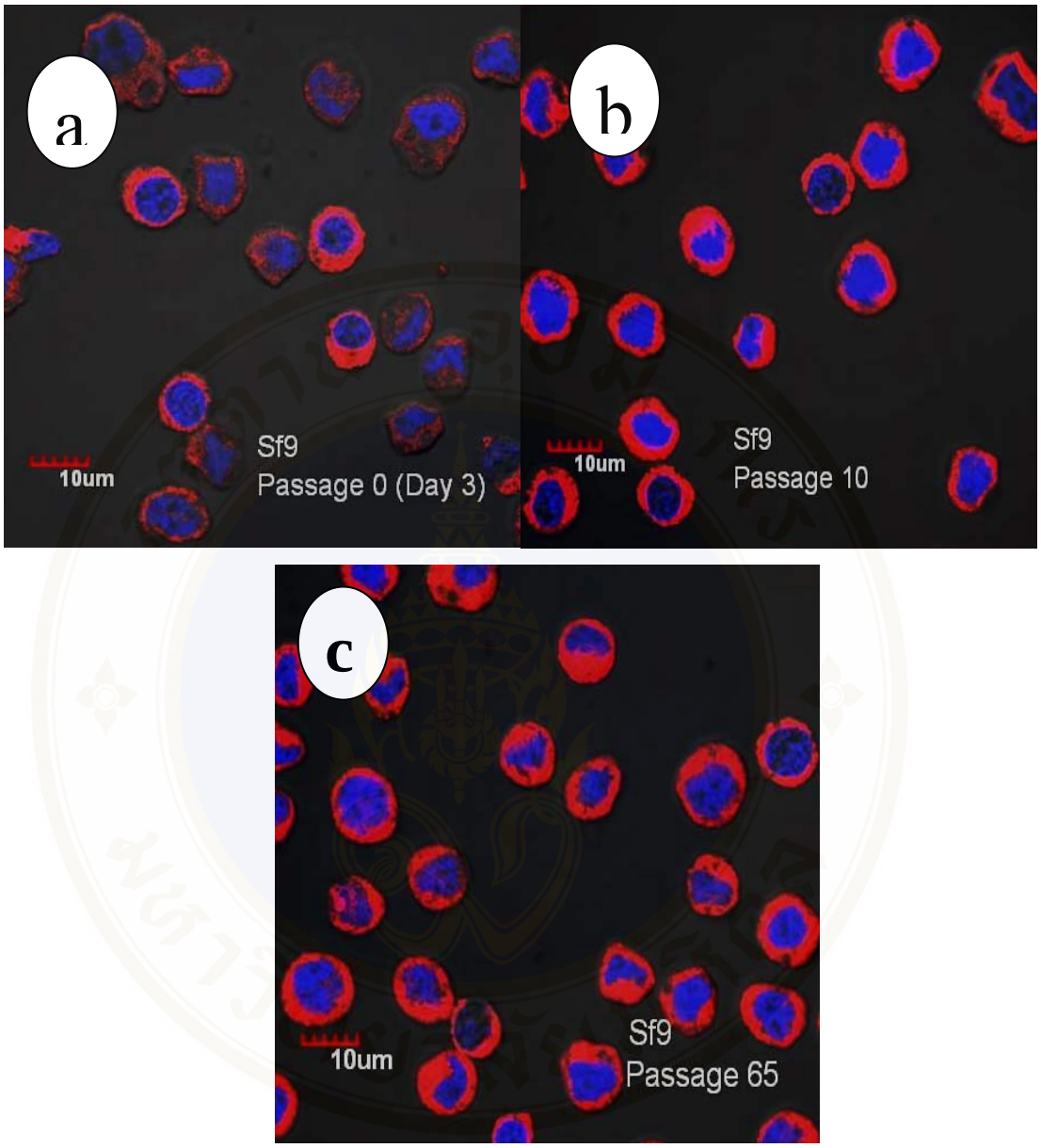

Fig.5.5. Examples of confocal microscopy images of insect cells challenged with YHV and stained with TOPO-3 for the nuclei (blue) and anti-p20 of YHV (red).(a) Initial challenge culture at day 3 post challenge showing positive immunofluorescence as discrete spots in the cell cytoplasm. (b) Cells from passage 10 showing more uniform immunopositive cytoplasm.(c) Cells from passage 65 that resemble those from passage10.

