FACULTY OF VETERINARY MEDICINE
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Laboratory of Virology

Department of Virology, Parasitology and Immunology

Faculty of Veterinary Medicine

Ghent University

Belgium

Development of Primary and Secondary Cell Cultures from the Lymphoid Organ of *Penaeus vannamei* to Study the Replication Cycle of White Spot Syndrome Virus (WSSV)

Wenfeng Li

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Prof. Dr. Hans J. Nauwynck

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List of Abbreviations

a.a.	Amino acids
BD	Billion dollars
bFGF	Basic fibroblast growth factor
CHH	Crustacean hyperglycaemic hormone
CPE	Cytopathic effect
CSC	Chinese Scholarship Council
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EGF	Epidermal growth factor
EM	Electron microscopy
EMA	Ethidium monoazide bromide
FAO	Food and agriculture organization of the united nations
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GSH	L-glutathione
GTP	Guanosine triphosphate
H&E	Haematoxylin and Eosin
hpi	Hours post inoculation
ICTV	International committee on taxonomy of viruses
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IIF	Indirect immunofluorescence
IMNV	Infectious myonecrosis virus
kDa	Kilo Dalton
L-15	Leibovitz's 15
LO	Lymphoid organ

LOS	Lymphoid organ spheroids
MBV	*Monodon*-type baculovirus
MEM	Minimum essential medium
MT	Million tones
NaCl	Sodium chloride
nm	Nanometre
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRDV	Penaeid rod-shaped DNA virus
rDNA	Ribosomal DNA
rER	Rough endoplasmic reticulum
RT-PCR	Reverse transcription PCR
SEMBV	Systemic ectodermal and mesodermal baculovirus
SG	Sinus gland
SID50	Shrimp infectious dose 50% end-point
SME	Shrimp muscle extract
SPF	Specific pathogen free
TEM	Transmission electron microscopy
TSV	Taura syndrome virus
UP	Ultra pure
USD	United states dollars
YHV	Yellow head virus
VP	Viral protein
WSS	White spot syndrome
WSSV	White spot syndrome virus
µg	Microgram
µl	Microlitre
µm	Micrometre

Chapter 1

Literature review

1.1 Aquaculture

Aquaculture most likely originated thousands of years ago when people started to live near coasts (Fast, 1992). Nowadays, aquaculture is considered as one of the fastest growing food resources in the whole world. According to the latest statistics released by the Food and Agriculture Organization of the United Nations (FAO), the world seafood production (fish, crustaceans, molluscs and other aquatic animals) reached 158 million tonnes (MT) (aquaculture production: 66.6 MT, capture production: 91.4 MT, Figure 1). 136.2 MT of the total seafood production was used for direct human consumption. Fish production has grown steadily in the last five decades, with food fish supply increasing at an average annual rate of 3.2 percent, outpacing world population growth at 1.6 percent (FAO, 2014). Globally, fish provide 3.0 billion people with almost 20% of their average per capita intake of animal protein, and 4.9 billion people with 10% of animal protein. World per capita fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012, with the share of aquaculture production in total food supply at 49%. The value of farmed food fish production was estimated at USD 137.7 billion. World food fish aquaculture production in 2012 (66.6 MT) consisted of 44.2 MT of finfish (66.3%), 15.2 MT of molluscs (22.8%), 6.4 MT of crustaceans (9.7%) and 0.9 MT of other aquatic animal species (1.3%) (FAO, 2012a).

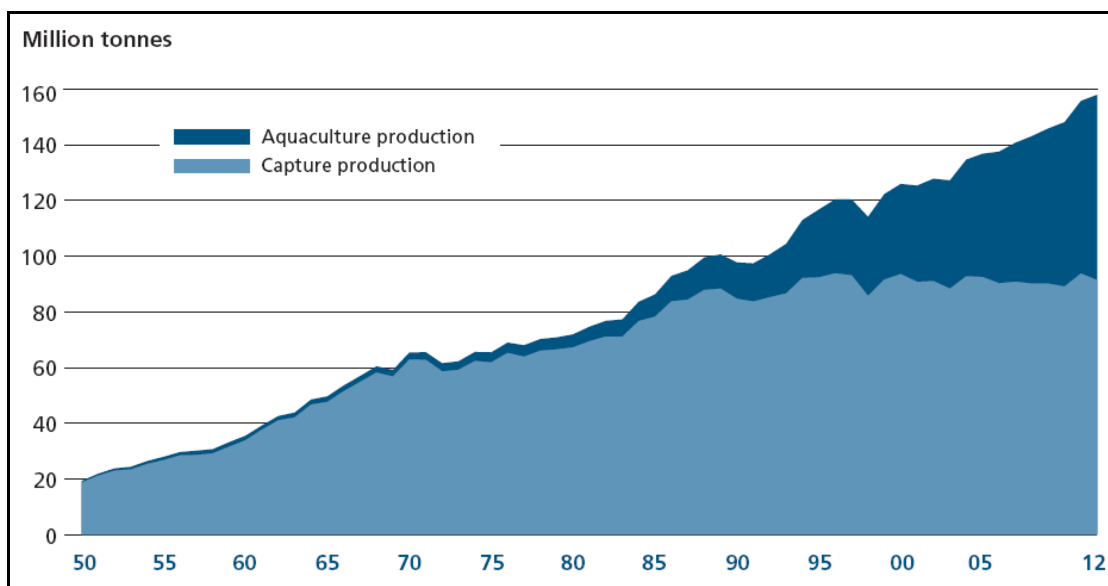

Figure 1 World seafood capture and aquaculture production.

Aquaculture has continued to show a strong growth, increasing at an average annual growth rate of 6.1% from 36.8 MT in 2002 to 66.6 MT in 2012. The contribution of aquaculture to total seafood production has risen steadily on all continents. The top ten aquaculture producers (excluding aquatic plants and non-food products) are China (41.1 MT), India (4.2 MT), Viet Nam (3.1 MT), Indonesia (3.1 MT), Bangladesh, Norway, Thailand, Chile, Egypt and Myanmar. They contribute 88% of the world seafood production (FAO, 2012b).

1.1.1 Aquaculture of crustacean

Until now, more than 60 crustacean species have been cultured in aquaculture farms in the world. The total production in 2012 was 6.4 MT, which represents an estimated value of 30.9 billion dollars (BD). This production was 60.8% from mariculture and 39.2% from inland aquacultures. The production from mariculture was dominated by white leg shrimp (*Penaeus vannamei*), which reached 3.18 MT (13.6 BD) and the second was another important species: the black tiger shrimp (*Penaeus monodon*) which reached 0.86 MT (3.6 BD). Shrimp continues to be the most important product traded in value terms, accounting for about 15% of the total value of internationally traded aquaculture products in 2012 (FAO, 2012a, c, d).

1.1.2 *Penaeus vannamei*

The white leg shrimp *Penaeus* (*Litopenaeus*) *vannamei* (Figure 2) belongs to the decapod crustaceans, which is probably the most complicated arthropod group. *Penaeus vannamei* is a native american shrimp species found along the western pacific coast from Sonora, Mexico in the North, through Central and South America as far south as Tumbes in Peru, where the water temperature is over 20 ºC the whole year.

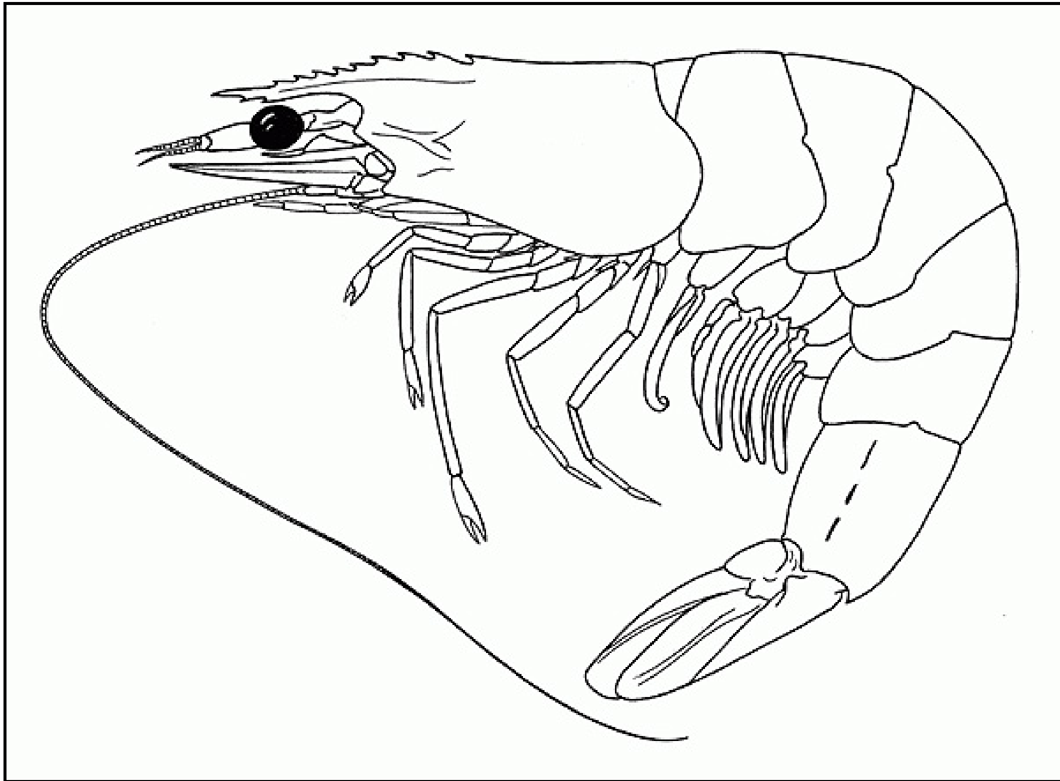

Figure 2 *Penaeus vannamei* black and white drawing (Boone, 1931).

The first spawning of this species was achieved in Florida in 1973, which was from a wild-caught mated female from Panama. From 1976 the commercial culture of *P. vannamei* began in South and Central America and subsequently expanded to Hawaii and mainland United States of America. From 1996, specific pathogen free (SPF) *P. vannamei* were successfully raised in Hawaii and soon were introduced in Taiwan and mainland of China. Then, a booming increase in the production of *P. vannamei* started in Asia. The production reached nearly 1.12 MT by 2004 and has now overtaken the production of *P. monodon* in China, Taiwan province of China and Thailand (Chamberlain, 2010). Afterwards, *P. vannamei* became one of the most important crustacean species in aquaculture industry. The commercial production of *P. vannamei* increased from 2,000 tonnes in 1976 worldwide to 194,000 tonnes in 1998 but decreased to 146,000 tonnes in 2000 due to an explosion of white spot syndrome (WSS). After that, with the booming spread of this species in Asian countries, the production of *P. vannamei* increased rapidly and reached 3.2 MT in 2012 (Figure 3; FAO, 2012d).

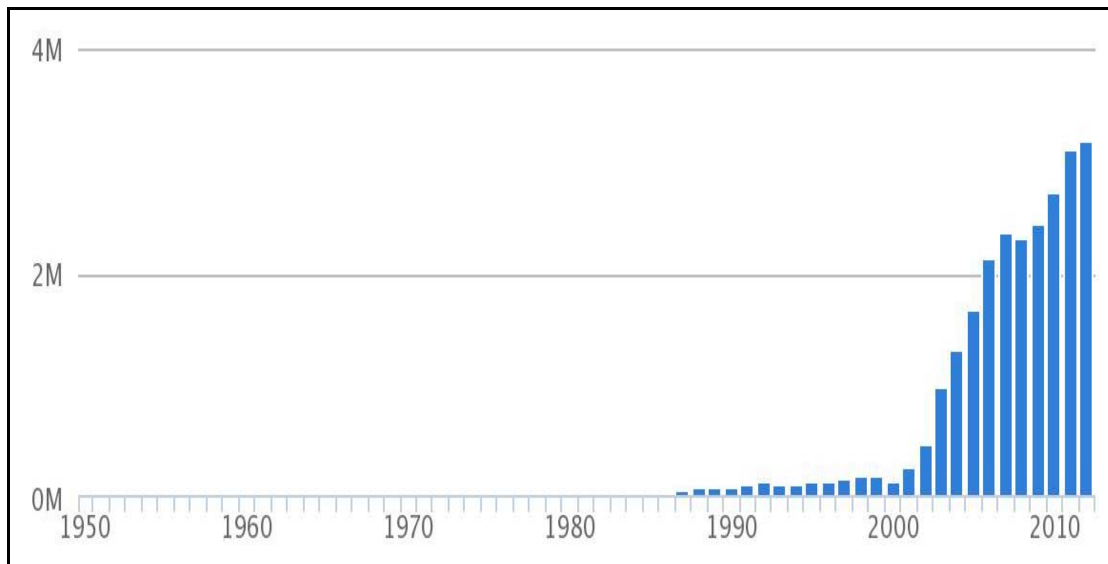

Figure 3 Global aquaculture production of *P. vannamei* from 1950 to 2012 (FAO - Fisheries and Aquaculture information and statistics service).

1.2 White spot syndrome virus (WSSV)

1.2.1 Morphology and classification

White spot syndrome virus (WSSV), a rapidly replicating and extremely lethal shrimp pathogen, was first detected in Taiwan in 1992 (Chou *et al.*, 1995), and then spread to Japan and almost all Asian countries in the following years (Huang *et al*., 2002). WSSV is an enveloped, non-occluded, double-stranded DNA virus (Chou *et al.*, 1995). WSSV virions show an ovoid to bacillar morphology with a long envelope extension at one extremity (Durand *et al*., 1997) (Figure 4). The WSSV size ranges between 210 and 420 nm in length and 70-167 nm in diameter (Lu *et al.*, 1997; Zheng *et al.*, 1997). The viral envelope is 6-7 nm thick and the area between the envelope and the nucleocapsid varies between 2 and 7.5 nm. The nucleocapsid dimensions are 180-420 in length and 54-85 nm in diameter indicating that it is tightly packed within the virion (Durand *et al*., 1997). The WSSV is considered to be one of the largest viruses that have been found worldwide because its genome contains a 300 kbp circular dsDNA (van Hulten *et al*., 2001, Yang *et al*., 2001).

Based on its unique morphological and genetic features, the International Committee on Taxonomy of Viruses (ICTV) assigned WSSV as the only member of the genus *Whispovirus* within a new virus family called *Nimaviridae*. This name refers to the thread-like extension at one end of the virus particle (*nima* is Latin for "thread") (Leu *et al*., 2009).

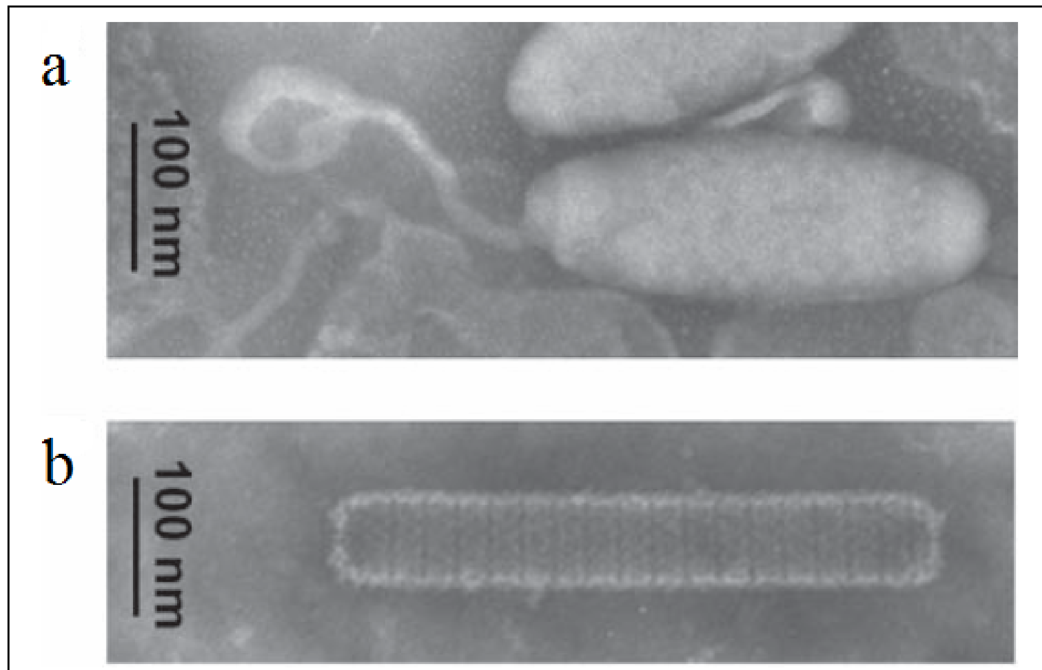

Figure 4 Morphology of the WSSV virion. Negative contrast electron micrographs of (a) an intact WSSV virion with tail-like extension and (b) nucleocapsid (Leu *et al*., 2005).

1.2.2 Genome

WSSV DNA was firstly isolated in 1997. The virus genome is a large double-stranded circular DNA molecule of approximately 300 kbp. To date, three WSSV isolates have been completely sequenced. The isolate from china (WSSV-CN, accession no. AF332093) and from Thailand (WSSV-TH, accession no. AF369029) was published in 2001 and the isolate from Taiwan (WSSV-TW, accession no. AF440570) was published in 2005. They had varying sizes: 305107, 292967 and 307287 bp for the China, Thailand and Taiwan isolates, respectively. Among them, the China isolate, WSSV-CN was chosen as the prototype strain by the ICTV whispovirus study group committee (Leu *et al*., 2009). In the WSSV-CN genome (Figure 5), at least 181

putative ORFs are likely to encode functional proteins and 80% of these ORFs have a potential polyadenylation site (AATAAA) downstream of the ORF. These predicted ORFs could encode proteins that range from 60 to 6,077 amino acids (a.a.; Leu *et al.* 2009). Until now, more than 50 ORFs encode proteins that resemble known proteins (>20% a.a. identity) or motifs (Leu *et al.*, 2009; Yang *et al.* 2001). A total of 27 ORFs can be classified into ten putative WSSV gene families and these families have been proposed to arise from gene duplications in the WSSV genome (van Hulten *et al.* 2001).

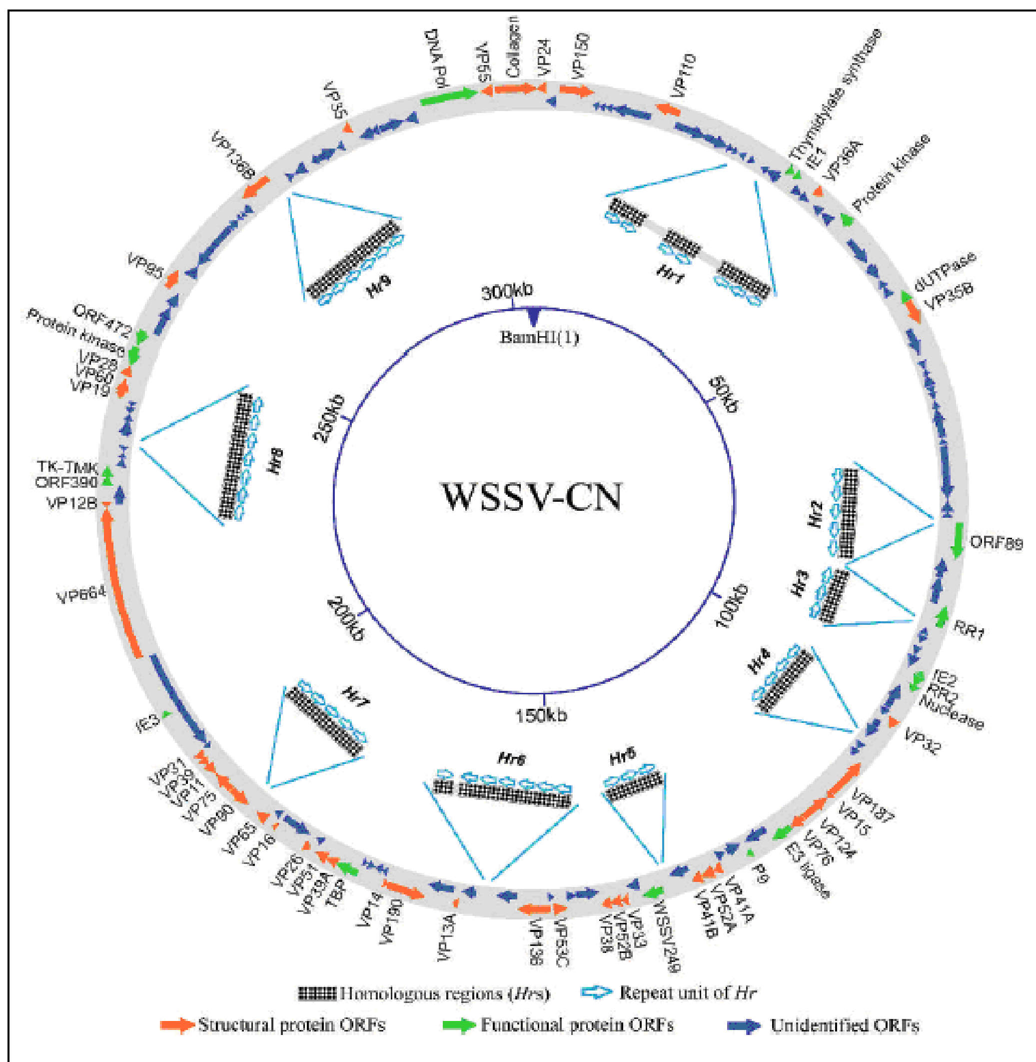

Figure 5 Schematic diagram showing the genomic organization of the circular double-stranded WSSV-CN genome. The positions and direction of transcription of corresponding genes are indicated with solid arrows (Leu *et al.*, 2009).

1.2.3 Major structural proteins

Up till now, at least 50 structural proteins of WSSV have been identified by proteomics, ranging in size from 60 a.a. to 6,077 a.a. Among them, about 7 proteins are located in the viral nucleocapsid, 5 in the tegument and more than 30 in the viral envelope (Huang *et al*. 2002; Leu *et al*., 2009; Li *et al*., 2007; Reddy *et al*., 2013; Tsai *et al*. 2004&2006; Xie *et al*. 2006; Zhang *et al*. 2004). However, the biological functions of most of WSSV's structural proteins are unknown. Electron microscopy analysis demonstrated that the WSSV virion consists of a nucleocapsid surrounded by an envelope. Tsai *et al*. (2006) suggested that, in addition to the envelope proteins (such as VP 28) and capsid proteins (such as VP664), there are a group of proteins that should be classified as tegument proteins, including VP26 and at least four other proteins (Table 1). The notion that VP26 is a tegument protein is supported by other reports (Xie and Yang 2005; Xie *et al*. 2006).