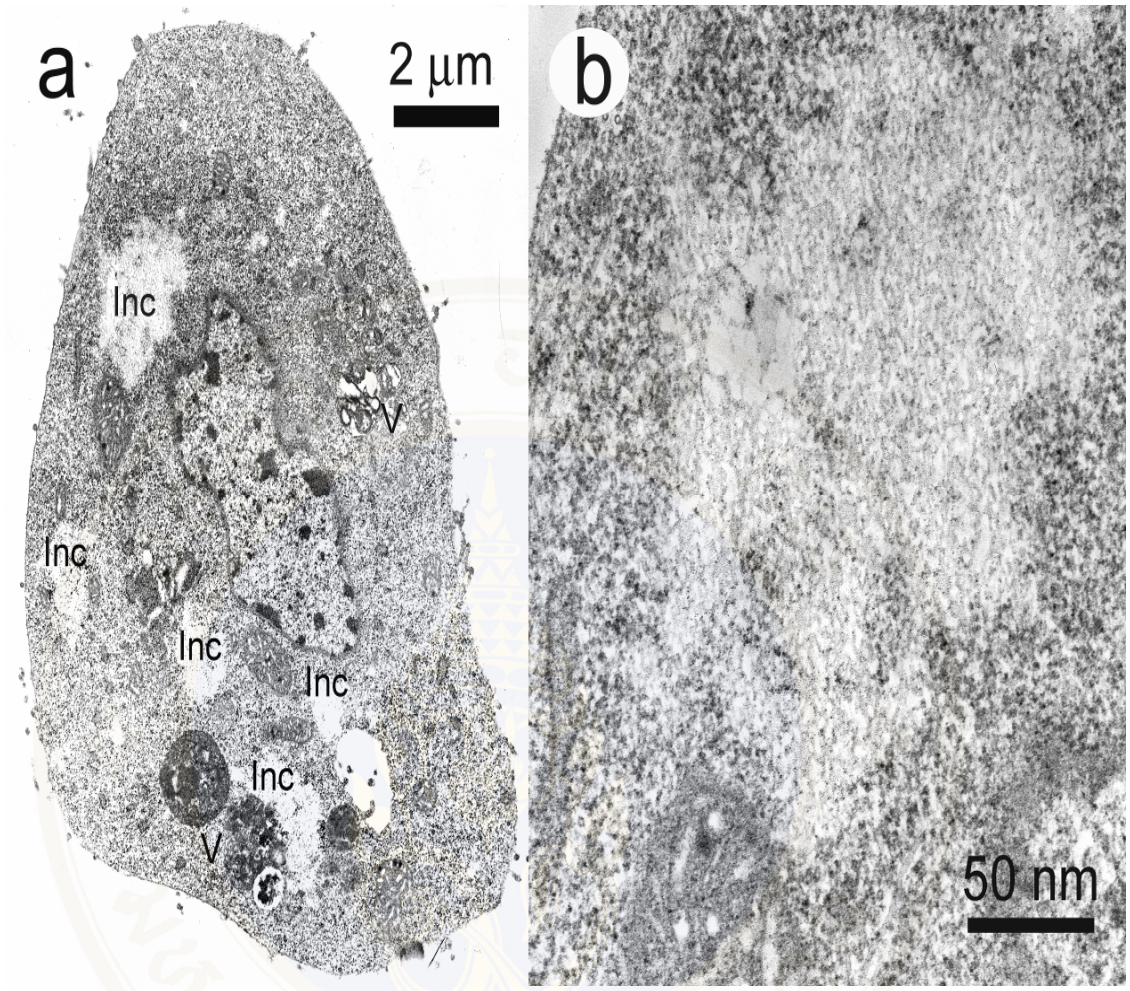

Figure5.6. Transmission electron micrographs of Sf9 cells persistently infected with YHV. (a) low magnification micrograph showing a cell containing unusual inclusions (Inc) together with numerous vacuoles (V). (b) High magnification of the inclusion (Inc) at the upper left position in (a) showing unusual structures that do not resemble virogenic material previously reported for YHV.

5.5 Infectious WSSV produced from persistently infected Sf-9 cells.

To investigate whether viruses produced from persistently infected cultures still express characteristic WSSV virulence for shrimp or not, we performed shrimp challenge tests. *P. vannamei*, 10-15 g each, were injected with concentrated culture medium harvested from culture passage 2 and passage 65 or injected with cell homogenates obtained from passages 2 and 65. The injection procedure is described in the materials and methods section. Hemolymph was collected in anticoagulant on days 2 and 7 post injection for immunocytochemistry using a confocal microscope. Moribund shrimp and surviving shrimp on the termination day were harvested and fixed with Davidson's fixative and subjected to histological examination (Table 1).

	Methods			
	Immunohistochemistry		**Histology**	
Treatment	**Day 2**	**Day 7**	**Moribund**	**Termination day**
Negative control	Negative	Negative	ND	Negative
Positive control	Positive (2/2)	ND	Positive (2/2)	ND
whole cells P2	Positive (2/2)	ND	Positive (3/3)	ND
supernatant P2	Negative (2/2)	Positive (2/2)	Positive (1/1)	Positive (1/1)
whole cells P65	Negative (2/2)	Positive (2/2)	Negative (1/1)	Negative(2/2)
supernatant P65	Negative (2/2)	Negative (2/2)	ND	Negative (2/2)

[1] ND = not done

[2] **(-/-)** = amount of result / amount of samples

Table.1. Results of confocal immuno-staning and histology examination for shrimp challenged with preparations from insect cells immunopositive for WSSV.

The cumulative mortality results for the WSSV challenge tests are shown in Fig.5.7. The mortality started in the experimental shrimp from day 2 post challenge. The highest percent cumulative mortality (90-100%) resulted from the positive control and from homogenates or supernatant solution of infected Sf9 cells at passage 2. Mortality from WSSV was confirmed by observation of typical WSSV histopathology (i.e., pathognomonic lesions of WSSV) (Fig.5.8a). By contrast, challenge with similar preparations from passage 65 gave low mortality rates (16.7-40%) and WSSV typical hypertrophied nuclei were not detected in H&E tissue sections. (Fig.5.8b). However, at day 7 post challenge, hemocytes of shrimp injected with homogenates of infected Sf-9 cells at passage 65 gave positive immunofluorescence for the WSSV envelope protein VP28 (Fig. 5.8c). Treatment differences were analysed using one way analysis of variance (SignaStat software version 3.5). The mortality from homogenates of infected Sf9 cells at passage 65 were significantly higher than those of the negative control ($p< 0.05$). But the mortality from the solution of infected cells at passage 65 was not significantly different from that of the negative control. Since the moribund shrimp in the group challenged with cell homogenates from passage 65 did not show typical WSSV lesions, there was some uncertainty as to the reason for the mortality in that test group.

Treatment / Day	Negative mean	Positive mean	Whole65 mean	Sup P65 mean	WholeP2 mean	SupP2 mean
D0	0.0	0.0	0.0	0.0	0.0	0.0
D1	0.0	1.0	0.0	0.0	4.5	3.5
D2	0.0	3.0	1.0	0.0	7.0	6.0
D3	0.0	8.0	1.5	1.5	10.5	9.5
D4	0.0	11.0	4.0	2.0	13.5	11.0
D5	1.0	13.5	6.0	2.5	14.5	11.5
D6	1.0	15.0	6.0	2.5	15.0	13.5
D7	1.5	15.0	6.0	2.5	15.0	13.5

Table.2. Mean cumulative number of shrimp dead in each day after injection of whole cell homogenates or concentrated supernatant solution from insect cell cultures immunopositive for WSSV.