Table 1 The WSSV structural protein genes (Leu *et al*., 2009)

ORF name	Size (a.a.)	Protein name	Function/characteristics
wsv009	95	VP95	Structural protein
wsv026	507	VP507	Structural protein
wsv115	968	VP53B	Structural protein
wsv129	357	VP357	Structural protein
wsv137	337	VP337	Structural protein
wsv198	278	VP32	Structural protein
wsv199	856	VP320	Structural protein
wsv249	216	VP216	Structural protein
wsv260	387	VP387	Structural protein
wsv269	489	VP53C	Structural protein
wsv284	100	VP13A	Structural protein
wsv293	60	VP14	Structural protein
wsv303	184	VP184	Structural protein
wsv332	786	VP75	Structural protein
wsv338	433	VP11	Structural protein
wsv390	321	ORF390	Structural protein
wsv465	1243	VP136B	Structural protein
wsv502	362	VP362	Structural protein

wsv526	448	VP448	Structural protein
wsv001	1684	VP1684, Collagen-like	Structural protein, envelope
wsv011	1301	VP53A, VP150	Structural protein, envelope
wsv035	972	VP110	Structural protein, envelope
wsv209	1606	VP187	Structural protein, envelope
wsv216	1194	VP124	Structural protein, envelope
wsv237	292	VP41A	Structural protein, envelope
wsv238	486	VP51A, VP52A	Structural protein, envelope
wsv242	300	VP300, VP41B	Structural protein, envelope
wsv254	281	VP281, VP36B, VP33	Structural protein, envelope
wsv256	384	VP384, VP51B, V52B	Structural protein, envelope
wsv259	309	VP38A, VP38	Structural protein, envelope
wsv321	117	VP13B, VP16	Structural protein, envelope
wsv325	465	VP60A, VP56	Structural protein, envelope
wsv327	856	VP90	Structural protein, envelope
wsv339	283	VP39B, VP39	Structural protein, envelope
wsv340	261	VP31	Structural protein, envelope
wsv386	68	VP68, VP12B	Structural protein, envelope
wsv414	121	VP19	Structural protein, envelope
wsv421	204	VP28	Structural protein, envelope
wsv002	208	VP24	Structural protein, tegument
wsv077	297	VP36A	Structural protein, tegument
wsv306	419	VP39A	Structural protein, tegument
wsv311	204	VP26	Structural protein, tegument
wsv442	800	VP95	Structural protein, tegument
wsv037	1280	VP160B	Structural protein, capsid
wsv214	80	VP15	Structural protein, capsid, DNA-binding
wsv220	674	VP76, VP73	Structural protein, capsid
wsv271	1218	VP136, VP136A	Structural protein, capsid
wsv289	1565	VP160A, VP190	Structural protein, capsid
wsv308	466	VP466, VP51C, VP51	Structural protein, capsid
wsv360	6077	VP664	Structural protein, capsid

1.2.3.1 VP28

Most of the WSSV structural proteins are envelope proteins (about 30 of at least 50 identified structural proteins, Li *et al*., 2007; Tsai *et al*. 2004&2006; Xie *et al*. 2006), and these envelope proteins play crucial roles in virus binding, entry and assembly (Chazal *et al*. 2003). VP28, encoded by open reading frame (ORF) 421 (wsv421), was identified by van Hulten *et al*. (2000). VP28 is the major protein in WSSV envelope. VP28 was observed as early as 3 hours post adsorption (Yi *et al*., 2004). It is generally considered to play an important role in the initial steps of systemic WSSV infection in shrimp (van Hulten *et al*., 2001). There is a strong hydrophobic region present at the N-terminus of VP28, including a putative transmembrane region (van Hulten *et al*., 2000). This biological structure of VP28 suggests that it might play a role as attachment protein. Furthermore, Yi *et al.* (2004) reported that VP28 plays an important role in the infection process as an attachment protein, binding the virus to shrimp cells, and helping it to enter into the cytoplasm. Sritunyalucksana *et al*. (2006) demonstrated that VP28 could interact with Rab7 protein which mainly functions in exocytosis and endocytosis in mammalian cells. Xie *et al*. (2006) found that the tegument proteins VP26 and VP24 bind to VP28 by far-Western analysis and co-immunoprecipitation. Liu *et al*. (2009) reported that VP28 has a direct interaction with VP37 *in vivo*. Li *et al*. (2015) identified the interaction domains of VP28 and VP24 (tegument protein, Figure 6, Chang *et al*., 2010; Watthanasurorot *et al*., 2014). Overall, these studies suggest that VP28 could not only bind to host membrane proteins, but also interact with VP24 and VP26 to anchor the envelope onto the underlying tegument layer (Leu *et al*., 2009; Li *et al*., 2015). In addition, antibodies against VP28 showed a significant delay of shrimp mortality (van Hulten *et al*., 2001; Yi *et al*., 2004).

1.2.3.2 VP26

VP26, the major tegument protein that was encoded by the wsv311 gene, was first identified as being associated to the nucleocapsid (van Hulten *et al*., 2001). Later, VP26 was demonstrated to be located in the space between the envelope and the nucleocapsid acting as a linker protein (Figure 6, Chang *et al*., 2010; Tsai *et al*., 2006). It is likely that the N-terminus of VP26 (a strongly hydrophobic region) anchors in the

envelope, while the C-terminus (containing a hydrophilic sequence) is bound to the nucleocapsid. Chang *et al*. (2008) reported that VP26 interacts with VP28 and VP51A, finally resulting in a VP51A-VP26-VP28 complex. Lin *et al*. (2015) reported that VP26 has a direct interaction with VP52B, which is located on the outer surface of WSSV virions. VP26 also interacts with envelope protein VP37 (Liu *et al*. 2009). In addition, VP26 was found to be capable of binding to actin or actin-associated proteins. Thus, it has been hypothesized that VP26 may be instrumental in trafficking the WSSV nucleocapsid into the host nucleus via interacting with actin or cellular cytoskeleton (Xie & Yang, 2005).

1.2.3.3 VP664

VP664 is one major capsid protein and consists of a remarkable long polypeptide of 6,077 a.a. It was encoded by wsv360 with a giant sequence of 18234 nt (Leu *et al*., 2005). This protein derived its name from the calculated molecular weight of 664 kDa, which is the largest viral structural protein ever found (Leu *et al*., 2009). This huge structural protein appears to form the stacked ring structures that are visible in the nucleocapsid under TEM (Leu *et al*. 2005). Time course analysis of VP664 by RT-PCR showed that this transcript was actively transcribed from 2 h post injection, suggesting that this protein should contribute to the assembly and morphogenesis of the virion (Leu *et al*. 2005).

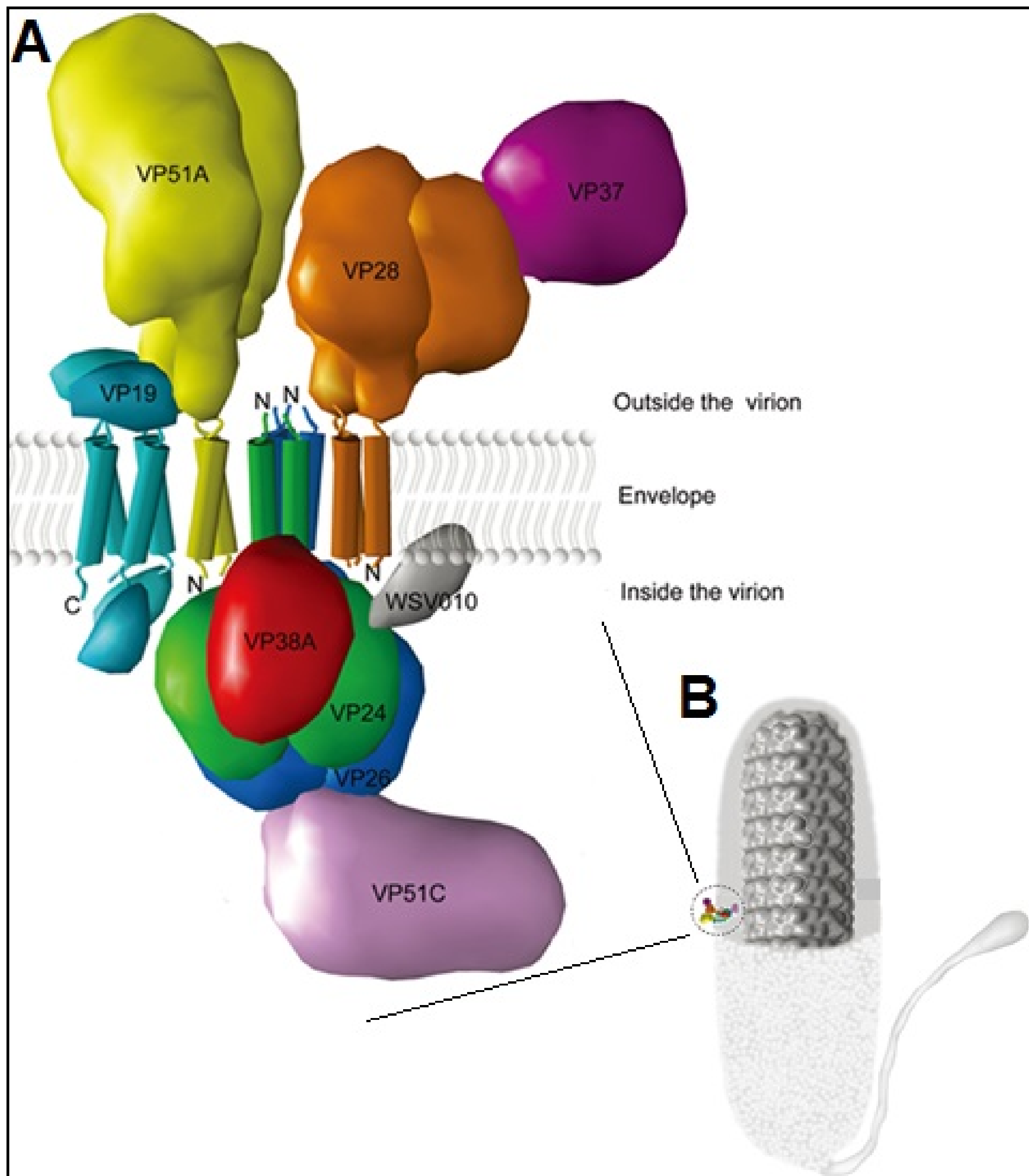

Figure 6 3D models of the membrane protein complex and the nucleocapsid. (A) Graphical representation of the putative relative localizations and interactions of the WSSV virion protein complex composed of VP19, VP24, VP26, VP28, VP37, VP38A, VP51A, VP51C and WSV010. Each protein is indicated with a unique color. The transmembrane helices are shown as cylinders. The oligomerization of VP19, VP24, VP51A (dimers), VP26 and VP28 (trimers) is also shown. (B) Refined model of the WSSV virion nucleocapsid. Note that the insert of the protein cluster is not drawn to the correct scale (Chang *et al*., 2010).

1.2.4 WSSV infection

1.2.4.1 Transmission

In general, transmission of viruses can occur through two pathways: horizontally (feeding of the infected organisms or contaminated food, through gills and/or other body surfaces by direct exposure to virus particles in water) (Leu *et al*., 2009), and vertically (virus is passed from an infected female parent to her F1 progeny) (Sánchez-Paz, 2010). Up till now, the transmission of WSSV by ingestion of infected tissue and direct exposure of body surfaces to virus particles in water has been reported (Durand et al., 2002; Chang *et al.*, 1996). However, feeding of the infected tissues has been proven to be much more effective to transmit virus among shrimp than immersion in the WSSV infected water (Lotz & Soto, 2002). Prior *et al.* (2003) tried to develop a controlled bio-assay by immersion of shrimp. Although very large amounts of infectious virus were added to the water, mortality rates stayed below 40%.

1.2.4.2 Host range

WSSV has a remarkably broad host range covering many species of shrimp, crayfish, crab and lobster. To date, more than 90 species of arthropods have been reported as hosts or carriers of WSSV either from outbreaks in culture facilities upon experimental infection or catched in the wild (Table 1). This virus was considered to be able to replicate in almost all crustaceans from marine, brackish and freshwater sources (Flegel, 1997; Flegel & Fegan, 2002; Lo *et al.,* 1996). Most commercially important penaeid species were included. Moreover, WSSV is especially highly pathogenic and virulent in penaeid shrimp. WSSV mainly infects cells in tissues of ectodermal and mesodermal origins, and the virus replicates and assembles the virions in the hypertrophied nuclei of infected cells without the production of occlusion bodies. WSSV undergoes lytic infection, and during the late stage of infection, the infected nuclei/cells disintegrate, which causes the affected tissues/organs to become seriously damaged and necrotic. Due to the wide host range, together with the frequent international trades of shrimp stocks and adults, WSSV has become one of the most widespread viruses, occurring in almost all shrimp-farming countries (Flegel

and Fegan, 2002). Except the mature eggs (Lo *et al*., 1997), almost all life stages are potentially susceptible to infection.

1.2.4.3 Clinical signs and pathology

White spots were observed on the carapace, appendages and the epidermis of diseased shrimp. The diameter of these spots ranges from 0.5 - 3.0 mm and sometimes these spots coalesce into larger plates (Lo *et al*., 1996). The exact mechanism of white spot formation was not clear until now. It is possible that WSSV infection causes abnormal deposition of calcium salts in the cuticular epidermis (Wang *et al*., 1997) or disruption in the transfer of exudates from the epithelial cells to the cuticle (Wang *et al*., 1999). However, the high alkalinity and bacterial diseases can also cause white spots on the carapace of shrimp (Leu *et al*., 2009). In the early stage of infection, the infected shrimp become lethargic. They gather unusually around the edges of ponds during the daytime and reduce their feed uptake. Most of the infected animals change their body color to reddish due to the expansion of cuticular chromatophores. In the late stage of infection, there is a systemic destruction of WSSV-target tissues. Most of the infected cells have homogeneous hypertrophied nuclei, which show amphophilic intranuclear inclusions and marginated chromatin (Wang *et al*., 2000). Moreover, karyorrhexis and cellular disintegration occurs, leading to the formation of necrotic areas characterized by vacuolization (Karunasagar *et al*., 1997; Kasornchandra *et al*., 1998). Cumulative mortality may reach 100% in 5 to 10 days after the onset of disease (Karunasagar *et al*., 1997).

Table 2 The known host species reported to be naturally or experimentally infected with WSSV (Sánchez-Paz, 2010).

Phylum	Order	Family	Species
Arthropoda	*Anostraca*	*Artemiidae*	*Artemia sp.*; *A. franciscana*
Arthropoda	*Calanoida*	*Pseudodiaptomidae*	*Schmackeria dubia*
Arthropoda	*Decapoda*	*Alpheidae*	*Alpheus brevicristatus*; *A. lobidens*
Arthropoda	*Decapoda*	*Astacidae*	*Astacus astacus*; *A. leptodactylus*; *Pacifastacus leniusculus*
Arthropoda	*Decapoda*	*Calappidae*	*Calappa lophos*; *C. philargius*
Arthropoda	*Decapoda*	*Callianassidae*	*Callianassa sp.*
Arthropoda	*Decapoda*	*Cancridae*	*Cancer pagurus*
Arthropoda	*Decapoda*	*Cambaridae*	*Orconectes limosus; O. punctimanus*; *Procambarus clarkii*
Arthropoda	*Decapoda*	*Dorippidae*	*Paradorippe granulata*
Arthropoda	*Decapoda*	*Eriphiidae*	*Menippe rumphii*
Arthropoda	*Decapoda*	*Grapsidae*	*Grapsus albolineatus*; *Metopograpsus messor*
Arthropoda	*Decapoda*	*Leucosiidae*	*Philyra syndactyla*
Arthropoda	*Decapoda*	*Lithodidae*	*Lithodes maja*
Arthropoda	*Decapoda*	*Majidae*	*Doclea hybrida*
Arthropoda	*Decapoda*	*Matutidae*	*Matuta miersi*; *M. planipes*
Arthropoda	*Decapoda*	*Ocypodidae*	*Gelasimus marionis nitidu*; *Macrophthalmus sulcatus; Uca pugilator*
Arthropoda	*Decapoda*	*Palaemonidae*	*Exopalaemon orientis*; *Macrobrachium idella; M. lamarrei*; *M. rosenbergii*
			Palaemon adspersus
Arthropoda	*Decapoda*	*Palinuridae*	*Panulirus homarus*; *P. longipes*; *P. ornatus; P. penicillatus*; *P. polyphagus*; *P. versicolor*
Arthropoda	*Decapoda*	*Parastacidae*	*Cherax destructor albidus*; *C. quadricarinatus*

Arthropoda	*Decapoda*	*Parathelphusidae*	*Parathelphusa hydrodomous*; *P. pulvinata*
Arthropoda	*Decapoda*	*Parthenopidae*	*Parthenope prensor*
Arthropoda	*Decapoda*	*Penaeideae*	*Metapenaeus brevicornis*; *M. dobsoni*; *M. ensis; M. lysianassa*; *M. monoceros*; *Parapeneopsis stylifera; Penaeus aztecus*; *P. chinensis*; *P. duorarum*; *P. indicus* *P. japonicus*; *P. merguiensis*; *P. monodon*; *P. penicillatus; P. schmitti*; *P. semisulcatus* *P. setiferus*; *P. stylirostris; P. vannamei*; *Trachypenaeus curvirostris*
Arthropoda	*Decapoda*	*Portunidae*	*Callinectes arcuatus*; *C. sapidus*; *Carcinus maenas*; *Charybdis annulata*; *Ch. cruciata*; *Ch. granulata*; *Ch. feriatus*; *Ch. japonica*; *Ch. lucifera*; *Ch. Natator;* *Liocarcinus depurator*; *Lio. puber*; *Podophthalmus vigil*; *Portunus pelagicus* *P. sanguinolentus*; *Scylla serrata*; *S. tranquebarica*; *Thalamita danae*
Arthropoda	*Decapoda*	*Scyllaridae*	*Scyllarus arctus*
Arthropoda	*Decapoda*	*Sergestidae*	*Acetes sp.*
Arthropoda	*Decapoda*	*Sesarmidae*	*Sesarmidae*
Arthropoda	*Decapoda*	*Solenoceridae*	*Solenocera indica*
Arthropoda	*Decapoda*	*Varunidae*	*Helice tridens*; *Pseudograpsus intermedius*
Arthropoda	*Decapoda*	*Xanthidae*	*Atergatis integerrimus*; *Demania splendida; Halimede ochtodes*; *Liagore rubromaculata*
Arthropoda	*Diptera*	*Ephydridae*	*Ephydrida sp.*
Arthropoda	*Stomatopoda*	*Squillidae*	*Squilla mantis*
Annelida	*Eunicida*	*Eunicidae*	*Marphysa gravelyi*
Chaetognatha	-	-	-
Rotifera	*Ploimida*	*Brachionidae*	*Brachionus urceus*

1.2.4.4 Pathogenesis

The pathways of WSSV entry into shrimp have not been clearly defined. However, some experiments showed that several tissues might be potential primary sites. Chang *et al*. (1996) reported that the primary sites of WSSV infection in early juvenile *P. monodon* were the subcuticular epithelial cells of stomach, cells in gills, in integument and connective tissue of the hepatopancreas. In the WSSV immersion challenged *P. monodon*, many WSSV-positive cells were found in gills (Arts *et al*., 2007). According to the experimental data from an oral route challenge, the primary sites of WSSV replication as determined with IHC were the epithelial cells in the foregut and cells in the gills (Escobedo-Bonilla *et al*., 2007). The portals of viral spread from the primary sites to other target organs were controversial (Escobedo-Bonilla *et al*., 2008). In shrimp *Penaeus merguiensis*, *Marsupenaeus japonicus* and *Palaemon sp.*, WSSV infects hemocytes and travel in these cells to reach target organs (Wang *et al*., 2002; Di Leonardo *et al*., 2005). However, In *Penaeus monodon*, *Procambarus clarkii* and *Penaeus vannamei*, circulating hemocytes are refractory to WSSV infection, which indicates WSSV might reach other target tissues through hemolymph circulation in a cell-free form (van de Braak *et al*., 2002; Shi *et al*., 2005, Escobedo-Bonilla *et al*., 2007). The mechanisms of spread may be host species-dependent.

In brief, WSSV usually causes systemic infections in tissues of ectodermal and mesodermal origin, including epidermis, gills, foregut, hindgut, antennal gland, lymphoid organ, muscle, eye-stalk, heart, gonads and haematopoietic cells. It does not affect most tissues of endodermal origin (e.g. hepatopancreas, anterior and posterior midgut caeca and midgut trunk). In moribund shrimp, the epithelia of stomach, gills and integument may be severely damaged, which may cause organ dysfunction and lead to death (Escobedo-Bonilla *et al*., 2008).

1.2.4.5 Time course of WSSV replication cycle

So far, the replication cycle of WSSV is still not clear. Due to the lack of available shrimp or other crustacean cell cultures and stable cell lines, the replication cycle of WSSV *in vitro* could not be studied up till now. However, data have been collected *in vivo*. In infected shrimp, WSSV gene expression and DNA replication have been detected by PCR. Wang *et al*. (2004) reported that 23.5% of ORFs of WSSV DNA

began to be expressed at 2 h post infection, 4.2% at 6 h post infection, 17.7% at 12 h post infection and 47.9% at 24 h post infection in gills of infected *P. monodon*. Most of the WSSV structural genes were expressed in between 12 and 24 h post infection. Hameed *et al*. (2006) tested WSSV infection and replication in gills of infected *P. monodon* and *P. indicus* by PCR. WSSV DNA was amplified from 12 h post injection and increased between 12 h post injection and moribund stage. Transcriptional analysis showed that the WSSV genes that encode the large and small subunits of ribonucleotide reductase, protein kinase, chimeric thymidine kinase-thymidylate kinase and DNA polymerase were identified in *P. monodon* at 2-4 h post infection (Liu *et al*., 2001; Marks *et al*., 2003; Tsai *et al*., 2000; Chen *et al*., 2002), which indicated that the immediate-early and early genes of WSSV started to be expressed as early as 2-4 h post infection. Lan *et al*. (2006) reported that 47 ORFs have been transcribed within 6 h post injection. Another WSSV infection experiment that was performed in *P. japonicas* showed that the number of copies of WSSV viral DNA in the pleopods of the challenged shrimp increased by around 350 times and reached a peak between 12 and 24 hpi, suggesting that 12 to 24 hpi was the interval during which viral replication occurred (Chen *et al*., 2011). In addition, Chang *et al*. (1996) proved that WSSV-positive cells were initially observed at 16 h post infection in the stomach, gill, cuticular epidermis and hepatopancreas of infected *P. monodon* by *in situ* hybridization. With TEM, it was revealed that the chromatin of infected nuclei of black tiger shrimp *P. monodon* was marginated as early as 6 h post injection and the nuclei were filled with enveloped and non-enveloped WSSV virions at 24 h post injection (Wongprasert *et al*., 2003), which might indicate that replication of WSSV virions went to a final stage at 24 h post injection *in vivo*. In brief, the *in vivo* studies indicated that the time course of one replication cycle of WSSV was approximately 24 hours.