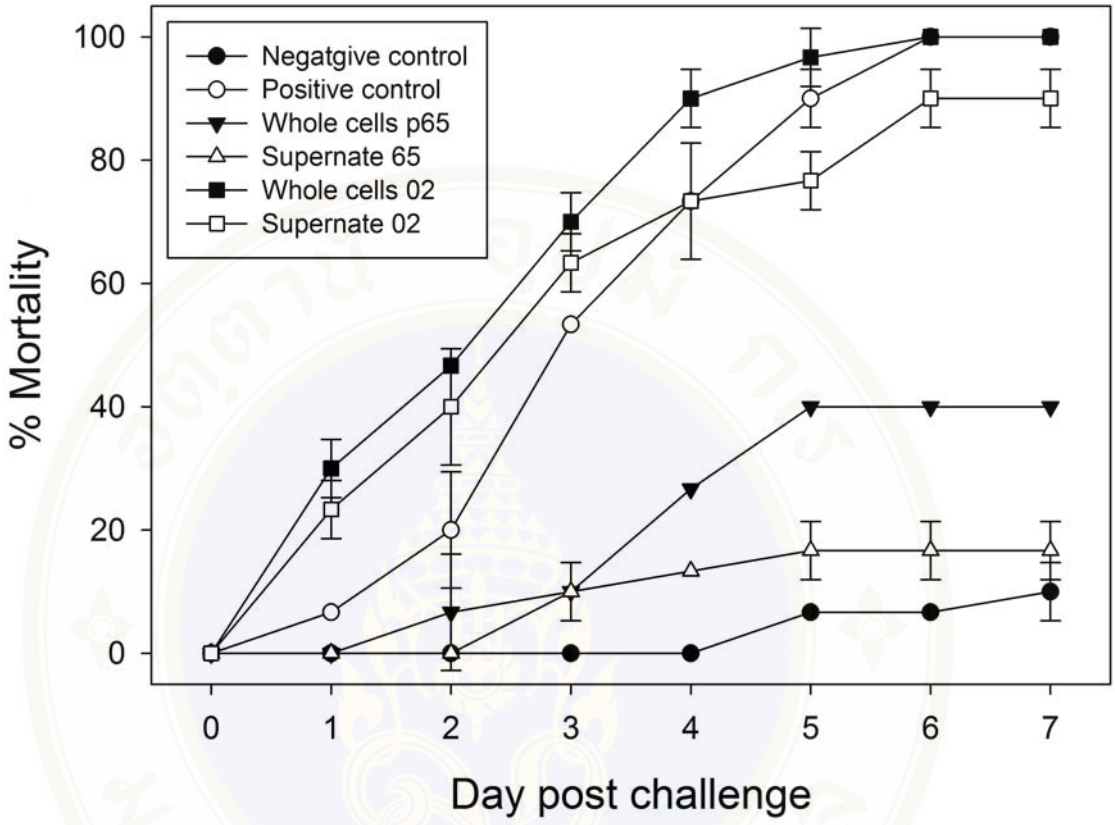

Figure.5.7 Cumulative mortalities of *P.vannamei* after challenged with inocula derived from Sf-9 cells immunopositive for WSSV. Mean cumulative mortality for the positive control, whole cells from P2 and supernatant from P2 were not significantly different, while mortality from whole cells at P65 was significantly different from that of both the negative control and supernatant from P65. Mortality for the latter group and the negative control group were not significantly different.

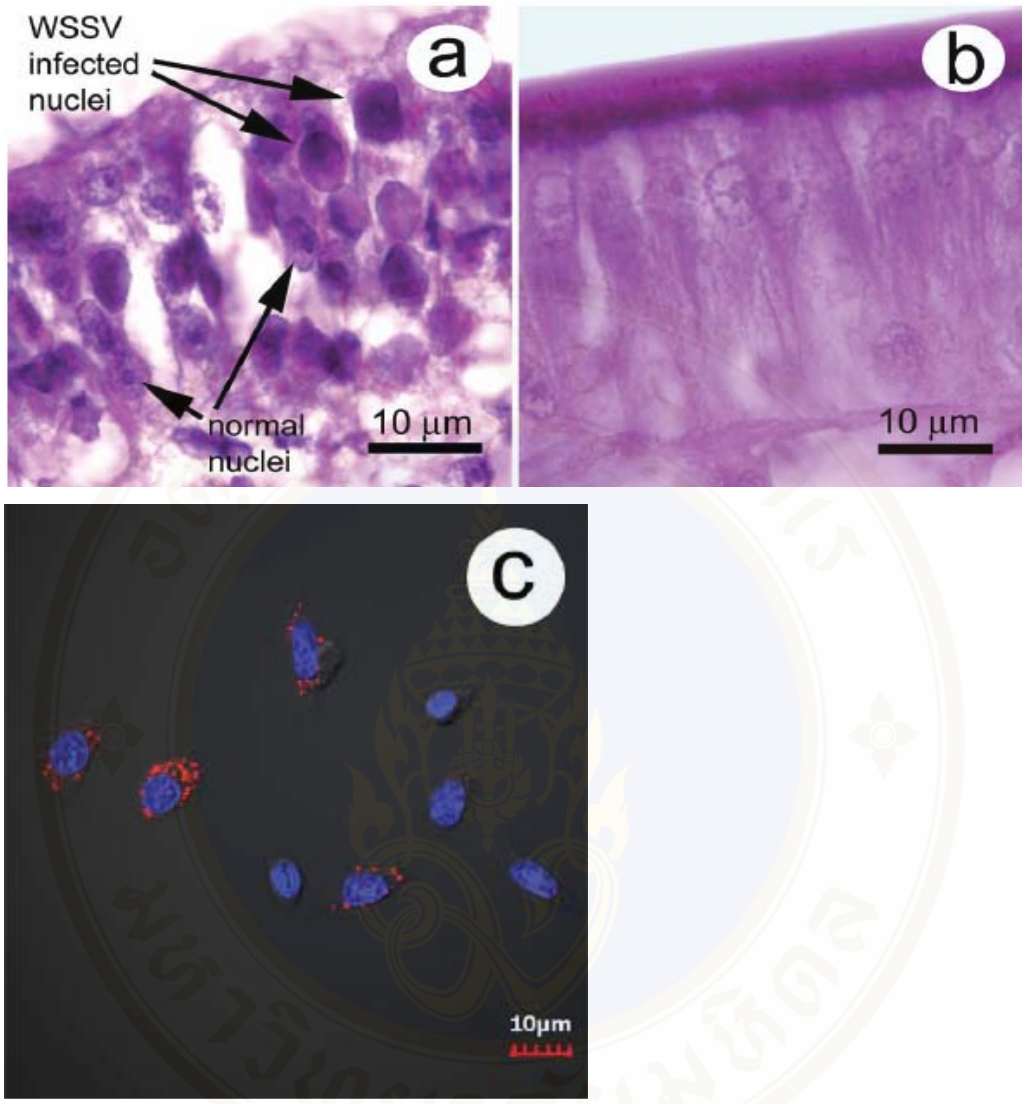

Figure.5.8. Histopathology and immunocytohemical analysis of shrimp challenged with cell and supernatant preparations from Sf9 insect cell cultures immunopositive for WSSV. (a) H&E stained sub-cuticular epithelium of *Penaeus vannamei* challenged with homogenate from WSSV-infected Sf9 cells at passage 2 and showing enlarged, basophilic nuclei pathognomonic for WSSV infection. (b) H&E stained subcuticular epithelium from *P. vannamei* challenged with homogenate from WSSV-infected Sf9 cells at passage 65 and showing normal histology. (c) Hemocytes from the shrimp specimen in (b) showing a positive immunohistochemical reaction (red) for WSSV antigen.