1.3 Cell culture of penaeid shrimp

The first cell culture system from tissues of penaeid shrimp was reported in 1986 (Chen *et al*., 1986). This cell culture was derived from gonad and heart tissues of *P. monodon*. Subsequently, especially when the white spot syndrome virus was first

detected in 1992 in Taiwan and soon in most southeast Asian countries (Kasornchandra *et al*., 1995), a huge amount of trials were focused on primary cell culture from penaeid shrimps (Assavalapsakul *et al*., 2003; Chen & Kou, 1989; Dantas-Lima *et al*., 2012; Ellender *et al.*, 1988; George *et al*., 2011; Hsu *et al*., 1995; Hu *et al.*, 1990; Jiang *et al*., 2006; Lang *et al*., 2002a, b; Li *et al*., 2014; Luedeman & Lightner 1992; Nadala *et al*., 1993; Owens *et al*., 1999; Toullec *et al*., 1996; Wang *et al*., 2000). Plenty of media (commercial or self-prepared) and medium supplements (sera, tissue extracts, growth factors…) were applied in primary cell culture practice. But until now, there is no cell line available in the world (Jose *et al*., 2012). Moreover, the subculture of primary shrimp cell cultures and their survival have been found to be the most difficult tasks (Fraser & Hall, 1999; Gao *et al*., 2003). Though subculture from shrimp primary cells has been reported (Claydon & Owens, 2008; Fan & Wang, 2002; Hu *et al*., 2008; Tapay *et al*., 1995), the capability to suspend shrimp cells to proliferate was questionable and needed to be verified (Han *et al*., 2013) because in many cases these cells were not shrimp cells but from contaminated micro-organisms (Hsu *et al*., 1995; Rinkevich, 1999). Only a few reports exist on the infection of cell cultures with white spot syndrome virus (WSSV) (Itami *et al*., 1999; Kasornchandra *et al*., 1998 and 1999; Tapay *et al*., 1995 and 1997). In brief, despite several decades of intensive research efforts, continuous cell lines from penaeid shrimp still have to be developed (Jose *et al*., 2010; Rinkevich, 2011). There is still a long way to go for the establishment of a cell line from the penaeid shrimps.

1.3.1 Species sources

Most cultures of crustacean tissues derived from penaeid shrimp. More than 80% of the publications about cell cultures were using tissues from penaeid shrimp. The most popular species of penaeid shrimp which was used for cell cultures is *Penaeus monodon* (Hsu *et al*., 1995; Jose *et al*., 2012; Owens *et al*., 1999). Since the culture of *P. vannamei* expanded rapidly over most parts of Asia after 1996 (Chamberlain, 2010), this species has become the most important cultured penaeid species. Within this period, *P. vannamei* has been applied in plenty of cell culture trails from 1999 (Dantas-Lima *et al*., 2012; George *et al*., 2011; Han *et al*., 2013). *P. japonicus* (Itami et al., 1999; Lang t al., 2002) and *P. stylirostris* (Shike *et al*., 2000, Shimizu *et al*., 2001a, b) were also popular in shrimp cell culture practice. Other species including *P.*

chinensis, P. indicus, P. merguiensis, P. orientates, P. aetecus and *P. ensis* have been barely used due to their limited living spaces and low production in aquaculture (Figure 7).

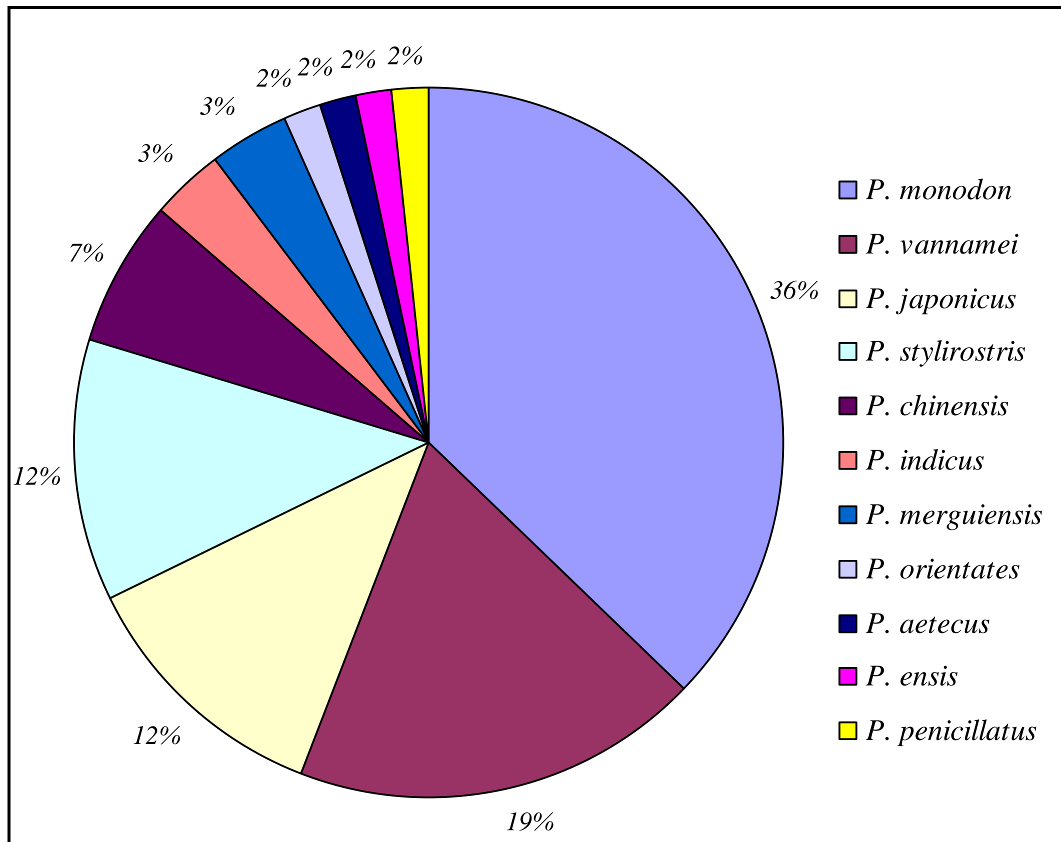

Figure 7 Penaeid shrimp species used in the production of primary cell cultures.

1.3.2 Tissue sources

The tissue that is used for primary cell culture is considered to be an important first selection step for a successful development of cell lines in the future. In the studies of penaeid shrimp cell cultures, tissue sources are very variable (Figure 8). The lymphoid organ is the most commonly-used cell source for making cell cultures which gave promising results (Assavalapsakul *et al*., 2003; Chen & Kou, 1989; Han *et al*., 2013; Tapay *et al*., 1995; Wang *et al*., 2000). It is followed by ovary (George & Dhar, 2010; Kasornchandra, 1999; Luedeman & Lightner, 1992), hepatopancreas (Chen *et al*., 1986; Ellener *et al*., 1988; Hu *et al*., 1990), hemocytes (Dantas-Lima *et al*., 2012; Jiang *et al*., 2006; Jose *et al*., 2010; Lang *et al*., 2002) and heart (Chen and Wang, 1999; Tapay *et al*., 1997). Cell cultures of shrimp derived from muscle (Chen *et al*., 1986; Wang *et al*., 2000), nerves (Nadala *et al*., 1993; Owens and Smith, 1999),

epidermis (Toullec, *et al*., 1996), hematopoiesis (Jiravanichpaisal *et al*., 2006; West *et al*., 1999) and gills (Hsu *et al*., 1995) have also been reported in some studies. Other tissues, such as gut (Nadala *et al*., 1993), eyestalks (George and Dhar, 2010), embryos (Frerichs *et al*., 1996) were just occasionally used as tissue sources in cell cultures.

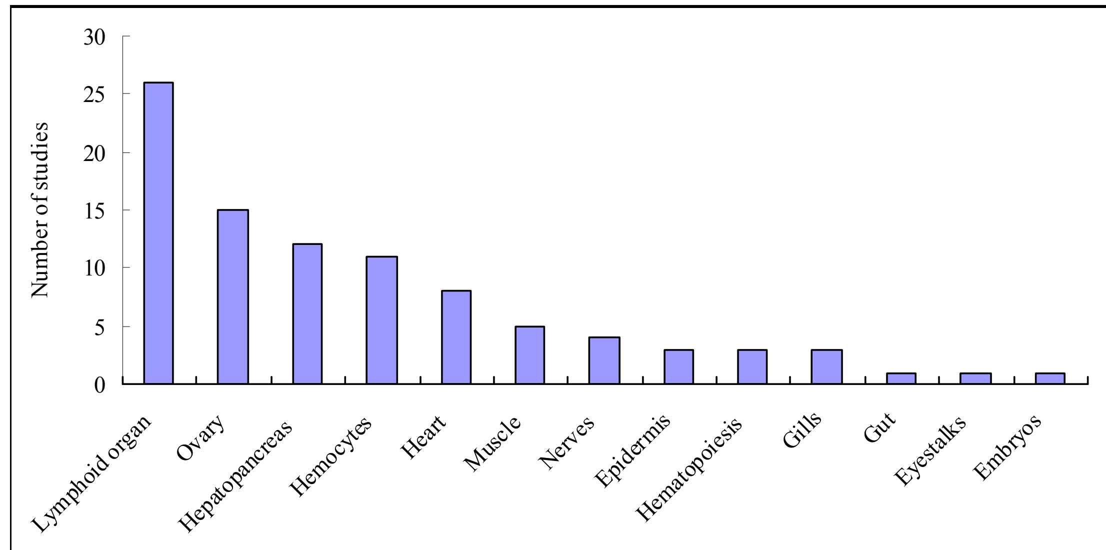

Figure 8 Tissues used for primary cell cultures of penaeid shrimp.

The lymphoid organ was the most commonly-used tissue in shrimp cell culture practice (Assavalapsakul *et al*., 2003; Chen and Kou, 1989; Han *et al*., 2013; Tapay *et al*., 1995; Wang *et al*., 2000). The lymphoid organ, which was originally named as "Oka" organ, was firstly described in *Penaeus orientalis* by Oka (1969). Later on, the lymphoid organ was found in the ginger prawn (*Penaeus japonicus* Bate; *Penaeus carinatus* Dana), the white prawn (*Penaeus indicus* de Man) and the banana prawn (*Penaeus merguiensis* de Man) (Oka, 1969). Soon after, it was reported to be found in *Penaeus monodon* (van de Braak *et al.*, 2002) and *Penaeus chinensis* (Shao *et al.,* 2004). The lymphoid organ (LO) is situated ventral to the stomach and slightly dorso-anterior to the ventral hepatopancreas (Duangsuwan *et al.*, 2008a, Figure 9). The position of the lymphoid organ differs slightly between the male and the female shrimp. The LO lies between the hepatopancreas and stomach in male prawn and between the ovary and the hepatopancreas in female prawn (Figure 10). Sex and gonad maturation may also contribute to the differences in the LO position. The ovary seems to press this organ onto the upper part of the hepatopancreas (Rusaini & Owens, 2010).

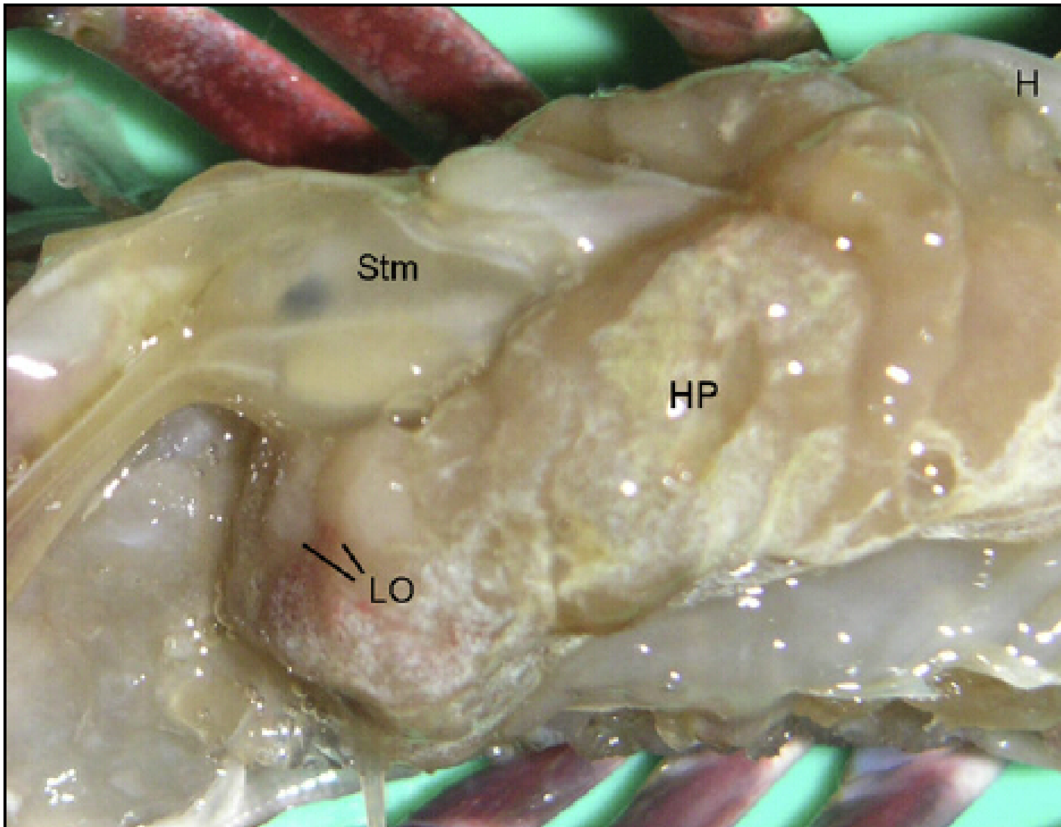

Figure 9 A photograph of the cephalothorax region of *Penaeus monodon* whose carapace was removed, showing the paired lymphoid organ (LO) lying ventral to the stomach (Stm) and dorso-anterior to the ventral hepatopancreas (HP); H: heart (Duangsuwan *et al.*, 2008a).

The size of the lymphoid organ (LO) is correlated with the developmental stage of the prawn. The LO is small in the postlarval stage but increases significantly in later stages. The prawn species and animal size may also affect the LO size (Rusaini & Owens, 2010). The bigger the prawn, the larger the LO size. The LO size was less than 2 mm in diameter in *Penaeus chinensis* sampled in August with a body length of 12 - 13 cm, but more than 2 mm in larger shrimp in November (Shao *et al.*, 2004). In the fully mature black tiger shrimp with a body length of 18 - 20 cm and body weight of 150 - 170 g, lymphoid organs measures around 3 - 4 mm long, 2 - 3 mm wide and 1 - 2 mm thick (Duangsuwan *et al.*, 2008a).

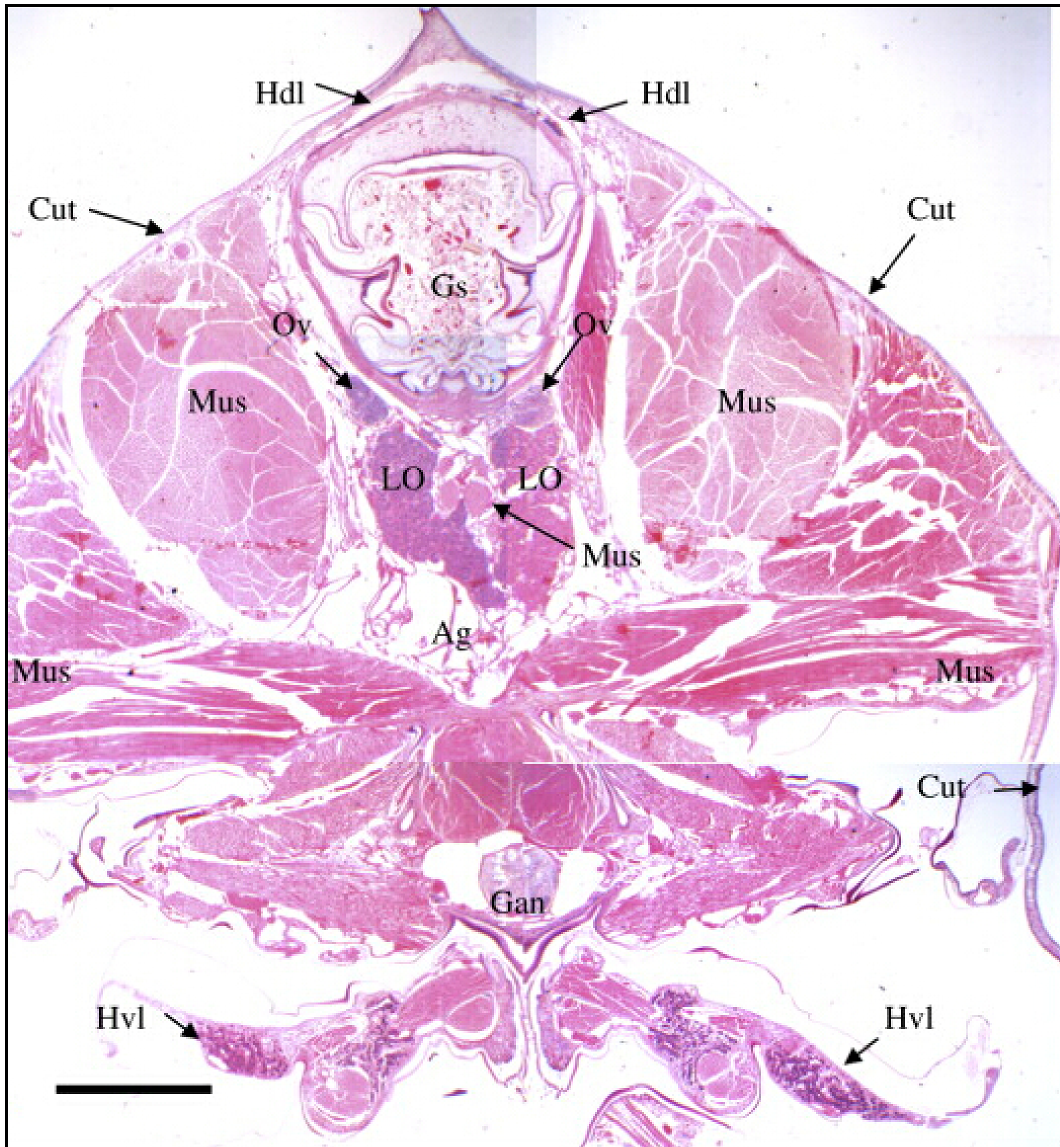

Figure 10 Cross-section of the lymphoid organ (LO) and surrounding tissues of *P. monodon*. The LO consists of two lobes located ventro-lateral of the gastric sieve and dorsal of the antennal gland. H & E stain. Ag, antennal gland; Cut, cuticle; Gan, ganglion; Gs, gastric sieve, Hdl, haematopoietic dorsal lobules; Hvl, haematopoietic ventral lobules; Mus, muscle; LO, lymphoid organ; Ov, ovary, scale bar = 100 µm. (Rusaini, 2006).

The lymphoid organ (LO) consists of two ovoid-shaped lobes. Each lobe of the LO is surrounded by a connective tissue capsule, which invaginate inwards together with antennal tubules and separate each lymphoid organ lobe into lymphoid lobules (Duangsuwan *et al.*, 2008a). Each LO lobe is connected with the heart via the subgastric artery. The subgastric artery originating from the heart, enters the LO lobes

and branches further 4-5 times to form vascular plexuses and finally terminal capillaries in the LO lobes. Each terminal capillary forms a haemal lumen of a lymphoid tubule (Figure 11(a), Duangsuwan *et al.*, 2008a). Spaces between the lymphoid tubules are occupied by haemal sinuses that are packed with large numbers of haemocytes. Each lobe of the LO consists of lymphoid tubules with haemal lumen and haemal sinuses. The lymphoid tubules with haemal lumen are lined by (i) flattened endothelial cells, (ii) stromal cells and (iii) ovoid-shaped capsular cells. There are two types of stromal cells: the stromal cells in the inner layer have a clear, unstained cytoplasm, whereas the stromal cells in the outer layer have more darkly stained cytoplasm (H and E stain, Bell & Lightner, 1996). The ovoid-shaped capsular cells form the outermost layer of the lymphoid tubule and they can run circumferentially around the lymphoid tubule (Figure 11(b)). Haemolymph enters each LO lobe via the subgastric artery and finally reaches the haemal lumen of lymphoid tubules. From the haemal lumens, haemocytes may penetrate the layer of endothelial cells, move into the space between stromal cells and later migrate into the haemal sinuses (van de Braak *et al.*, 2002).