CHAPTER VI
DISCUSSION

Shrimp virus propagation and persistent infection in insect cell lines

Stable, persistent infections of 2 very different shrimp viruses were produced easily in Sf9 insect cells by serial split passage, suggesting that propagation and antigen expression occurred by non-specific processes that might work with other shrimp viruses and other insect cell lines. The mechanism by which viral antigen expression occurred was not studied at the molecular level. However, the facts that antigens were expressed in 100% of the passaged cells for over 100 passages suggested that viral genes were persistently replicated and maintained. Certainly they must have been replicated in order for 100% infection of cells to be maintained for over 100 serial passages. Whether the genes were replicated and maintained as intact viral genomic material or in some host-associated cDNA form was not determined, but we cannot exclude the possibility that the whole viral genomes or perhaps modified forms of them were maintained, as has been reported for insect viruses in stable, persistent, single and dual infections of insect cell cultures (127).

It was surprising that transmission of the shrimp viruses or portions of them occurred so easily in these insect cells, since the originating insects (lepidopteran) are not known to be infected with WSSV and YHV. As such, it would seem unlikely that the cells would have specific receptors for these viruses. Also, given the absence of even partial forms of WSSV and YHV in the antigen-positive insect cells by TEM, it would seem unlikely that maintenance of the antigens in the cell cultures resulted from export and re-entry of genomic material via WSSV and YHV viral particles. It seems more likely that gene transmission occurred by sub-division of the relevant genes between mother and daughter cells during cell division. Alternatively, it could have occurred in whole or in part by non-specific endocytic processes. Endocytosis might also account for the introduction of viral genes during the initial shrimp-virus challenge. Answers to these questions must be left to further

research. Even though we currently do not know the modes of initial infection and maintenance during passaging, the process for obtaining the stable, antigen-positive cultures can be put to good practical use.

The results are similar to those described previously for stable, single and dual insect virus cultures (127). A major difference was that the culture supernatant solution from shrimp virus cultures was not infectious for shrimp. This suggested that viral maintenance in the insect cells occurred by transmission to daughter cells during cell division and/or by non-specific endocytic processes and not via specific receptors.

It is possible that the non-specific capacity for viral propogation in insect cell cultures was previously overlooked because of the general lack of any obvious morphological change resulting from infection (i.e., no cytopathic effects) and because of the lack of detectable virions in culture supernatants. We ourselves erred in this manner in our earlier attempts to propagate shrimp viruses in insect cells (unpublished). If this process does turn out to be generic for a wide spectrum of viruses, it may also prove useful for viral isolation.

Split passaging of whole insect cells may possibly be useful also for non-specific propagation of viruses from other invertebrates and perhaps even from vertebrates and plants. It is well known that arboviruses shuttle between insects and vertebrate hosts and that some plant viruses shuttle between insect and plant hosts (128). This indicates that insect cells are capable of supporting propagation of viruses that infect cells in greatly divergent phylogenetic lines (e.g., vertebrates and plants). Although a single insect cell type has not previously been shown to support propagation of both plant and vertebrate viruses, our results suggest that it may be possible by split passaging of whole cells.

Infectivity of shrimp viruses from persistent cultures

Injection challenge of experimental white shrimp *Penaeus (Litopeaneus) vanamei* with extracts from the culture supernatant solution or homogenates of infected Sf9 cells from passage 2 resulted in typical WSSV mortality, confirmed by histological analysis showing pathognomonic WSSV lesions. Since no recognizable WSSV or YHV virion structures were seen in TEM of infected cells, it is possible that genes important for virion assembly may be present in Sf-9 but be unexpressed or

poorly expressed. This was supported by recent work indicating that a structural gene of WSSV was not expressed in Sf-9 cells infected with WSSV (129). In addition, homogenates from cells at passage 2 did cause normal WSSV infections and mortality in histologically positive shrimp, indicating that the viral genome was intact, at least at that passage. Curiously, shrimp injected with the 10X concentrated supernatant from passage 2 (P2) cultures also showed high mortality and typical WSSV lesions, despite the fact that the supernatant solution had previously tested negative for WSSV by real-time PCR. These results suggested that the supernatant solution from the P2 cultures did contain a low quantity of WSSV virions and that they could cause disease in shrimp once concentrated by membrane filtration.