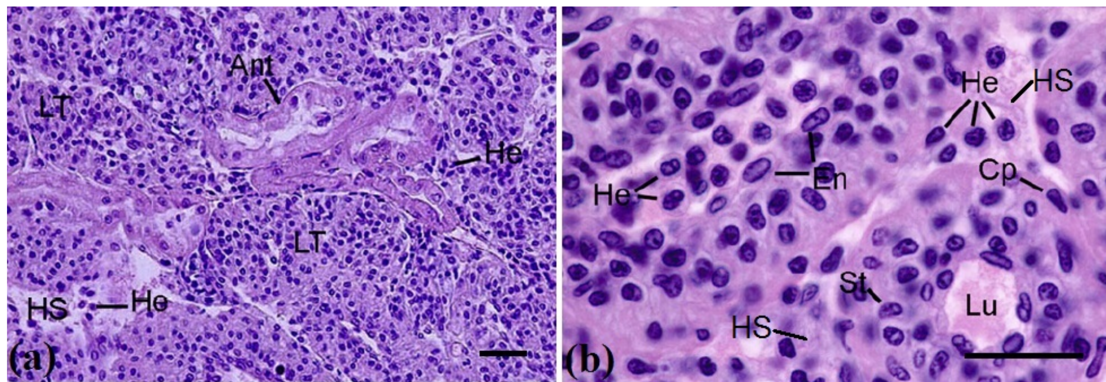

Figure 11 (a): Light micrograph of a paraffin section of the lymphoid organ of *P. monodon* stained with hematoxylin and eosin. Ant, antennal tubule; LT, lymphoid tubule; He: haemocytes; HS: haemal sinuses; scale bar = 25 µm. (b): Light micrograph of lymphoid tubules (LT). The luminal surface of lymphoid tubule is lined by endothelial cells (En). Capsular cells (Cp) are located at the outermost layer of the lymphoid tubule. Stromal cells (St) are located between endothelial and capsular cells, a large number of haemocytes (He) are packed in haemal sinuses (HS), scale bar = 25µm (Duangsuwan *et al.*, 2008a).

The lymphoid spheroids (or lymphoid organ spheroids, Figure 12) were first described by Lightner *et al*. (1987). Later on, this structure has been described by many other researchers as possible multinucleate giant cells (Owens *et al*., 1991), proliferative centres (Nadala *et al*., 1992), nodular structures (Kondo *et al*., 1994), lobular hyperplastic proliferation and degeneration of lymphoid organ cells (Turnbull *et al*., 1994) and abnormal cell foci (Spann *et al*., 1995). The lymphoid spheroids (or lymphoid organ spheroids) are formed in haemal sinuses (Rusaini & Owens, 2010) or located between the tubules, surrounded by fibrous connective tissue and lacking a central lumen (van de Braak *et al*., 2002). Now growing evidence indicated that the lymphoid spheroids (or lymphoid organ spheroids) might be a sign of infection by certain viruses, such as lymphoid organ vacuolization virus (LOVV, Bonami *et al*., 1992); lymphoid parvo-like virus (LPV, Owens *et al*., 1991); lymphoid organ virus (LOV, Spann *et al*., 1995); rhabdovirus of penaeid shrimp (RPS, Nadala *et al*., 1997); yellow head virus (YHV, Flegel *et al*., 1992) or Taura syndrome virus (TSV, Hasson *et al*., 1999). Duangsuwan *et al*. (2008a&b) reported that the lymphoid organ spheroids are not observed in the lymphoid organ of *P. monodon* that tested negative for WSSV and YHV but are found in the lymphoid organ of YHV infected *P. monodon*.

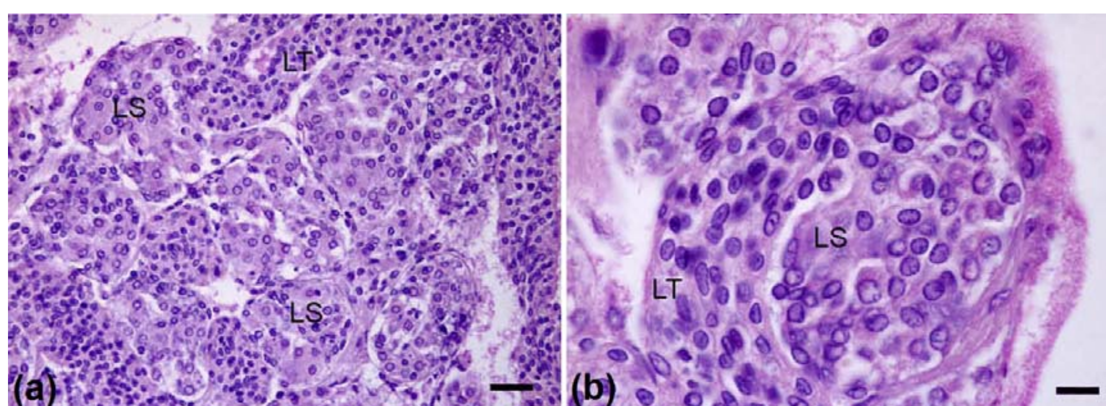

Figure 12 Light micrograph of sections of the LO of YHV infected *P. monodon* (H & E staining). (a): The transitional zone from lymphoid tubule (LT) to the more disorganized lymphoid spheroid (LS), scale bar = 25µm. (b): A longitudinal section of lymphoid spheroid (LS) that lacked lumen, scale bar = 10µm. (Duangsuwan *et al.*, 2008b).

The lymphoid organ is believed to play an important role in the defense during infections especially viral infections. This organ has a filtering function for removing foreign materials and infectious agents from the hemolymph (Duangsuwan *et al.*, 2008a; Kondo *et al.*, 1994). The lymphoid tubules are essentially the blind-ending capillaries of the subgastric artery, in which the hemolymph of the shrimp is passing. Once in the lumen of the lymphoid tubule, the hemolymph percolates through the endothelial cells and the tubular walls. Under normal conditions, some haemocytes also routinely pass into the tubular wall and then traverse into the haemal sinuses with the filtrate. In addition, it is believed that the lymphoid organ might be connected with the antennal gland via lymphoid associated-antennal tubules to control the electrolyte balance, osmotic regulation and disposal of wastes (Duangsuwan *et al.*, 2008a).

1.3.3 Media

Culture medium plays a crucial role in developing primary cell cultures and cell lines. A proper culture medium can significantly improve cell conditions. Different media have different characteristics with regard to the concentrations of salts, carbohydrates, amino acids and vitamins, and the buffer. In the field of *in vitro* cell cultures which are derived from penaeid shrimps, there is very limited knowledge on the detailed nutritional requirements. Thus, specifically modified media designed for mammals and insects have been applied for cell cultures from penaeid shrimps in most cases (Toullec, 1999). Leibovitz's 15 (L-15), M-199 and Grace's insect medium has been reported to be fit for cell cultures from penaeid shrimp (George and Dhar, 2010; Jose et al., 2010; Luedeman & Lightner, 1992). Besides them, plenty of other media such as DMEM, MEM, CMRL, MPS, F12, NCTC, Excell 401 and TC-100 have been tested in various studies of shrimp cell cultures but the results were not satisfactory (Figure 13).

L-15 medium has a strong buffering capacity (KH_2PO_4/Na_2HPO_4) and high amino acid concentration. Among the media used, L-15 medium was the most popular choice for penaeid shrimp cell cultures. The 2x L-15 medium was reported to be the best for primary cell culture from the lymphoid organ of *P. monodon* (Assavalapsakul *et al.*, 2003; Catap & Nudo, 2008; Chen & Wang, 1999; Hsu *et al.*, 1995; Wang *et al.*, 2000) and *P. vannamei* (Jose *et al.*, 2010; Lu *et al.*, 1995; Tapay *et al.*, 1995).

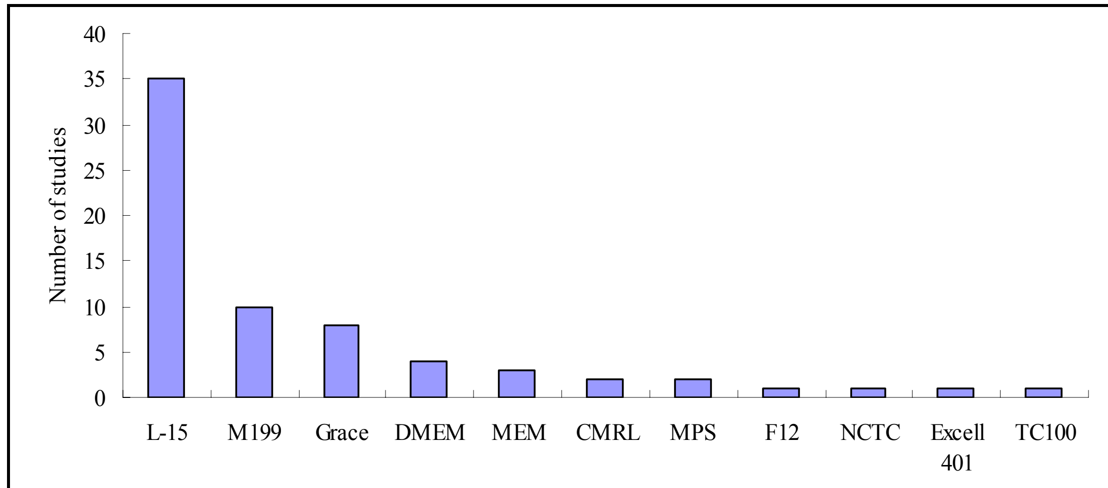

Figure 13 The media used for penaeid shrimp cell culture studies.

1.3.4 Serum and muscle extract

Fetal bovine serum (FBS) is the most commonly used medium supplement (Mothersill *et al.*, 2000) in mammal, insect and fish cell cultures. FBS provides minerals, amino acids, lipids, hormones, growth factors, cytokines and adhesion factors to promote cell attachment and proliferation (Freshney, 2005). The results of many studies indicated that FBS is essential for cell replication, although it seems that FBS does not provide all of the specific hormones and growth factors needed by the cultured cells (Mothersill and Austin, 2000). In penaeid shrimp cell cultures, FBS (10% - 20%) significantly enhanced the cell migration and survival of primary cell cultures (Fan and Wang, 2002; Itami et al., 1999; Shike et al., 2000; Shimizu *et al.*, 2001; Tapay et al., 1997).

Shrimp muscle extract (SME) was another medium supplement specifically used in cell culture from shrimps. Many reports has supported that the shrimp muscle extract could enhance the survival and performance of primary cell cultures (Chen *et al.*, 1986; Ludeman & Lightner, 1992; Nadala *et al.*, 1993). Although there are conflicting reports on the effects of SME on shrimp primary culture, the reasons for these discrepancies are not known. According to George & Dhar (2010), the nature of growth factors present in the tissue extract vary depending on tissue type, animal age and species, the extraction procedure and other unknown factors. Moreover, the composition of hormones and growth factors varies according to the molting and reproductive cycles (Mulford & Villena, 2000).

1.3.5 Growth factors

The effects of specific growth factors have been investigated in various studies. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-1 and IGF-2) are common growth factors that have been tested in shrimp cell cultures.

Epidermal growth factor (EGF) is a 6100-dalton polypeptide containing 53 amino acids (Savage *et al.*, 1972). The addition of epidermal growth factor (EGF) at a concentration of 20 µg/ml was found to markedly enhance growth of cells extracted from lymphoid organ of *P. stylirostris* and *P. vannamei* (Nadala *et al*., 1993). But in contrast, other authors did not obtain any significant effect on growth and multiplication of the cells upon addition of EGF (Fraser & Hall, 1999; Jose et al., 2012).

Basic fibroblast growth factor (bFGF) is a 17500-dalton protein that stimulates plasminogen activator production, collagenase synthesis, DNA synthesis and motility (Presta *et al.*, 1986). For cell cultures of penaeid shrimp, some authors observed enhanced proliferation of cells after administration of bFGF (Fan & Wang, 2002; Hsu *et al*., 1995), while others did not (Fraser & Hall, 1999; Jose *et al.*, 2012; Nadala *et al.*, 1993).

The insulin-like growth factor types 1 and 2 (IGF-1; IGF-2) are both members of the insulin superfamily of peptide hormones (Dupont & Holzenberger, 2003). Jose *et al*. (2009 & 2012) checked various growth factors and observed a better cell growth with 10 ng/ml IGF-1. On the other hand, it has been also reported that insulin growth factor 1 (IGF-1) did not enhance cell growth (Fan & Wang, 2002; Nadala *et al*., 1993). IGF-2 only obtained enhanced growth and proliferation of embryonic cells of *Penaeus chinensis* after administration of IGF-2 in combination with bFGF (Fan & Wang, 2002).

1.3.6 Attachment factors

Attachment factors such as laminin, fibronectin, gelatin, collagen and poly-L-lysine have been tested in cell cultures of penaeid shrimp. Jose *et al.* (2009) found that

fibronectin (20 ng/ml) and poly-L-lysine (200 ng/ml) coated wells were better than laminin (20 ng/ml) coated wells for cell attachment. However, Frerichs (1996) did not observe any increase in adhesiveness of embryonic cells of Macrobrachium rosenbergii in poly-L-lysine and collagen Type I (Sigma) coated culture surfaces. It has been reported that laminin and fibronectin coating did not improve cell attachment for *P. vannamei* hemocytes (Cooke *et al.*, 1989).

1.3.7 Cell separation methods

A proper cell separation method can improve the quality of the cell culture significantly from the very beginning. Different methods that were applied for cell dissociation in shrimp primary cell cultures have been reported with variable results. In most cases, the researchers minced the target tissues as 1 mm^3 cube and seeded them in culture vessels. Finally cell monolayers were gained by cell migration out of these tissue explants (Chen *et al.*, 1986; Han *et al.*, 2013). Another method for cell dissociation was enzymatic digestion. Mulford *et al.* (2000) found that pronase (100 μg/ml) could dissociate the hematopoietic (hpt) cells from Dublin Bay prawn (*Nephrops norvegicus)* well. Jiravanichpaisal *et al.* (2006) prepared the haematopoietic cell cultures from crayfish (*Pacifastacus leniusculus*) via dissociation of the cells with 0.1% collagenase (type I and IV). George and Dhar (2010) used different cell separation methods to compare and evaluate different tissue dissociation and finally they found that cell separation and cell attachment were better in collagenase treatment compared to trypsin treatment. Han *et al.* (2013) reported that non-mammalian-derived enzyme complex of HyQTase could dissociate primary cells from the lymphoid organ of *P. vannamei* and 54% of cells attached again after treatment. But the enzymes might be toxic for shrimp cells. Toullec (1999) found that the pronase and trypsin could damage crustacean cells while dispase and collagenase were less aggressive. It was reported that cells of lymphoid organs from *P. japonicus* failed to survive after treatment with trypsin for cell dissociation (Itami *et al.*, 1999). Toxicity of trypsin has also been observed on the primary cell cultures of *P. vannamei* (George & Dhar, 2010).

1.3.8 Osmolality, pH and temperature

1.3.8.1 Osmolality

Most of the penaeid shrimp live in marine environments and are completely adapted to high osmolality. But in the field of penaeid shrimp cell culture, the osmolality of culture media varies greatly in between studies, ranging from 470 $mOsmol.kg^{-1}$ (Hsu *et al.*, 1995), up to 770 $mOsmol.kg^{-1}$ (Nadala *et al.*, 1993). It is advisable to keep the osmolality close to the physiological values in the haemolymph, 768 $mOsmol.kg^{-1}$ in *P. duorarum*, 699 $mOsmol.kg^{-1}$ in *P. stylirostris*, 718 $mOsmol.kg^{-1}$ in *P. vannamei* (Castille & Lawrence, 1981) and 780 $mOsmol.kg^{-1}$ in *P. indicus* (Paradoestepa *et al.*, 1987) and in *P. chinensis* (Huang *et al.*, 1999). It was also found that the osmotic values differ according to the age. For *P. monodon*, the osmolality varies from 698 $mOsmol.kg^{-1}$ in 10 gram shrimp to 752 $mOsmol.kg^{-1}$ in 30 gram shrimp (Ferraris *et al.*, 1986). McFarland & Lee (1963) reported that the osmotic pressure of sub-adult shrimp is between 830 and 850 $mOsmol.kg^{-1}$, while it is just 745 $mOsmol.kg^{-1}$ and 680 $mOsmol.kg^{-1}$ for juvenile of *P. aztecus* and *P. setiferus*, respectively (Castille & Lawrence, 1981).

The ionic and osmotic parameters of the culture media should be adjusted individually depending on the species and living environments (Najafabadi *et al.*, 1992). The osmolality of the culture medium is adjusted by simply adding extra salt solutions. While most of the authors used simple sodium chloride (NaCl), a few researchers used a complex mixture of salts (Chen & Kou, 1989; Itami *et al.*, 1999). However, the osmotic pressure of shrimp hemolymph is not only evaluated by its salt concentration but also by the amounts of dissolved proteins (Weber & Van marrewijk, 1972).

1.3.8.2 pH

The pH of growth medium is crucial as inappropriate pH may result in poor cell maintenance and growth. The physiological pH of the hemolymph of crustacea ranges from 7.5 to 8.0, which is higher than that of vertebrates (Mulford & Villena, 2000). In the field of shrimp cell cultures, the pH used in growth media is within the range of 7.0 - 7.5 (Fan & Wang, 2002; Gao *et al.*, 2003; Han *et al.*, 2013; Mulford & Austin, 1998; Tapay *et al.*, 1995; Toullec *et al.*, 1996). It varies from 7 (Chen & Kou 1989;

Toullec *et al*., 1996), through 7.2 (Fan & Wang 2002; Jiang *et al*., 2006; Tong & Miao, 1996) and 7.4 (Wang *et al*., 2000), up to 7.5 (Dantas-Lima *et al*., 2012; Gao *et al*., 2003).

1.3.8.3 Temperature

The optimal temperature for cell cultures depends on the body temperature of the host (Freshney, 2005). It is known that shrimp are cultured in water with a temperature between 25°C and 32°C. Therefore, for most authors have incubated cultures of penaeid tissues at 25°C - 28°C (Chen, 1986; Han *et al*., 2013; Itami *et al*., 1999; Jose *et al*, 2012).

1.3.9 Cell cultures for the investigation of the replication cycle of shrimp viruses

After WSSV was firstly detected in Taiwan in 1992, reliable detection and diagnostic techniques have been set up. Most of these techniques were based on PCR and histopathology. A cell culture is a basic tool for the study of pathogen-cell interactions, especially for those pathogens that replicate intracellularly, such as viruses (Jiravanichpaisal *et al*., 2006). For better understanding of the replication cycle of WSSV and other aquatic viruses cell cultures are needed. At present, there are no continuous crustacean cell lines available. That is why primary cell cultures from various species and different tissues have been used. Plenty of these cultures have been tested for their susceptibility to WSSV.

Chen and Kou infected cultured cells from lymphoid organ of *P. monodon* by monodon-type baculovirus (MBV) in 1989. Although there were only parts of the cell monolayer susceptible to MBV, this might be the first successful attempt to culture virus from an aquatic invertebrate *in vitro* (Chen and Kou, 1989). Tapay *et al.* set up a 50% tissue culture infectious dose assay protocol for yellow head baculovirus (1995) and non-occluded baculo-like virus (1997) based on the primary cell culture from lymphoid organ of *P. stylirostris* and *P. vannamei* (Tapay *et al*., 1995 and 1997). Kasornchandra and Boonyaratpalin (1998) established a primary cell culture system from the lymphoid organ of *P. monodon* and this cell culture system was successfully

used for titration of white spot syndrome virus (WSSV) from different tissues of infected *P. monodon.* They proved that the cuticular epidermis gave the highest titer (Kasornchandra *et al*., 1998). In 1999, Itami *et al*. infected primary cell cultures from the lymphoid organ of *P. japonicus* by penaeid rod-shaped DNA virus (PRDV) and reported that the cytopathic effect (CPE) was observed within 8 days (Itami *et al*., 1999). Chen and Wang reported that no CPE was obtained from white spot syndrome virus (WSSV) incubated lymphoid organ cell monolayers (Chen and Wang, 1999) whereas Wang *et al*. (2000) reported that a CPE was observed at 2 days after inoculation. Although there were different experimental conditions, CPE might not be a proper evaluation factor for the virus-incubated primary cell culture system. Kasornchandra *et al.* (1999) established primary cell cultures from the lymphoid organ of *P. monodon* for testing the susceptibility for yellow head virus (YHV) and systemic ectodermal and mesodermal baculovirus (SEMBV). Wang *et al*. (2000) investigated the *in vitro* propagation and morphogenesis of white spot syndrome virus (WSSV) in primary cell cultures from the lymphoid organ of *P. monodon.* Their results showed that six different cell types existed in the primary cell culture and four of these cell types could be infected with WSSV (Wang *et al*., 2000). Maeda *et al*. infected the primary ovarian cultures from *P. japonicus* with WSSV and reported that the levels of WSSV antigens in culture supernatant gradually increased during the period between 24 and 120 h after virus inoculation (Minoru Maeda *et al*., 2004). Jiravanichpaisal *et al*. investigated the replication of WSSV in the haematopoietic cells derived from haematopoietic tissue of freshwater crayfish, *Pacifastacus leniusculus*. They concluded that the infection of WSSV was dependent on the temperature. Replication was not observed at 4 °C. In the following years, Jose *et al.* (2012) and Han *et al*. (2013) proved that the primary cell cultures from the lymphoid organ of *P. monodon, Metapenaeus ensis* and *P. vannamei* were susceptible to WSSV.

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Chapter 2

Aims of Thesis

With the development and expansion of penaeid shrimp culture industry worldwide, viral diseases have become the most serious threat. As one of the most lethal viral pathogens, white spot syndrome virus (WSSV) has already caused huge economic losses in shrimp aquaculture industry. Although the techniques for diagnosis and research have been established based on PCR and histopathology, a better understanding and ultimately antiviral cure of WSSV relies on the development of new-technologies to maintain cell cultures and to examine the WSSV replication in these cells. However, up till now, no cell line is available. A standardized and reproducible technology to produce primary cell culture from shrimp might be a good tool for further research on WSSV replication in its host cells.

The general aims of this thesis were to develop and optimize an *in vitro* cell culture and to study the replication cycle of white spot syndrome virus (WSSV).

The specific aims of this thesis were:

(1) To develop a basic primary cell culture system from the lymphoid organ of *P. vannamei*.

(2) To improve the culture medium of the primary cell culture system and to develop a technology to split primary cells.

(3) To investigate the replication cycle of white spot syndrome virus (WSSV) in cell cultures from the lymphoid organ of *P. vannamei.*

Chapter 3

Characterization of a primary cell culture from lymphoid organ of *Penaeus vannamei* and use for studies on WSSV replication

Wenfeng Li, Van Thao Nguyen, Mathias Corteel, João José Dantas-Lima, Khuong Van Thuong, Vo Van Tuan, Peter Bossier, Patrick Sorgeloos, Hans Nauwynck

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Abstract

Shrimp aquaculture is a booming agro-industry worldwide. Due to intensification of shrimp farming, pathogens emerged. Control of these pathogens especially viral pathogens is essential for a further expansion of this industry. Until now, the lack of shrimp cell lines has limited research on shrimp viral pathogens. In this context, a primary culture from the lymphoid organ of *Penaeus vannamei* was developed and standardized as a platform for further research on white spot syndrome virus (WSSV). Explants from the lymphoid organ of *P. vannamei* were cultured in 2 x L-15 (Leibovitz-15) medium supplemented with 20% fetal bovine serum (FBS), 10% Chen's salt, penicillin (100 IU/ml) & streptomycin (100 μg/ml), gentamycin (50 μg/ml) and fungizone (0.25 μg/ml) with a pH of 7.5. Gelatin (0.1%)-coated culture plates promoted the migration of cells from the explants and cell survival. 600 μg/ml cholesterol and 1000 μg/ml L-glutathione (GSH) both enhanced cell survival and performance *in vitro*. Susceptibility of lymphoid organ cells for infection with white spot syndrome virus (WSSV) was determined by indirect immunofluorescence (IIF) staining by monoclonal antibodies against VP28 (W29) and goat anti-mouse IgG-FITC. FITC positive signals in the nuclei starting from 9 hours post inoculation (hpi) demonstrated the susceptibility of the cells for WSSV infection in these cultures. This cell culture system will be used in the future as a tool for studying host-virus interactions in WSSV pathogenesis.

Introduction

Penaeid shrimp, the most important cultured crustacea, are threatened by diseases worldwide. Mainly viral diseases have resulted in disastrous economic losses to the shrimp agro-industry with white spot syndrome virus as the main shrimp killer (Kasornchandra *et al*., 1995; Wongteerasupaya *et al*., 1995). PCR, immunohistochemistry, immunofluorescence and electron microscopy have been of great value for diagnosis and research. However, lack of shrimp cell cultures and stable continuous cell lines has limited research on this virus. A better understanding of WSSV-associated problems relies on the development of standardized techniques to maintain and culture host cells. Plenty of publications on primary cell cultures of various organs from different shrimp species and using different media appeared already in literature in the past 27 years. The most commonly used species were *Penaeus. monodon* (Hsu *et al*., 1995; Jose *et al*., 2012; Owens *et al*., 1999) and *P. vannamei* (George *et al*., 2011; Lu *et al*., 1995; Nadala *et al*., 1993; Toullec *et al*., 1996). Explant and dissociation methods have been used to obtain cells from diverse tissues and organs, such as lymphoid organ (Assavalapsakul *et al*., 2003; Chen and Kou, 1989; Han *et al*., 2013; Tapay *et al*., 1995; Wang *et al*., 2000), ovary (George and Dhar, 2010; Kasornchandra *et al*., 1999; Luedeman and Lightner, 1992), heart (Chen and Wang, 1999; Goswami *et al*., 2010; Tapay *et al*., 1997), haemocytes (Dantas-Lima *et al*., 2012; Jiang *et al*., 2006; Jose *et al*., 2010; Lang *et al*., 2002), hepatopancreas (Ke *et al*., 1990), nerve cord (Lang *et al*., 2002; Nadala *et al*.,1993; Owens and Smith,1999), muscle (Chen *et al*., 1986; Wang et al., 2000b), hematopoietic tissue (Jiravanichpaisal *et al*., 2006; West *et al*., 1999), gills (Chen *et al*., 1986; Hsu *et al*.,1995; Nadala *et al*.,1993), gut (Nadala *et al*., 1993), eye stalk (George and Dhar, 2010) and embryonic cells (Frerichs *et al*., 1996). Primary cells from lymphoid organ have been used in WSSV research and some could be infected by WSSV (Itami *et al*., 1999; Kasornchandra and Boonyaratpalin, 1998; Kasornchandra *et al*., 1999; Tapay *et al*., 1995 and 1997). Until now, there is no report on primary cell cultures from the lymphoid organ of *P. vannamei* used for white spot syndrome virus (WSSV) research. In the present study, a reproducible protocol was developed for a primary cell culture from the lymphoid organ of

L.vannamei and the susceptibility of these cultured cells for an infection with WSSV was examined.

Materials and Methods

Experimental animals

Penaeus (*Litopenaeus*) *vannamei* post-larvae which were imported from Syaqua Siam Co. Ltd. (Thailand) were certified to be specific pathogen-free for the virus WSSV, TSV, YHV, IHHNV and IMNV. After arrival, the post-larvae were stocked in a recirculating aquaculture system with 3.5% salinity in Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium. These shrimp were reared with commercial pelleted feed. After 4 months, shrimp in premolt stage weighing 20 ± 2 g were used for the collection of lymphoid organ.

Primary cell culture of lymphoid organ

The shrimp were disinfected by immersion in 4.0% hypochlorite solution and 70% ethanol prepared in cold seawater (3.5% salinity) for 2 min, respectively. Finally the shrimp were rinsed several times in sterile cold seawater. The lymphoid organ consists of two white small ovoid-shaped tissues between the lateral side of the stomach and the anterior edge of the hepatopancreas. The organs were rinsed in washing medium (2 x L-15 (Sigma Aldrich), supplemented with 20% FBS, 10% Chen's salt (Chen and Kou, 1989), penicillin (1000 IU/ml, Gibco) & streptomycin (1000 µg/ml, Gibco), gentamycin (500 µg/ml, Gibco) and fungizone (2.5 µg/ml, Gibco) (pH: 7.5)) four times and chopped into 0.5 mm^3 cubic explant. All the explants were transferred into wells of 24-well plates with 150 µl of culture medium per well and after 12 h post seeding another 750 µl of culture medium was added into each well. Basic culture medium consisted of 2 x L-15 supplemented with 20% fetal bovine serum, 10% Chen's salt, penicillin (100 IU/ml) & streptomycin (100 µg/ml), gentamycin (50 µg/ml) and fungizone (0.25 µg/ml) (pH: 7.5). The cultures were incubated at 27 °C and examined every day with an inverted microscope. One third of the culture medium was changed every 3 days.

Effect of coating on primary cell cultures of lymphoid organ

Gelatin (0.1%, w/v) and poly-L-lysine (0.005%, Sigma Aldrich) were tested as attachment factors. In brief, the 24-well plate (with glass insert) was coated with 1000 µl per well gelatin or poly-L-lysine for 2 h at 37 °C. Then, the excess of fluid was removed and all coated wells were washed twice by UP water. Explants were introduced into the coated wells and cultured with 1 ml basic culture medium at 27 °C. The control wells were without coating. The number of living cells per explant was counted at 48 h, 96 h and 144 h.

Effect of cholesterol on primary cell cultures of lymphoid organ

Cholesterol is an essential molecule for living shrimp. In the hemolymph of *P. vannamei*, the concentration of cholesterol is 1480 µg/ml (Najafabadi *et al*., 1992), which is much higher than in FBS (330 µg/ml). Therefore, the effect of different concentrations of cholesterol was examined. Cells from lymphoid organ were cultured with different concentrations of cholesterol (66 µg/ml, 200 µg/ml, 600 µg/ml and 1800 µg/ml, Sigma Aldrich) in 24-well plates at 27 °C. The number of living cells per explant was counted at 48 h, 96 h and 144 h.

Effect of L-glutathione on primary cell cultures of lymphoid organ

L-glutathione is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species. The cells from the lymphoid organ were cultured with different concentrations of L-glutathione (0 µg/ml, 500 µg/ml, 1000 µg/ml and 2000 µg/ml, Sigma Aldrich) in 24-well plates at 27 °C. The number of living cells per explant was counted at 48 h, 96 h and 144 h.

Cell counting

Hoechst and EMA stainings were performed to count total and dead cells. The cultures on glass inserts were subsequently (i) incubated with 200 µl EMA solution (20 µg/ml in medium, Invitrogen) for 30 min in dark and on ice, (ii) exposed

to candescent light for 10 min on ice, (iii) fixed with 500 µl of 4% paraformaldehyde for 10 min, (iv) incubated with 200 µl Hoechst solution (10 µg/ml in PBS, Sigma Aldrich) for 10 min, (v) washed once with PBS and once with UP and finally mounted upside down on top of 2 µl of glycerin-DABCO on slides. All samples were checked by fluorescence microscopy.

Identification of hemocytes in primary cell cultures of lymphoid organ

The cells from the lymphoid organ were cultured on glass inserts in 24-well plates at 27 °C and hemocytes were stained with the hemocyte-specific monoclonal antibody WSH8 (Van de Braak *et al*., 2001) every 24 h post seeding to determine the proportion of haemocytes. The procedure was as follows: the glass inserts with cells were immersed in 200 µl of 4% paraformaldehyde for 10 min and in 200 µl of 0.1% Triton X-100 for 5 min at room temperature. After washing in PBS for 5 min, the glass inserts were incubated in 200 µl of monoclonal antibody WSH8 (1:100) at 37 °C for 1h. Then after three washings with PBS for 5min, they were incubated with goat anti-mouse IgG-FITC (1:100, Sigma Aldrich) at 37 °C for 1 h. Afterwards, the cultures were washed again three times with PBS and incubated with Hoechst solution (10 µg/ml in PBS, Sigma Aldrich) in dark for 10 min. After a washing with PBS and UP, they were mounted on a slide with glycerin-DABCO and stored at 4 °C.

Authentication of cells cultured *in vitro*

Especially in invertebrate cell culture, contaminations from protozoa and thraustochytrids occur. Authentication is hence needed. After 6-day culture, the cultured cells were collected for DNA extraction. DNA extraction was done according to Bossier *et al.* (2004). DNA was also extracted from shrimp tissue. The primer combinations Vanna16S (forward) / Vanna12S (reverse) were used in order to amplify the 1800 bp fragment of mitochondrial rDNA by PCR in a Hybaid PCR Express (Labsystemsk, Belgium). The primer sequences were as follows: Vanna16S: 5'-CCGGTCTGAACTCAGATC-3',Vanna12S: 5'-AACCAGGATTAGATACCC-3'. The thermal cycler PCR conditions were as follows: 1 cycle of 94 °C for 2 min, 34 cycles of 1 min 15 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C and a final extension cycle

of 72 °C for 10 min. After purification, the PCR fragments were sequenced with primers Vanna16S / Vanna12S and blasted against the EMBL-EBI database.

Preparation of WSSV inoculum

50 g shrimp body without stomach and hepatopancreas from moribund WSSV (Thailand strain) infected shrimps was minced in 100 ml shrimp PBS on ice. The extract was centrifuged at 5500 g for 30 min at 4 °C. Then, supernatant was collected and passed through a 0.45 µm membrane (Sarstedt). This WSSV stock (10^8 SID_{50} / ml) was diluted 10 times in 2 x L-15 containing 10% Chen's salt and 500 µg/ml lactalbumin hydrolysate before use. The negative stock was prepared from healthy shrimp following the same procedure.

Detection of WSSV infected cells in lymphoid cell cultures by indirect immunofluorescence (IIF)

The lymphoid cell cultures (15 explants / well, 96 h old) were prepared on gelatin (0.1%) coated glass inserts in 24-well plates. 200 µl of WSSV solution ($10^{6.3}$ SID_{50}) was added to each well with lymphoid cell culture and incubated at 27 °C for 1 h. At the same time, the negative control was inoculated with 200 µl of healthy shrimp stock per well. Then, the inoculum was removed and cells were washed three times with medium. 1 ml of medium was added to each well and the plates were incubated at 27 °C. Cell cultures were fixed at 3 h, 6 h, 9 h, 12 h and 24 h post inoculation and stained. In brief, the samples were fixed in 200 µl of 4% paraformaldehyde for 10 min and then in 200 µl of 0.1% Triton X-100 for 5 min at room temperature. After one washing with PBS for 5 min, the cell cultures were incubated in 200 µl of monoclonal antibody w29 (1:100 in PBS, Chaivisuthangkura *et al.*, 2004) which is directed against WSSV viral protein VP28 at 37 °C for 1 h. Then, after three washes with PBS for 5 min, they were incubated in goat anti-mouse IgG-FITC (1:100 in PBS, Sigma Aldrich) at 37 °C for 1 h. After three washes with PBS for 5 min, the cell cultures were incubated in Texas Red-X Phalloidin (1:40 in PBS, Invitrogen) at 37 °C for 1 h. Then after three washes with PBS for 5 min, the cell cultures were incubated in Hoechst solution (10 µg/ml in PBS, Sigma Aldrich) for 10 min. After washings with PBS and twice with UP water for 5 min, the cultures were mounted on slides with

glycerin-DABCO and stored at 4 °C. The inoculated cell cultures were analyzed with a confocal fluorescence microscope. All images were processed and merged by ImageJ software.

Statistical analysis

All results given in this paper were average values from three independent replicates with standard deviation. The effects of different treatments were statistically analyzed by ANOVA in SPSS 19.0. Differences were considered significant at $P < 0.05$.

Results

Characteristics of a cell culture from lymphoid organ

The lymphoid organ explants of *P. vannamei* stuck well to the polystyrene surface of culture wells and once attached, cells started to migrate out of the explants. The first migrating cells had a round appearance and these cells stayed in the surroundings of the explants in suspension or semi-suspension at 24 h post seeding (Figure 1A). These cells attached to the surface of the culture well and changed their shape. They became fusiform with branches (Figure 1B). Afterwards, fibroblast-like cells were migrating from the explants. Many round cells were seen between and on top of the fibroblast-like cells (Figure 1C). Islands of epitheloid cells were observed in the cultures at 72 h post seeding (Figure 1D). The final cell monolayer consisted of three cell types: round cells, fibroblast-like cells and epitheloid cells.

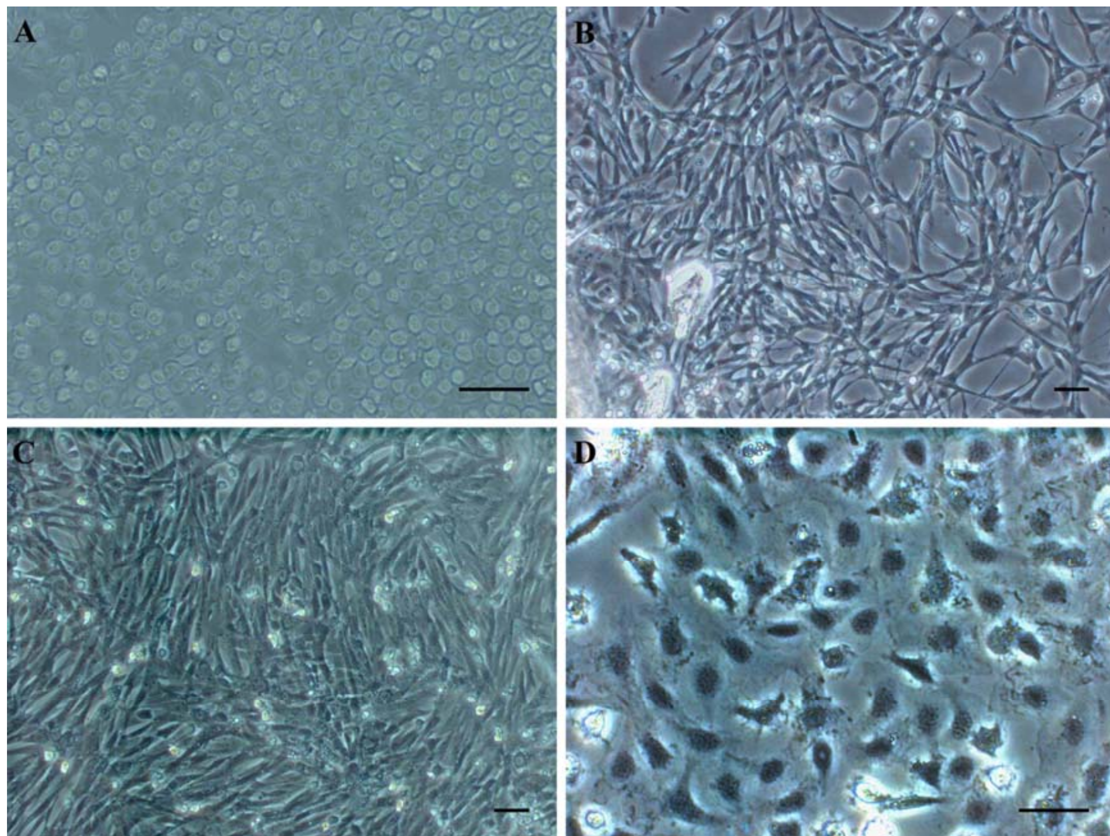

Figure 1. Monolayer of primary cell culture developed from lymphoid organ of *P. vannamei*. A: round cells migrating from explants at 24 h post seeding; B: fusiform cells with branches transformed from round cells at 48 h post seeding; C: fibroblast-like cells that grew out from explants at 48 h post seeding; D: epitheloid cells at 72 h post seeding (scale bar = 30 μm).

Effect of coating on lymphoid cell culture

The effect of coating materials (gelatin and poly-L-lysine) on growth of primary lymphoid cell cultures was evaluated (Figure 2). Significant differences in number of living cells/explant between gelatin (0.1%) coated wells and control wells were obtained at 48 h ($p < 0.05$), 96 h ($p < 0.001$) and 144 h ($p < 0.001$). The number of living cells/explant in gelatin (0.1%) coated wells and poly-L-lysine coated wells was also significantly different at 96 h ($p < 0.001$) and 144 h ($p < 0.001$). No significant difference was obtained between poly-L-lysine group and control group.

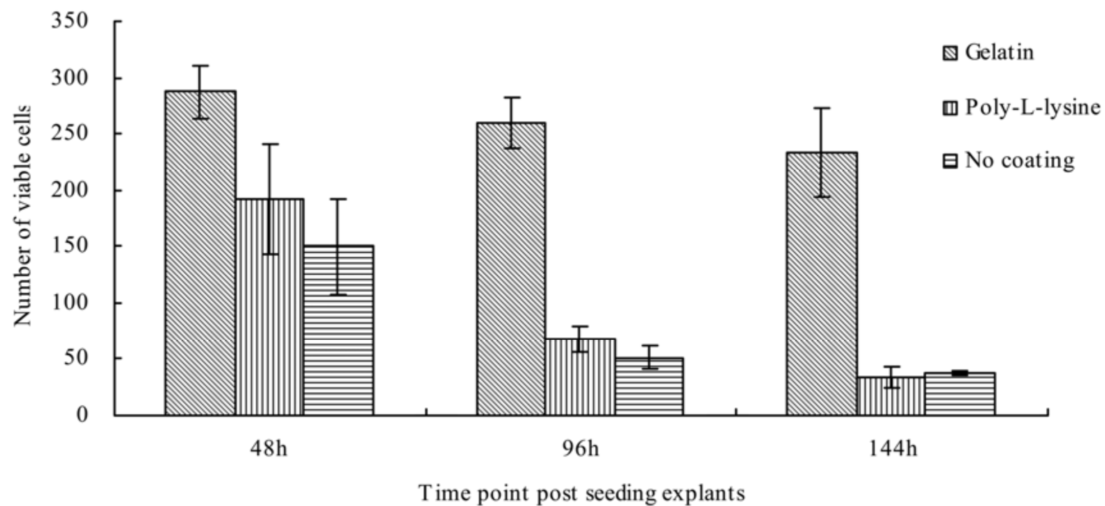

Figure 2. Effect of coating (gelatin and poly-L-lysine) on the performance of primary cell cultures developed from lymphoid organ of *P. vannamei* at 48 h, 96 h and 144 h post seeding.

Effect of cholesterol on lymphoid cell culture

The effect of different concentrations of cholesterol (66 µg/ml (control), 200 µg/ml, 600 µg/ml and 1800 µg/ml, Sigma Aldrich) on the growth of primary lymphoid cell cultures was evaluated (Figure 3). The highest number of living cells/explant was obtained with 600 µg/ml cholesterol, followed by 200 µg/ml cholesterol. Significant differences in number of living cells/explant between 600 µg/ml and 66 µg/ml cholesterol were obtained at 48 h ($p < 0.01$), 96 h ($p < 0.001$) and 144 h ($p < 0.001$). The number of living cells/explant cultured in the presence of 200 µg/ml cholesterol was also significantly different with the controls at 48 h ($p < 0.05$), 96 h ($p < 0.01$) and 144 h ($p < 0.001$) post seeding. Between 200 µg/ml and 600 µg/ml cholesterol there was no significant difference. No significant difference was obtained between 1800 µg/ml and 66 µg/ml (control).

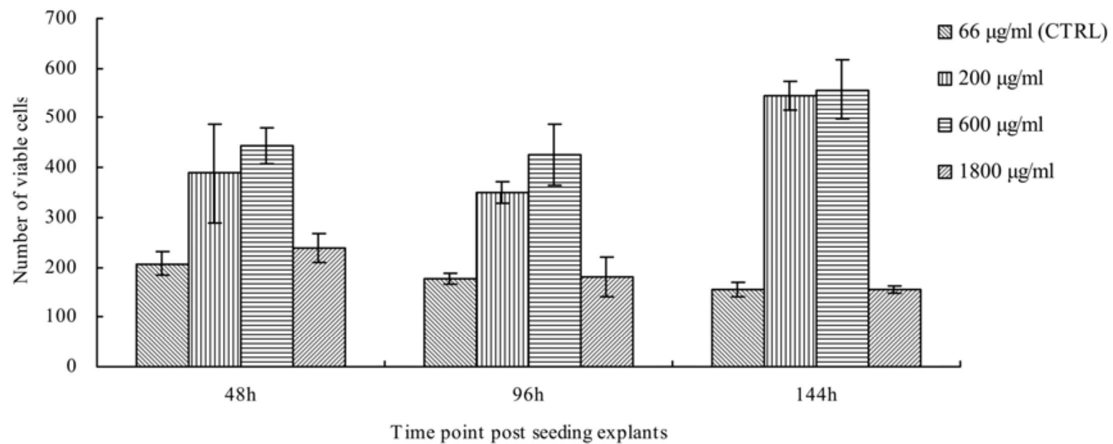

Figure 3. Effect of cholesterol on the performance of primary cell cultures developed from lymphoid organ of *P. vannamei* at 48 h, 96 h and 144 h post seeding.