In contrast to the P2 cultures, challenge with supernatant solutions from passage 65 (P65) did not cause significant shrimp mortality or give any indications of WSSV infection by immunohistochemistry or histology. On the other hand, injection of shrimp with whole cell homogenates from P65 did result in significant shrimp mortality (40%) when compared to the negative control. However, the moribund shrimp did not show typical WSSV lesions characteristic of shrimp dying from white spot disease. At the same time, they did show hemocytes immunopositive for WSSV envelope protein VP28. These results suggested that the shrimp challenged with the P65 homogenates may have been infected with an attenuated form of WSSV that developed during the long term passage of the WSSV immunopositive insect cells. This phenomenon is known to occur with other viruses (130). If so, our results suggest that an attenuated form of WSSV can still cause mortality in shrimp despite the inability to produce typical lesions. This might be a direct effect of the virus or an indirect effect that predisposes the shrimp to death by a different pathogen, as has been proposed for some relatively tolerant mud crab species infected with WSSV (131). It is not yet known whether the surviving shrimp from these challenges with high passages of insect cells immunopositive for WSSV would be protected from a subsequent lethal-dose challenge with a respective virulent stock preparation of WSSV. Such protection has been reported for shrimp pre-treated with heterologously expressed WSSV envelope protein VP28 (78,132,133) or inactivated WSSV (134). If so, insect cell cultures might find additional application in the production of living attenuated viruses for protection against viral pathogens.

In a similar study with YHV, (135) it was shown that shrimp injected with culture supernatants from C6/36 mosquito cells immunopositive for YHV could not be infected. However, shrimp injection-challenged with whole-cell homogenates from Passage 5 (early-passage) of such cultures died with histological and clinical signs typical for yellow head disease (YHD), while homogenates of mock-passaged cells did not. By contrast, shrimp challenged with cell homogenates of late-passage cultures became infected with YHV, but survived, suggesting that YHV attenuation had occurred during its long-term serial passage in insect cells. Again, it is not yet known whether surviving shrimp from such a challenge would survive subsequent challenge with a lethal dose of YHV.

Overall, the results of this work and those from work done subsequently with YHV in C6/36 cells have shown that insect cells can support the replication of intact genomic material of WSSV and YHV at low passage numbers, since homogenates from these cultures can cause typical diseases of these viruses from low passage numbers. Such cultures have also been used to successfully study interactions between shrimp and viral genes *in vitro* (136). The nature of shrimp viral antigen persistence in high passage cultures of insect cells remains to be explored, as does the possibility of using such cultures to prepare protective vaccine-like reagents for protection of shrimp against WSSV and YHV.

CHAPTER VII
CONCLUSIONS

The fact that we have been able to achieve stable, persistent expression of antigens from two very different shrimp viruses in Sf-9 insect cell lines by serial split passaging suggests that antigen expression occurred by non-specific processes that might work with other shrimp viruses and other insect cell lines. The mechanism by which viral antigen expression occurred was not studied at the molecular level.

In summary, the work revealed that WSSV and YHV could not propagated successfully by serial split passaging of lepidopteran cells although the cells were immortally immunopositive for viral antigens. The reason for the absence of complete virions by TEM was probably due to the lack of expression of key viral proteins necessary for virion formation. Despite the lack of virions, the complete viral genome was clearly propagated for low passage numbers since homogenates of whole cells were capable of transmitting viral infections to challenged shrimp. By contrast, homogenates from high passage numbers resulted in shrimp infections by not mortality. The insect cells positive for some antigens of WSSV and YHV could be passaged indefinitely and could also be stored at -80^{o}C and revived at will. Such immortal cells may be used for further study on the nature of viral antigen maintenance and the mechanism for replication of shrimp viral genetic material that supports perpetual antigen production. As already demonstrated, the cells may also be useful for study of shrimp and viral gene interations *in vitro*.

Finally, we would like to close with the speculative question as to whether split passage of whole insect cells might possibly be useful also for non-specific maintenance of genes from viruses of other invertebrates and perhaps even from vertebrates and plants.

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