Effect of L-glutathione on lymphoid cell culture

In this study, different concentrations of L-glutathione (0 μg/ml, 500 μg/ml, 1000 μg/ml and 2000 μg/ml) were added to the culture medium to evaluate their effect on cell performance. Overall, cells treated with 1000 μg/ml and 500 μg/ml of L-glutathione showed better results on the number of living cells/explant (Figure 4). Significant differences on living cell number/explant between the 1000 μg/ml L-glutathione group and controls were obtained at 48 h ($p < 0.01$), 96 h ($p < 0.05$) and 144 h ($p < 0.01$) after incubation. The living cell number in the 500 μg/ml L-glutathione group was also significantly different from that of the controls at 48 h ($p < 0.05$) and 144 h ($p < 0.01$) post seeding. With 2000 μg/ml L-glutathione, no significant difference with the control was recorded at 144 h post seeding.

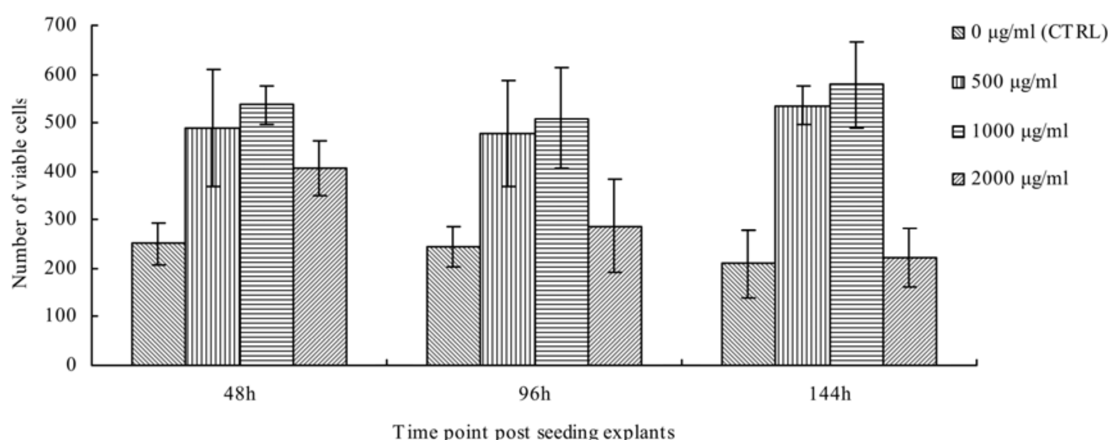

Figure 4. Effect of L-glutathione on the performance of primary cell cultures developed from lymphoid organ of *P. vannamei* at 48 h, 96 h and 144 h post seeding.

Final culture medium formula

In this experiment, basic culture medium supplemented with either 1000 µg/ml L-glutathione or 600 µg/ml cholesterol or 1000 µg/ml L-glutathione + 600 µg/ml cholesterol were evaluated on their effect on cell performance at 48 h, 96 h and 144 h post seeding explants (Figure 5). Significant differences on number of living cells/explant between the medium with cholesterol (600 µg/ml) + L-glutathione (1000 µg/ml) and medium with cholesterol (600 µg/ml) were obtained at 48 h ($p < 0.05$), 96 h ($p < 0.05$) post seeding. Significant differences on number of living cells/explant between the medium with cholesterol (600 µg/ml) + L-glutathione (1000 µg/ml) and medium with L-glutathione (1000 µg/ml) were obtained at 48 h ($p < 0.05$), 96 h ($p < 0.001$) and 144 h ($p < 0.05$) post seeding.

Based on these results above, the final culture medium was defined: 2 x L-15 (Sigma Aldrich) supplemented with 20% FBS, 10% Chen's salt, 600 µg/ml cholesterol, 1000 µg/ml L-glutathione, penicillin (100 IU/ml) & streptomycin (100 µg/ml), gentamycin (50 µg/ml) and fungizone (0.25 µg/ml) (pH: 7.5). The cell monolayer was formed completely or partly in 4 - 6 days post seeding explants.

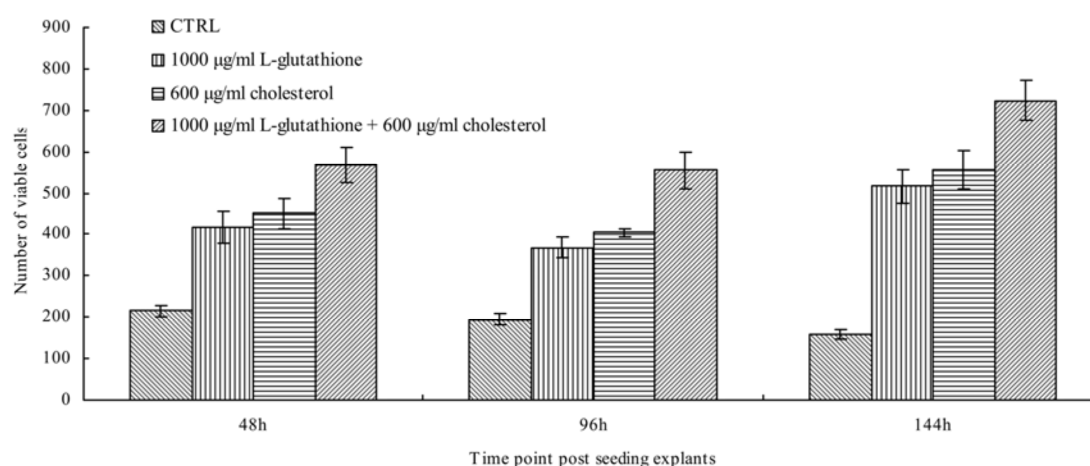

Figure 5. Effect of basic culture medium with L-glutathione (1000 µg/ml) and / or cholesterol (600 µg/ml) on performance of primary cell cultures developed from lymphoid organ of *P. vannamei* at 48 h, 96 h and 144 h post seeding.

Proportion of WSH8-positive cells (hemocytes) in primary lymphoid cell culture

WSH8 is a monoclonal antibody directed against a component of granules of hemocytes (van de Braak *et al*., 2001). It is a marker for haemocytes in shrimp. In order to determine the proportion of WSH8-positive cells in primary cell cultures from the lymphoid organs of *P. vannamei*, an IIF staining was performed using monoclonal antibody WSH8. The data showed that the proportion of WSH8 positive cells decreased gradually from 81 ± 4% at 24 h of cultivation to 49 ± 5% at 72 h post seeding and 12 ± 1% at 96 h post seeding (Figure 6).

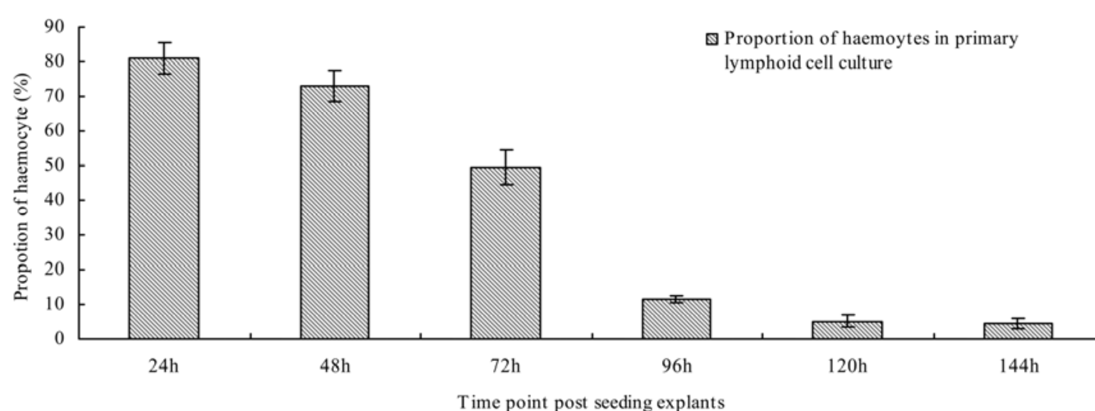

Figure 6. Proportion of WSH8-positive cells (hemocytes) in primary cell cultures developed from lymphoid organ of *P. vannamei* at different time points post seeding.

Authentication of the cells cultured *in vitro*

The first 20 hits from the blast of the sequence of the PCR fragment obtained from cultured cells against the EMBL-EBI database were all from *P. vannamei*. The sequence of the PCR fragment obtained from cultured cells showed 99% identity with the sequence of mitochondrial rDNA of *P. vannamei* from EMBL-EBI (DQ534543.1: *P. vannamei* mitochondrion, complete genome), indicating that the cultured cells were from the shrimp *P.vannamei* , and not from contaminations.

WSSV replication in primary lymphoid cell cultures

Upon WSSV inoculation, WSSV VP28-positive signals were observed in some nuclei of infected cells starting from 9 h post virus inoculation (Figure 7 9 hpi) and became more widespread at 24 h post inoculation (Figure 7 24 hpi).

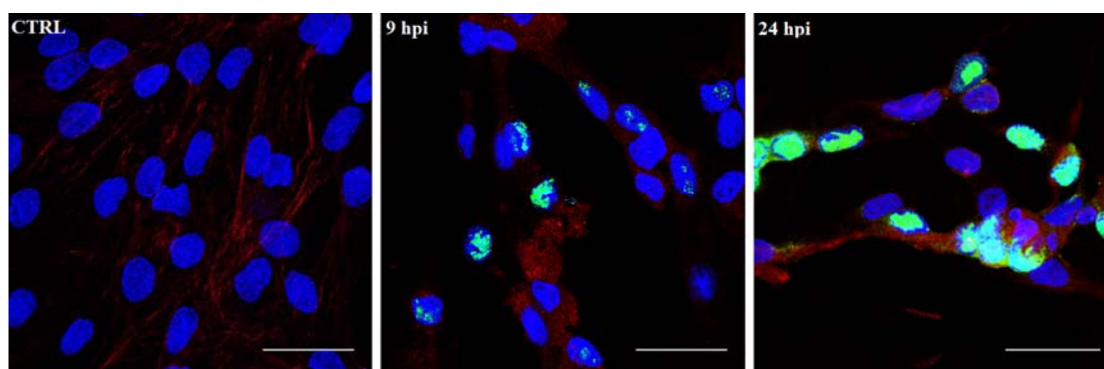

Figure 7. Indirect Immunofluorescence (IIF) staining of WSSV VP28 in primary lymphoid cell cultures (4-day old) from *P. vannamei* at 9 h and 24 h post inoculation (scale bar = 30 μm). Blue: Hoechst staining for nuclei; Green: W29 and F2761-FITC staining for WSSV VP 28; Red: Texas Red-X Phalloidin staining for cytoplasm.

Discussion

In the present study, the coating and composition of the medium for making primary cell cultures from the lymphoid organ of *L.vannamei* were developed. Compared with other tissues and organs of penaeid shrimp, the lymphoid organ was preferred by most researchers due to its good performance *in vitro* (Assavalapsakul *et al.*, 2003; Chen and Kou, 1989; Chen and Wang, 1999; Hsu *et al.*, 1995; Hu *et al.*, 2008; Itami *et al.*, 1999; Jose *et al.*, 2012; Lang *et al.*, 2002; Nadala *et al.*, 1993; Shike *et al.*, 2000; Tapay *et al.*, 1997; Tong *et al.*, 1996; Tong and Miao, 1996). A few of those primary cell cultures from lymphoid organ of *P. monodon* (Kasornchandra *et al.*, 1998 and 1999), *Penaeus stylirostris* (Tapay *et al.*, 1995 and 1997) and *Marsupenaeus japonicus* (Itami *et al.*, 1999) were reported to be susceptible to white spot syndrome virus. Up till now, there was no report on the susceptibility of primary cell cultures of lymphoid organ of *L.vannamei in vitro.*

In primary cell cultures of the lymphoid organ, the cells migrating from the explants changed in time post seeding. In the first 24 h, round cells dominated in the proximity

of explants, which were reported by Jose *et al*. (2012), Tsing *et al.* (1989) and Wang *et al*. (2000). In the present study these cells were confirmed to be haemocytes by monoclonal antibody WSH8, a marker for granules of shrimp haemocytes (Van de Braak *et al*., 2001). After 24 h cultivation, fibroblast-like cells started to grow out from explants and epitheloid cells were usually observed after 48 h. These two cell types were also reported before (Itami *et al*., 1999; Wang *et al*., 2000). The percentage of haemocytes in the cultures decreased gradually during the experiment, dropping from 81 ± 4% at 24 h post seeding to 12 ± 1% at 96 h post seeding. This drop may be explained by (i) the increase of non-haemocyte cells by migration and proliferation, (ii) death of haemocytes and (iii) losing the granule marker by differentiation.

Double strength L-15 medium with 20% FBS gave good results on cell survival and performance of primary cell cultures of lymphoid organ in the present study, which supported earlier reports (Ellender *et al*., 1992; Fan and Wang, 2002; Itami *et al*., 1999; Jose *et al*., 2010; Nadala *et al*., 1993; Shike *et al*., 2000; Shimizu *et al.*, 2001; Tapay *et al*., 1997). The osmolality of growth media varied greatly in between studies, ranging from 470 $mOsmol.kg^{-1}$ (Hsu *et al.*, 1995), up to 770 $mOsmol.kg^{-1}$ (Nadala *et al.*, 1993). The osmolality of adult *P. vannamei* shrimp weighing around 20 gram, cultured in 35 ppt seawater, was determined to be 900 $mOsmol.kg^{-1}$. In culture medium, the osmolality was increased to about 900±20 $mOsmol.L^{-1}$ by 10% Chen' Salt mixture (Chen *et al*., 1989). The pH of growth medium is crucial as inappropriate pH may result in poor cell maintenance and growth (Mulford and Villena, 2000). In the field of shrimp cell cultures, the pH used in growth media is within the range of 7.0 - 7.5 (Fan and Wang, 2002; Gao *et al.*, 2003; Mulford and Austin, 1998; Tapay *et al.*, 1995; Toullec *et al.*, 1996). During earlier studies in our laboratory, pH values were tested for culturing haemocytes and 7.5 was chosen to be the best (Dantas-Lima *et al*., 2012). In the present study, culture medium with a pH of 7.5 gave good results.

Gelatin and poly-L-lysine are both commonly used coating materials in cell culture research. In our experiment, gelatin (0.1%) and poly-L-lysine (0.005%) both showed good results at the first 48 h post seeding explants. However, gelatin performed significantly better than poly-L-lysine at 96 h and 144 h post seeding explants. Gelatin contained higher concentrations of glycine (27.5%) and proline (16.4%) (Eastoe, 1955), which were also much higher than other amino acids in shrimp body

(Vázquez-Ortiz et al., 1995). This might be helpful for the attachment and migration of lymphoid cells from *P. vannamei*.

Cholesterol is an important precursor of steroid hormones and is the major sterol in shrimp (Gong *et al*., 2000). Like other crustaceans, shrimp are unable to synthesize sterols and thus require cholesterol from dietary source for growth, development and survival (Fox *et al*., 1994). Kanazawa *et al*. (1971) reported that cholesterol is required in the shrimp diet for normal growth. Kasornchandra *et al*. (1999) reported that 0.01% cholesterol (100 μg/ml) enhances growth and performance of cells from lymphoid organ of *P. monodon.* In the present study, 600 μg/ml cholesterol significantly enhanced survival and performance of lymphoid cells from *P. vannamei in vitro*.

L-glutathione (GSH) is very important for cell growth and viability. Addition of GSH has been reported to improve mammalian tissue and cell cultures (Ozawa *et al.*, 2006; Wang and Day, 2002). However, in the field of cell cultures of crustaceans, there has been no report on the effect of GSH towards cell growth. GSH has been proven to be helpful for hemocyte survival of *P. vannamei in vitro* (Dantas-Lima *et al*., 2012), which might be due to the strong anti-oxidant activity of GSH. In the present study, a concentration of 1000 μg/ml in culture medium was found to give the highest viable cell number.

In the present study, 96-hr-old primary lymphoid cell cultures were inoculated with WSSV. VP28 was detected in nuclei of lymphoid cells staining for 9 hpi and positive signals became more widespread at 24 hpi. In the near future, more work will be done on the replication cycle of WSSV in the lymphoid cell cultures of *P. vannamei*.

Acknowledgements

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Chapter 4

Eye extract improves cell migration out of lymphoid organ explants of *P. vannamei* and viability of the primary cell cultures

Wenfeng Li, Vo Van Tuan, Khuong Van Thuong, Peter Bossier, Hans Nauwynck

In Vitro Cellular & Developmental Biology - Animal

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Abstract

Since no cell line from shrimp has been established up till now, an optimization of the primary cell culture protocol is necessary. In this context, the effect of extracts (supernatant of a 1:50 (w/v) suspension) from different shrimp organs (muscle, brain, ganglia, eyestalk, ovary and eye) on the performance of primary lymphoid cell cultures was evaluated. 10% of eye extract and 3% of ovary extract enhanced maximally the migration and survival of cells of lymphoid organ of *P. vannamei* significantly at 48, 96 and 144 h post seeding. Extracts from eyestalk (10%), muscle (10%) and brain (1%) significantly promoted the migration and survival of cells at 48 and 96 h post seeding but not anymore at 144 h post seeding. In conclusion, it may be advised to add 10% of eye extract or 3% of ovary extract to cells for the maximal health of primary cell cultures from the lymphoid organ of *P. vannamei*.

Introduction

Viral pathogens are serious threats for shrimp culture industry. Lack of continuous cell lines hampers better insights in the pathogenesis and control of viral diseases (Lightener and Redman 1998). In the last 30 years, plenty of attempts were focusing on the development of primary cell cultures from different organs of different shrimp species (Chen *et al*. 1986; Li *et al.* 2014). Some of these primary cell culture systems were already reported to be useful for studying virus-cell interactions (Itami *et al*. 1999; Kasornchandra *et al*. 1998). However, lack of knowledge on shrimp physiology and endocrinology limited the further development of shrimp cell cultures. Serum is generally used as a powerful medium supplement in cell cultures. It is a source of growth factors, hormones, proteins, lipids, additional amino acids, vitamins and trace elements (Gstraunthaler 2003). In the beginning of the development of insect cell cultures, plasma and hemolymph from insects were used with the same purpose (Grace, 1962). In shrimp cell culture, hemolymph and muscle extract have also been reported to be beneficial for the growth of cultures (Han *et al*. 2013; Tapay *et al*. 1995). In this context, extracts from different organs were tested on the behavior of primary cell cultures from the lymphoid organ of *P. vannamei* that were prepared following the technology of Li *et al*. (2014).

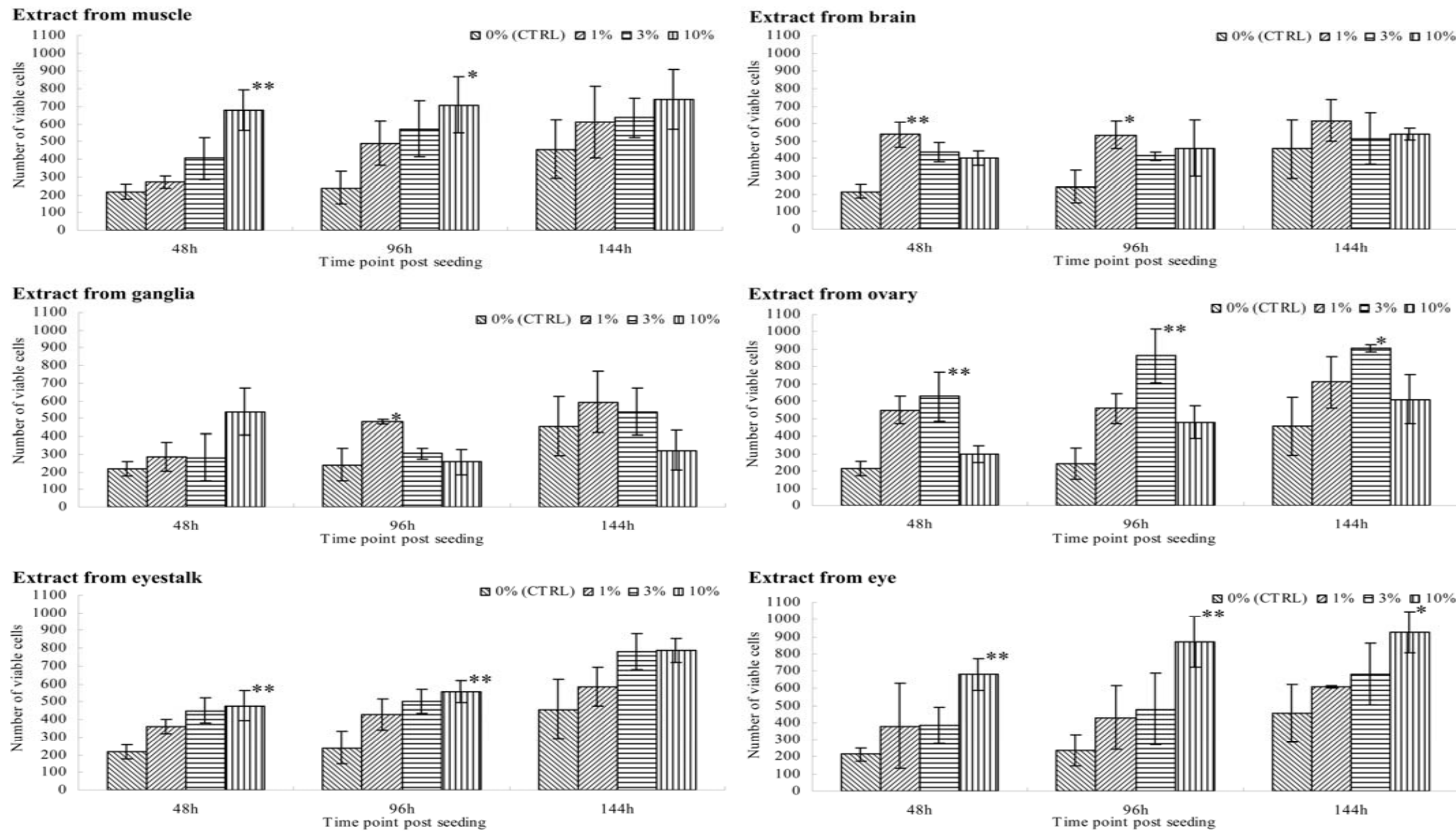
Materials and Methods

Specific pathogen-free *Penaeus vannamei* in premolt stage weighing 20 ± 2 g were used for the collection of lymphoid organs. The primary cell cultures were prepared as described previously (Li *et al.* 2014). Extracts were obtained from different organs of *P. vannamei*: brain, ganglia, muscle, eyestalk, ovary and eye. In general, the organs were weighed, minced and brought into 2x L-15 at a ratio of 1:50 (w/v), respectively. Then, the mixtures were centrifuged at 5500g for 30 min at 4°C. Finally the supernatants were collected and passed through a 0.45 μm filter (Sarstedt) and stored at -70 °C before use. Cells from lymphoid organ were cultured in basic culture medium (2 x L-15 supplemented with 20% fetal bovine serum (FBS), 10% Chen's

salt, penicillin (100 IU/ml) & streptomycin (100 µg/ml), gentamicin (50 µg/ml) and fungizone (0.25 µg/ml) (pH: 7.5)) with different concentrations (0% (CTRL), 1%, 3% and 10%) of extracts from different organs in 24-well plates at 27 °C. The number of viable cells per explant was counted at 48 h, 96 h and 144 h. The Hoechst and ethidium monoazide bromide (EMA, Invitrogen) stainings were performed to count total and dead cells as described before (Li *et al.* 2014). All results given in this context were average values from three independent replicates with standard deviation. The effects of different treatments were statistically analyzed by ANOVA in SPSS 19.0. Differences were considered significant at $P < 0.05$.

Results

The effect of different concentrations of extracts from shrimp organs (brain, ganglia, muscle, eyestalk, ovary and eye) on the performance of primary lymphoid cell cultures was evaluated. All the results are presented in Figure 1. At 48 h post seeding, significant differences in number of living cells/explant were obtained with the extracts from muscle, brain, ovary, eyestalk and eye of *P. vannamei* compared with the control culture. The highest number of living cells/explant was observed with 10% muscle extract, followed by 10% eye extract, 3% ovary extract, 1% brain extract and 10% eyestalk extract. No significant differences were found with ganglia extract. At 96 h post seeding, the number of living cells/explant was significantly different with the extracts from muscle, brain, ovary, eyestalk, eye and ganglia of *P. vannamei* compared with the control culture. The highest number of living cells/explant was obtained with 10% eye extract, followed by 3% ovary extract, 10% muscle extract, 10% eyestalk extract, 1% brain extract and 1% ganglia extract. At 144 h post seeding, the number of living cells/explant with the extracts from ovary and eye of *P. vannamei* differed significantly with the control culture. The highest number of living cells/explant was reached with 10% eye extract, followed by 3% ovary extract. No significant difference was detected with extracts from 10% muscle, 1% brain, 1% ganglia and 10% eyestalk of *P. vannamei* (Figure 2). In conclusion, for the primary lymphoid cell culture, the 10% extract from eye and 3% extract from ovary showed the best results on cell migration and performance.



In comparison with the control culture, *: significantly different ($p<0.05$); **: very significantly different ($p<0.01$).

Figure 1 Effect of extracts from different organs of *P. vannamei* (muscle, brain, ganglia, ovary, eyestalk and eye) on the performance of primary cell cultures from the lymphoid organ of *P. vannamei* at 48 h, 96 h and 144 h post seeding.

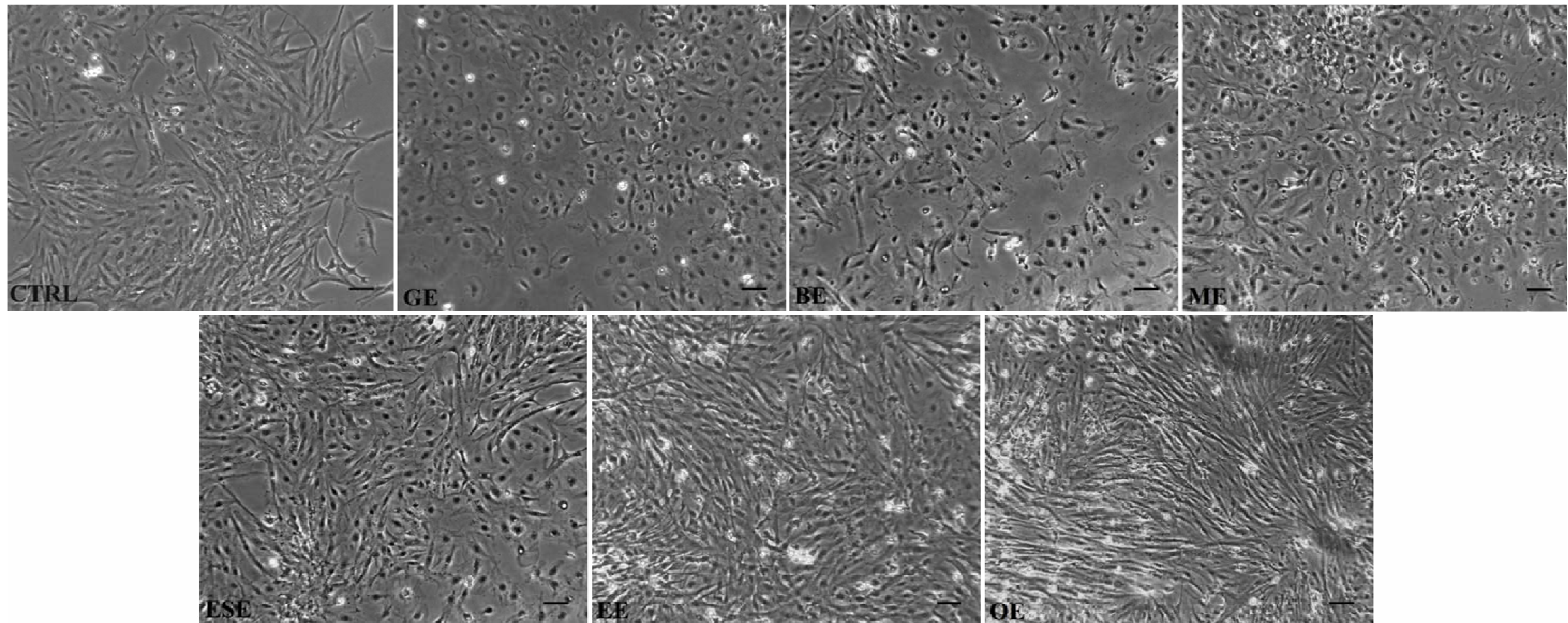


Figure 2 Cell cultures from the lymphoid organ of *P. vannamei* supplemented with different organ extracts (ganglia, brain, muscle, eyestalk, eye and ovary) at 144 h post seeding (Scale bar = 30 μm). CTRL: control group; GE: 1% extract from ganglia; BE: 1% extract from brain; ME: 10% extract from muscle; ESE: 10% extract from eye stalk; EE: 10% extract from eye; OE: 3% extract from ovary.

Discussion

The development of shrimp cell cultures began in the 1980s (Chen *et al*. 1986). Until now, almost all media and media supplements that were applied in shrimp cell culture came from mammal and insect cell cultures (George and Dhar 2010; Jose *et al*. 2012). At present, the mammal cell culture medium Leibovitz-15 is the most popular medium used in shrimp cell culture (Han *et al*. 2013). FBS is an essential supplement in shrimp cell culture (Chen and Kou 1989; Jose *et al*. 2012). Shrimp hemolymph and muscle extract were the most common additional supplements for shrimp cell culture practices up till now. However, the results obtained with hemolymph were quite contradictory in between different researchers. Chen (1986) proved that the hemolymph from shrimp enhanced the cell migration out of explants but Tong and Miao (1996) reported that hemolymph had an inhibitory effect. This difference might be due to the different shrimp species or the methods for collecting hemolymph. Muscle extract has generally been proven to promote the performance of cells *in vitro* (Han *et al.* 2013; Nadala *et al.* 1993), which was in agreement with the results from the present study.

The extracts from different shrimp organs (brain, ganglia, muscle, eyestalk, ovary and eye) could supply cells with certain unknown essential factors such as proteins, amino acids, lipids, growth factors and hormones. For example, in ovary, during the gonadotrophic cycle, ovarian proteins increase from virtually undetectable levels in undeveloped ovaries to > 400 mg in mature ovaries (Rankin *et al.* 1989). Further, dramatic accumulation of lipids occurs during vitellogenesis, up to a concentration of 18% - 41% (in various species) of the total ovarian dry mass at the end of ovarian maturation (Teshima and Kanazawa 1983; Castille and Lawrence 1989). By the end of oocyte development, approximately 30% of the total fatty acid content of both phospholipids and triacylglycerols is made up of polyunsaturated fatty acids in the ovary (Ravid *et al.* 1999).

Kamemoto (1985) reported that the physiological processes in crustaceans are mainly regulated by neuroendocrines of natural peptides, which are produced in brain, thoracic ganglion, X-organ-sinus gland system and pericardial organ. The X-organ and sinus gland (SG) are located in the optic ganglia in the eyestalk (Wanlem et al.

2011). In the present study, the brain (1%) and eyestalk extracts (10%) promoted the survival and performance of cells cultured *in vitro*. It is possible that the neuroendocrines produced in brain and X-organ-sinus gland are involved in the stimulation of the cell metabolism. Eyestalk extract was tested before by Nadala (1993) and the eyestalk extract showed less ability to enhance the performance of cells *in vitro* than the shrimp head extract, which contained brain and eye extract. In the present study, the eye extract promoted the performance of cells cultured *in vitro* better than eyestalk extract which supports the power of eye components. The crustacean hyperglycemic hormone (CHH), which regulates various aspects of growth, reproduction, and metabolism (Le Blanc 2007), has been detected in the retina of the crayfish *Procambarus clarkia* (Escamilla Chimal *et al.* 2001) but no information is available for *P. vannamei*. In addition, Fisher et al. (1952) found a very large quantity of retinoid (vitamin A) in the eyes of euphausiid species. 11-cis or all-trans retinol were found in most mantis shrimp species (Goldsmith and Cronin 1993). Retinoid and retinol are a class of molecules derived from vitamin A that are considered to be crucial for growth, development, and homeostatic processes in animals. Shiau and Chen (1999) reported that the shrimp fed diets supplemented with 300 retinol equivalent vitamin A/kg had a significantly greater weight gain (Shiau and Chen 1999). The effects of retinoid in shrimp can be related to the reported role of this metabolite, essential for normal health and life functions, such as growth, development, and reproduction in animals. Till now, the function of retinoid (vitamin A) and their precursors for invertebrates is unclear.

In conclusion, the extracts from eye, ovary, muscle, eyestalk and brain of *P. vannamei* significantly enhanced the cell migration out of explants at the first 96 h post seeding. The best results were obtained with 10% of eye extract and 3% of ovary extract of *P. vannamei*.

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Chapter 5

Virus replication cycle of white spot syndrome virus (WSSV) in secondary cell cultures from the lymphoid organ of *Penaeus vannamei*

Wenfeng Li, Lowiese M. B. Desmarets, Gaëtan M. A. De Gryse, Sebastiaan Theuns, Vo Van Tuan, Khuong Van Thuong, Peter Bossier, Hans J. Nauwynck

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Abstract

The replication cycle of white spot syndrome virus (WSSV) was investigated in the secondary cell cultures from the lymphoid organ of *Penaeus vannamei*. The secondary cells formed a confluent monolayer at 24 h post reseeding and this monolayer could be maintained for 10 days with a viability of 90%. Binding of WSSV to cells reached a maximum (73 ± 3% of cells and 4.84 ± 0.2 virus particles per virus-binding cell) at 120 min at 4 °C. WSSV entered cells by endocytosis. The co-localization of WSSV and early endosomes was observed starting from 30 min post inoculation. Double indirect immunofluorescence staining showed that all cell-bound WSSV particles entered these cells in the period between 0 to 60 min post inoculation and that the uncoating of WSSV occurred in the same period. After 1 hr inoculation at 27 °C, the WSSV nucleocapsid protein VP664 and envelope protein VP28 started to be synthesized in the cytoplasm from 1 and 3 hour post inoculation (hpi) and were transported into nuclei from 3 and 6 hpi, respectively. The percentage of cells that were VP664 and VP28 positive in their nuclei peaked (50 ± 4%) at 12 hpi. Quantitative PCR test showed that WSSV DNA started to be synthesized from 6 hpi. *In vivo* titration of the supernatants showed that the progeny WSSV became released from 12 hpi and peaked at 18 hpi. In conclusion, the secondary cell cultures from the lymphoid organ were proven to be ideal to examine the replication cycle of WSSV.

Introduction

Of all cultured crustaceans worldwide, penaeid shrimp are considered to be one of the economically most important species. With this development, diseases, especially viral diseases, threatened this booming aqua-industry and caused huge economic losses (Flegel, 1997). White spot syndrome virus (WSSV) is a killer for shrimp and other crustaceans (Kasornchandra *et al*., 1995; Wongteerasupaya *et al*., 1995). Cell cultures are basic and useful tools for the study of replication cycles of viruses and the development of antivirals. Plenty of trials have been performed on the development of cell cultures from different organs of different crustaceans (mainly shrimp) with increasing frequency in the last 30 years (Assavalapsakul *et al*., 2003; Han *et al*., 2013; Wang *et al*., 2000; Kasornchandra, 1999; Li *et al*., 2014). In brief, cells derived from lymphoid, ovary and haematopoietic tissues have been maintained for a certain period and some of these cell cultures were reported to be susceptible to WSSV (Li *et al*., 2014; Jiravanichpaisal *et al*., 2006). However, up till now, the WSSV replication cycle has not been fully understood.

WSSV is now considered as one of the most prevalent and widespread viruses (Flegel, 1997). The size of WSSV ranges from 210 to 420 nm in length and from 70 to 167 nm in diameter (Lu *et al*., 1997). The WSSV envelope consists of more than 35 different proteins (Lin *et al*., 2002), of which VP28 is the most abundant. VP28 is the major envelope protein (van Hulten *et al*., 2001) and was reported to play a crucial role in the infection process as attachment protein, helping the virus to enter into the cytoplasm (Yi *et al*., 2004). The proteins constituting the WSSV nucleocapsid are still mostly unknown (Leu *et al*., 2009). VP664, which consists of a long polypeptide of 6077 amino acids encoded by an intron-less giant open reading frame (ORF) of 18234 nucleotides, is a major nucleocapsid protein (Sánchez-Paz, 2010). VP664 appears to form the stacked ring structures that are visible in the nucleocapsid under the TEM (Leu *et al.*, 2005). To date, the different steps of the replication cycle are still not very well understood.

In the present study, binding, entry, disassembly, protein and genome synthesis, assembly and production and release of WSSV were investigated in secondary cells of the lymphoid organ cells of *P. vannamei*.

Materials and Methods

Experimental animals

Penaeus (*Litopenaeus*) *vannamei* post-larvae were imported from Syaqua Siam Co. Ltd. (Thailand). They were certified to be specific pathogen-free for WSSV, TSV, YHV, IHHNV and IMNV. After arrival, the post-larvae were stocked in a recirculating aquaculture system with 3.5% salinity in the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium. After 4 months, shrimp in premolt stage, weighing 20 ± 2 g, were used for the collection of lymphoid organ.

Primary and secondary cell cultures from cell culture from the lymphoid organ of *P. vannamei*

The primary cell cultures were prepared as described previously (Li *et al.* 2014). The eye extract was prepared as described by Li *et al.* (2015). The cultures were incubated at 27 °C and one third of the culture medium was changed every 3 days. After 9 days of culture, cells were harvested by flushing the wells with a pipette. After the explants were removed, cells were pelleted by centrifugation at 400 g for 10 min and resuspended in 1ml of fresh culture medium. After counting with a Bürker-Türk counting chamber, cells were diluted and 1.1 x 10^5 cells were seeded in each of 24-well plates (containing a glass cover slip coated with 0.1% gelatin). After 24 h post seeding, the secondary cell cultures (1x10^5 cells per well in 24-well plate) were ready for following experiments.

Determination of the percentage of viable cells in the secondary cell cultures

The percentage of viable cells was determined in the secondary cell cultures every 3 days. One third of the culture medium was changed every 3 days. The ethidium monoazide bromide (EMA) and Hoechst stainings were performed to count dead and total cells as described previously (Li *et al.* 2014).

WSSV purification and titer determination

A previously studied WSSV strain (WSSV-Thai-1, Escobedo-Bonilla *et al.*, 2005) was used in the purification experiments. The purified WSSV inoculum and its titration test were prepared as described previously by Dantas-Lima *et al*. (2014). Titer of WSSV inoculum was calculated using the Reed & Muench formula (Reed and Muench, 1938). The titer of WSSV inoculum was $10^{7.7}$ SID_{50}/ml. The mock inoculum was prepared from healthy shrimp.

Analysis of WSSV binding to cells

To characterize the binding of WSSV to the secondary cells of the lymphoid organ, direct virus-binding studies were carried out. The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 0, 30, 60, 90, 120, 150 and 180 min at 4 °C. After three washings with cold culture medium, cells were fixed in cold 4% paraformaldehyde for 10 min at room temperature. After one washing with PBS for 5 min, the cells were incubated in 200 µl of monoclonal antibody w29 (1:100 in PBS, Chaivisuthangkura *et al*., 2004) which is directed against WSSV protein VP28 at 37 °C for 1 h. After three washings with PBS for 5 min, they were incubated in 200 µl of goat anti-mouse IgG-FITC (1:100 in PBS, Sigma Aldrich, USA) at 37 °C for 1 h. After three washings with PBS for 5 min, the cells were incubated in 200 µl of Phalloidin Texas Red (1:40 in PBS, Life Technologies, Belgium) at 37 °C for 1 h. After three washings with PBS for 5 min, the cultures were incubated in 200 µl of Hoechst 33342 solution (10 µg/ml in PBS) for 10 min. After washings with PBS and UP water, the cells were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analyzed with a confocal fluorescence microscope. The percentage of virus-binding cells (750 cells were chosen randomly) and the number of cell-bound virions on each virus-binding cell (150 cells were chosen randomly) at every time point were calculated.

Analysis of WSSV entry process by confocal microscopy

The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 2 hour at 4 °C. After three washings with cold culture medium, 500 µl of culture medium were added into each well and the cells were incubated at 27 °C. The cells

were collected at 0, 30, 60, 90, 120, 150 and 180 min post inoculation, fixed in cold 4% paraformaldehyde for 10 min and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. After one washing with PBS for 5 min, the cells were incubated in 200 µl of polyclonal antibody EEA1 (C-15) (1:100 in PBS, Santa Cruz, USA) which is directed against early endosomes at 37 °C for 1 h. After three washings with PBS for 5 min, the cells were incubated in 200 µl of rabbit anti-goat Alexa Fluor 594 (1:100 in PBS, Sigma Aldrich, USA) at 37 °C for 1 h. Then, after three washings with PBS for 5 min, the cells were incubated in 200 µl of w29 (1:100 in PBS) at 37 °C for 1 h. After three washings with PBS for 5 min, they were incubated in goat anti-mouse IgG-FITC (1:100 in PBS) at 37 °C for 1 h. After three washings with PBS for 5 min, the cell cultures were incubated in 200 µl of Hoechst 33342 solution (10 µg/ml in PBS) for 10 min. After washings with PBS and UP water, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analyzed with a confocal fluorescence microscope. The percentage of virus binding cells, percentage of cells containing WSSV particle-positive endosomes (750 cells were chosen randomly) and the number of WSSV particle-positive endosomes per virus-positive cell (150 cells were chosen randomly) were calculated.

Analysis of WSSV disassembly in cytoplasm

The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 2 hour at 4 °C. After three washings with cold culture medium, 500 µl of fresh culture medium were added into each well and immediately the cells were incubated at 27 °C. The cells were collected at 0, 30, 60, 90, 120, 150 and 180 min, fixed in cold 4% paraformaldehyde for 10 min and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. After one washing with PBS for 5 min, the cells were incubated in 200 µl of polyclonal antibody WSSV419 (1:200 in PBS, Leu *et al.*, 2005) which is directed against WSSV nucleocapsid protein VP664 at 37 °C for 1 h. Then, after three washings with PBS for 5 min, the cells were incubated in 200 µl of goat anti-rabbit Alexa Fluor 594 (1:100 in PBS, Sigma Aldrich, USA) at 37 °C for 1 h. After three washings with PBS for 5 min, the cells were stained with w29 (1:100 in PBS), goat anti-mouse IgG-FITC (1:100 in PBS) and Hoechst 33342 solution (10 µg/ml in PBS) as described above. After washings with PBS and UP water, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were

analyzed with a confocal fluorescence microscope. The numbers of entire WSSV particles and nucleocapsids without envelope per virus-positive cell (150 cells were chosen randomly) were calculated.

Analysis of synthesis and transport of WSSV envelope VP28 and nucleocapsid VP664 proteins in cells

The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 1 hour at 27 °C. After three washings with culture medium, 500 µl of fresh culture medium were added into each well and the cells were further incubated at 27 °C. The cells were collected at 0, 1, 3, 6, 9, 12, 15, 18 and 24 hours post inoculation (hpi) at 27 °C. At the same time, the supernatant was collected and stored at -70 °C after 2000 g centrifugation for 10 min. The cells were fixed in cold 4% paraformaldehyde for 10 min and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. After one washing with PBS for 5 min, the cells were incubated in 200 µl of WSSV419 (1:200 in PBS) at 37 °C for 1 h. Then, after three washings with PBS for 5 min, the cells were incubated in 200 µl of goat anti-rabbit Alexa Fluor 594 (1:100 in PBS) at 37 °C for 1 h. After three washings with PBS for 5 min, the cells were stained with w29 (1:100 in PBS), goat anti-mouse IgG-FITC (1:100 in PBS) and Hoechst 33342 solution (10 µg/ml in PBS) as described above. After washings with PBS and UP water, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. All samples were analyzed with a confocal fluorescence microscope. The percentages of cells in which only VP664 or both VP664 and VP28 were detected in the cytoplasm and the percentage of cells in which only VP664 or both VP664 and VP28 were found in the nucleus were calculated (750 cells were chosen randomly).

Real time PCR for the quantification of intra- and extracellular WSSV DNA load

The secondary cell cultures were incubated with 200 µl WSSV inoculum (10^7 SID_{50}) for 1 hour at 27 °C. After three washings with cold culture medium, 500 µl of fresh culture medium were added into each well. The cells were further incubated at 27 °C and collected at 0, 1, 3, 6, 9, 12, 15, 18 and 24 hours post inoculation (hpi) at 27 °C. Meantime, the supernatant was collected and stored at -70 °C after 2000 g

centrifugation for 10 min. The cells were scraped and stored together with the cells from supernatant at -70 °C. Then DNA was extracted from the supernatant and the cell fraction using the QIAamp DNA mini kit (Qiagen, California, USA). Primers were designed in a conserved region of the VP19 coding sequence using the Primer3Plus website. A 20 µl PCR mixture was used per reaction, which contained 10 µl PrecisionPLUS 2x qPCR MasterMix with SYBR Green and ROX (PrimerDesign Ltd., Southampton, UK), 50 nM forward primer 5'-ATTGGTATCCTCGTCCTGGC-3', 200 nM reverse primer 5'-GTTATCGTTGGCAGTGTCGTC-3', 6.5 µl DNase/RNase free H_20, and 3 µl sample DNA or diluted DNA standard (see below). An enzyme activation step at 95 °C for 2 min was followed by 40 cycli of each 15 s at 95 °C and 60 s at 58 °C. A first-derivative melting curve analysis was performed by heating the mixture to 95 °C for 15 s, then cooling to 60 °C for 1 min, and heating back to 95 °C at 0.3 °C increments. Amplification, monitoring, and melting curve analysis were carried out in a Step One Plus™ real-time PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA).

Synthetic DNA standards for absolute quantification

DNA was extracted from WSSV-Viet stock viruses using the QIAamp DNA mini kit (Qiagen, California, USA). The VP19 DNA fragment was amplified using the above-described primers in a 50 µl PCR reaction mixture, composed of 10 µl OneTAQ Standard reaction buffer (New England Biolabs Inc., Massachusetts, USA), 1 µl dNTPmix, 1 µl WSSV_VP19Fw primer, 1 µl WSSV_VP19Rv primer, 0.25 µl OneTAQ DNA-polymerase (New England Biolabs Inc.), 32.75 µl DNase/RNase free H_20, and 4 µl DNA. After an enzyme activation step at 94 °C for 30 s, 35 cycles of amplification, each 20 s at 94 °C, 20 s at 55 °C, and 60 s at 68 °C, were followed by a terminal elongation of 5 min at 68 °C. Fragment length was controlled by agarose gel electrophoresis and fragments with the correct length were excised and purified from the gel using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Specificity of the fragments was controlled by assessment of the sequence (performed by the GATC Biotech Company, Konstanz, Germany). The amount of DNA was determined using the Nanodrop 2000 system. Ten-fold serial dilutions of

the DNA were made over a range of 6 log units (10^6-10^1) for the generation of the standard curve (Efficiency: 97.28%; R^2: 0.99).

Titration of the supernatant from WSSV-inoculated secondary cell cultures from 0 to 24 hpi at 27 °C.

The supernatant collected from the WSSV-inoculated secondary cell cultures from 1 to 24 hpi were diluted tenfold (10^{-1} to 10^{-4}). 50 µl of each dilution were injected into one shrimp (5.0 g) and 5 shrimp were used per dilution. All moribund shrimp were stored at -70 °C. After 7 days, all surviving shrimp were euthanized. The cephalothoraxes were dissected longitudinally, embedded in 2% methycellulose (Fluka, Belgium), frozen and cryosectioned. Cryosections were stained with w29 (1:100 in PBS), goat anti-mouse IgG-FITC (1:100 in PBS) and Hoechst 33342 solution (10 µg/ml in PBS) as described above. The number of WSSV positive shrimp in each dilution was counted and finally the titer of each supernatant from 0 to 24 hpi at 27 °C was evaluated as previously described by Escobedo-Bonilla *et al.* (2005).

Statistical analysis

All results were average values from three independent replicates. The data were statistically analyzed in SPSS 19.0. Differences were considered to be significant at $P < 0.05$.

Results

Behavior of the secondary cell cultures from the lymphoid organ of *P. vannamei*

A full cell monolayer was formed within 24 h post seeding (Fig. 1(a)). The cell monolayer contained two cell types: fibroblast-like cells and epitheloid cells (Fig. 1(b)). In the first 7 days, the fibroblast-like cells formed networks. The epitheloid cells were present in these networks as cell islands (Fig. 1(b)). The epitheloid cells degenerated after 7 days (Fig. 1(c)) and fibroblast-like cells occupied all available

spaces and became the dominating cell type in the cell monolayer (Fig. 1(d)). After 10 days, the fibroblast-like cells degenerated (Fig. 1(e) and (f)).

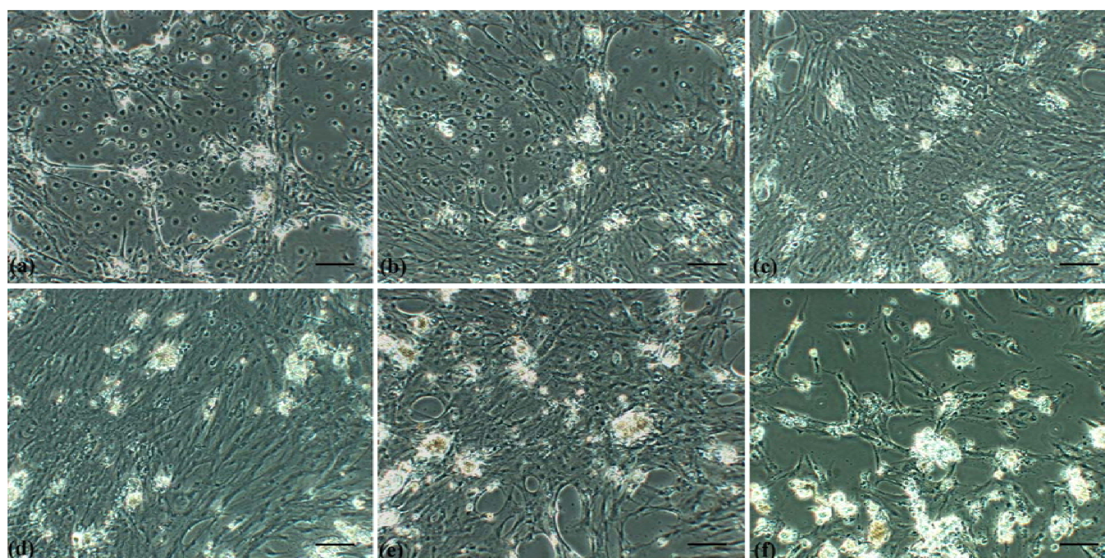

Fig. 1 The secondary cell cultures from the primary cell cultures at 1d (a), 4d (b), 7d (c), 10d (d), 13d (e) and 16d (f) post reseeding (scale bar = 100 μm).

Viability of the secondary cell cultures

The percentage of viable cells in the secondary cell cultures from the lymphoid organ of *P. vannamei* was determined at 1, 4, 7 and 10 d post seeding by an EMA & Hoechst staining. The proportion of viable cells in cell monolayer culture was 95 ± 3% at 1 d, 92 ± 4% at 4 d, 90 ± 2% at 7 d and 92 ± 2% at 10 d post seeding. The high proportion of viable cells during the first 10 days post seeding indicated that the secondary cell cultures from the lymphoid organ of *P. vannamei* survived well and were ready for the investigation of the WSSV replication cycle.

Kinetics of WSSV binding

The percentage of virus-binding cells and the number of virus particles bound per virus-binding cell were determined. Both the percentage of virus-binding cells and the number of virus particles bound per virus-binding cell rose with increasing time at 4 °C. Especially during the first 60 min post inoculation at 4 °C, WSSV attached quickly to cell. The percentage of virus-binding cells reached 56 ± 3% (Fig. 2(a)) and the number of virus particles bound per virus-binding cell reached 3.66 ± 0.19 at 60

min (Fig. 2(b)). From 120 min post inoculation, the percentage of virus-binding cells (73 ± 3%, Fig. 2(a)) and the number of virus particles bound per virus-binding cell (4.84 ± 0.2, Fig. 2(b)) reached a maximal level.

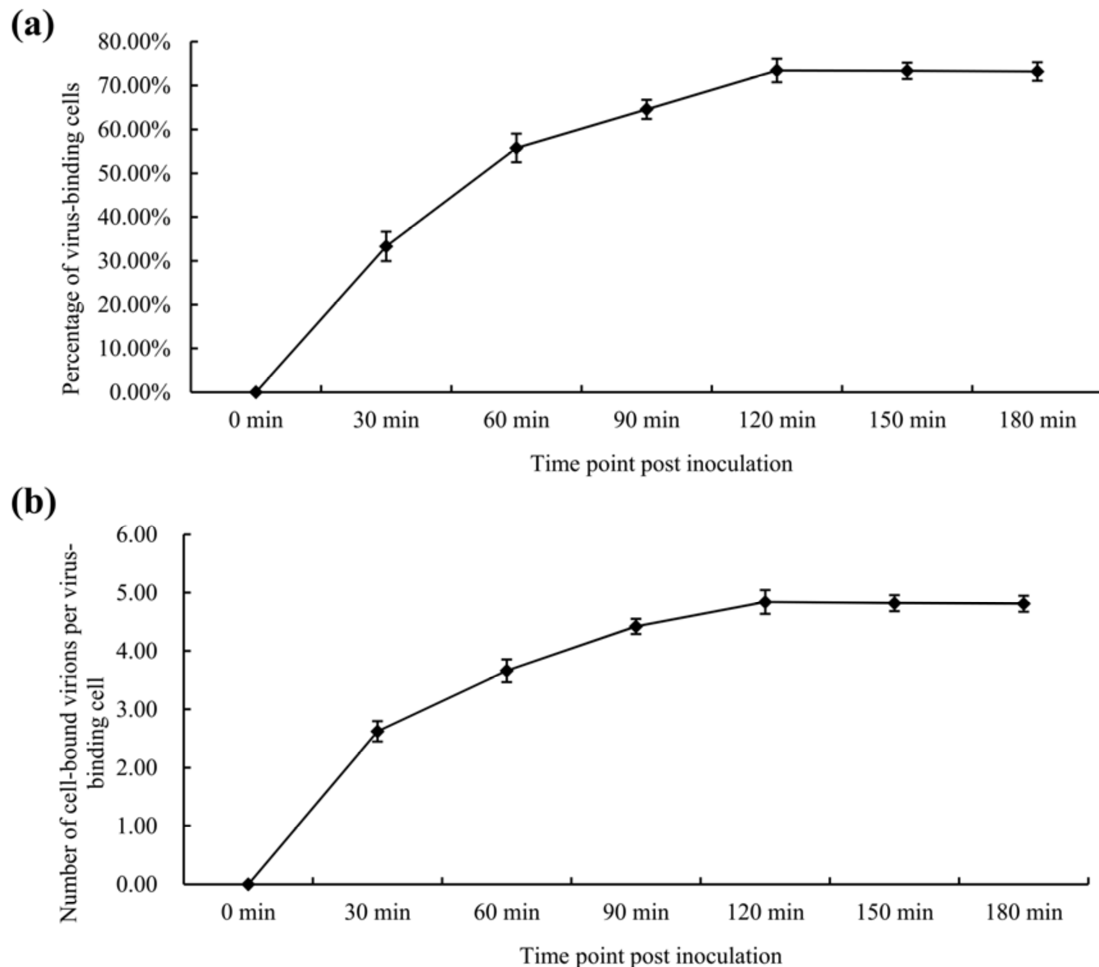

Fig. 2 The percentage of virus-binding cells (a) and the number of cell-bound virus particles per virus-binding cell (b) in the secondary cell cultures of the lymphoid organ of *P. vannamei* at different time points during WSSV incubation at 4 °C.

Colocalization of WSSV and early endosomes during entry

After the secondary cell cultures were incubated with WSSV at 4 °C until the maximum virus binding was reached (120 min, as described above), the cells were transferred to 27 °C, subsequently fixed at different time points and labeled for VP28 and EEA1 and observed under the confocal microscope. Green fluorescent spots representing bound virus were observed on the membrane of cells (Fig. 3(a)). After a further incubation at 27 °C, the percentage of cells with virus attached to the outside

of the cell decreased and the cells with internalized virus increased (Fig. 3(i)). The virus particles were widely distributed inside the early endosomes (Fig. 3(b), (c)). The percentage of cells with WSSV-containing endosomes reached a maximum (62 ± 3%) at 60 min at 27 °C (Fig. 3(i)). At the same time (30 - 60 min post inoculation), the number of virus-positive endosomes per virus-positive cell raised to a maximum number of particles per positive cell (Fig. 3(ii)). From 60 min to 180 min, the percentage of cells with WSSV-containing endosomes and the number of virus particles inside endosomes per virus-positive cell decreased gradually (Fig. 3(i), (ii)).

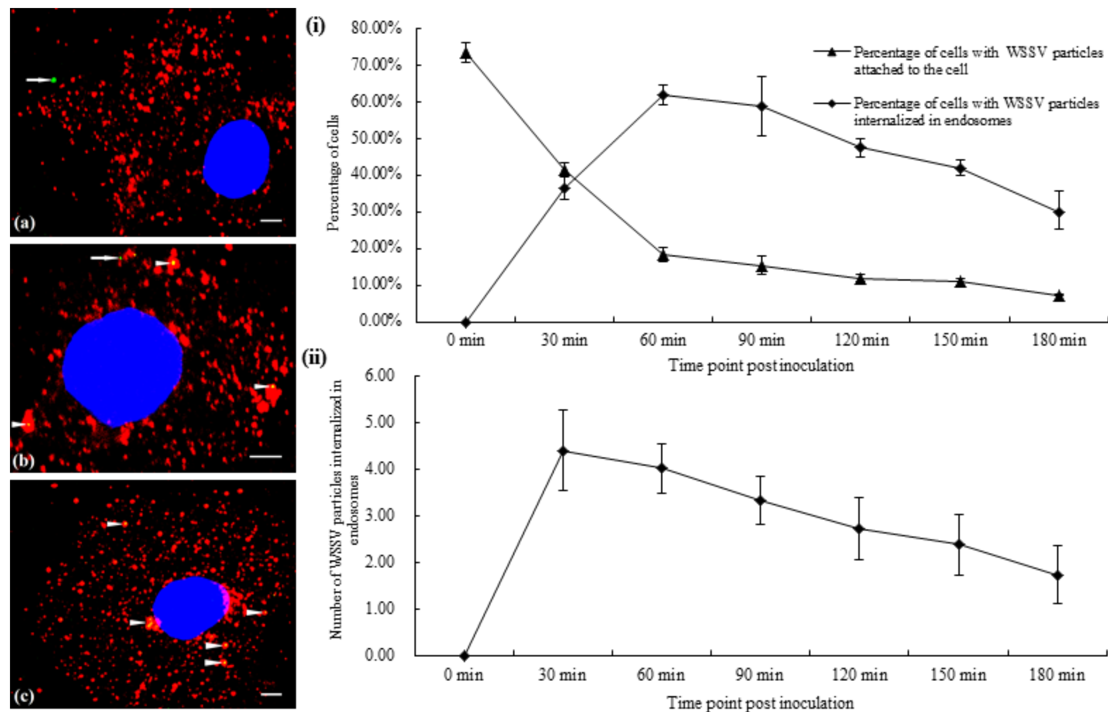

Fig. 3 (i): The percentage of WSSV-binding cells (white arrow: plasma membrane bound WSSV particle) and the percentage of cells with WSSV internalized endosomes (white arrow head: internalized WSSV particle) from 0 to 180 min after inoculation. (ii): The number of WSSV particles internalized endosomes per virus-infected cell from 0 to 180 min post inoculation. (a): Overlay picture at 0 min after inoculation; (b): Overlay picture at 30 min after inoculation; (c): Overlay picture at 60 min after inoculation. Blue: Hoechst 33342 - staining for nuclei; Green: w29 & goat anti-mouse IgG FITC - staining for WSSV particles; Red: EEA1 (C-15) & rabbit anti-goat Alexa Fluor 594 - staining for early endosomes; Yellow: co-localization (scale bar = 3 μm).

Kinetics of uncoating and disassembly of WSSV in the cytoplasm

After a 2-h-inoculation at 4 °C, the number of WSSV particles bound to the cell surface reached a maximum. Once the cells were incubated at 27 °C, WSSV particles started to enter cells. From 0 to 150 min at 27 °C, the number of complete virions (co-localizing VP28 with VP664) decreased (Fig. 4), especially during the first 60 min at 27 °C, (4.84 ± 0.20 to 0.55 ± 0.25 per virus-positive cell, Fig. 5). At the same time, the number of single VP28 and single VP664 signals started to rise. At 60 min at 27 °C, the number of single VP28 and single VP664 signals increased to 4.25 ± 0.86 per virus-positive cell and 4.95 ± 0.27 per virus-positive cell, respectively (Fig. 5). After 150 min at 27 °C, VP664 started to become expressed all over the cytoplasm.

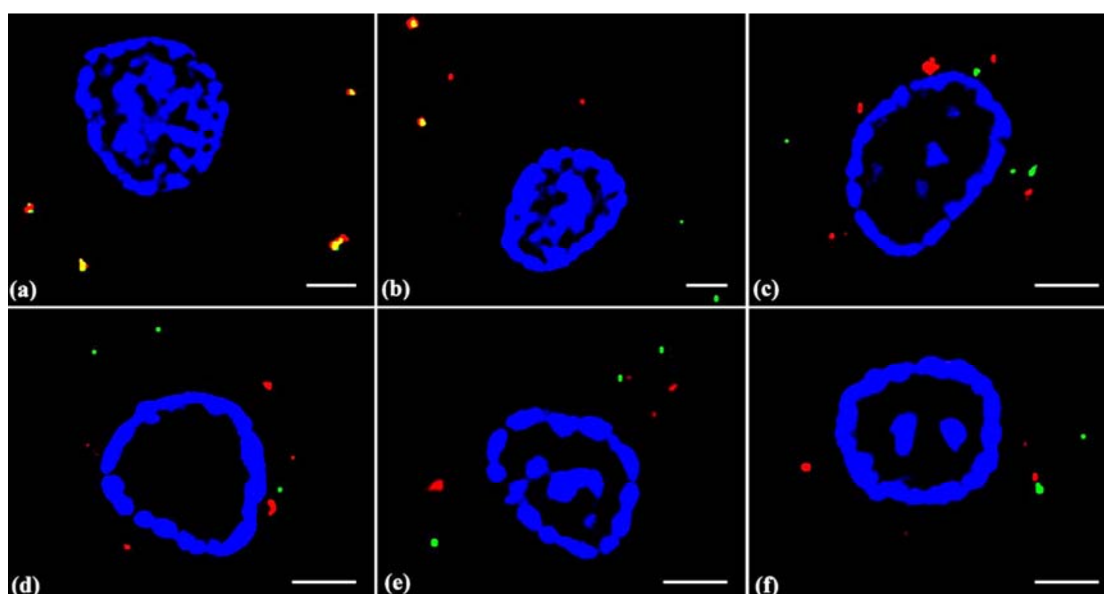

Fig. 4 Layer-scan pictures of the double IIF staining of the uncoating and disassembly of WSSV particles from 0 to 150 min after inoculation. (a): 0 min after inoculation; (b): 30 min after inoculation; (c): 60 min after inoculation; (d): 90 min after inoculation; (e): 120 min after inoculation; (f): 150 min after inoculation. Blue: Hoechst 33342 - staining for nuclei; Green: w29 & goat anti-mouse IgG FITC (F2761) - staining for WSSV envelope; Red: WSSV419 & goat anti-rabbit Alexa Fluor 594 - staining for WSSV nucleocapsid; Yellow: co-localization (scale bar = 3 μm).

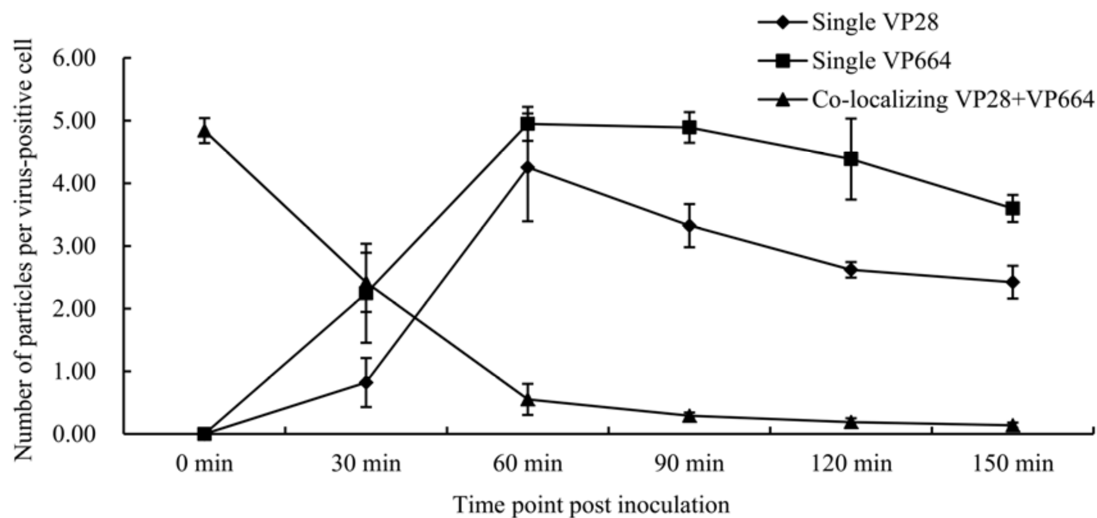

Fig. 5 The kinetics of uncoating of internalized WSSV particles from 0 to 150 min post inoculation.

Kinetics of VP28 and VP664 expression and transport to the nucleus

To assess the kinetics of VP28 and VP664 expression, cells were inoculated with WSSV at 27°C for 1 h, after which cells were fixed at different time points (0, 1, 3, 6, 9, 12, 15, 18, 24 hpi), and the percentage of VP28 and VP664 positive cells was determined (Fig. 6). New nucleocapsid protein VP664 started to become synthesized in the cytoplasm from 1 hpi (17 ± 3%). The newly produced VP664 was spread all over the whole cytoplasm (Fig. 6(a)). From 3 hpi, VP664 became transported into the nucleus (40 ± 4% of cells, Fig. 6(b)). This expression and transport of VP664 kept on going during the whole WSSV virion assembly procedure. Starting from 3 hpi, the envelope protein VP28 became expressed in the cytoplasm (5 ± 2%, Fig. 6), in which VP664 was already present. The newly produced VP28 was highly concentrated in some regions in the cytoplasm which were close to the nucleus and clearly separated from the areas where VP664 were synthesized (Fig. 6(c)). From 6 hpi, the VP28 was transported to the nucleus (10 ± 3%). From that time point, VP664 and VP28 became both concentrated in the nucleus (Fig. 6(d)). The percentage of cells which were both VP664 and VP28 positive in the nucleus reached a maximum (50 ± 5%) at 12 hpi and then dropped until 24 hpi at 13 ± 2%.

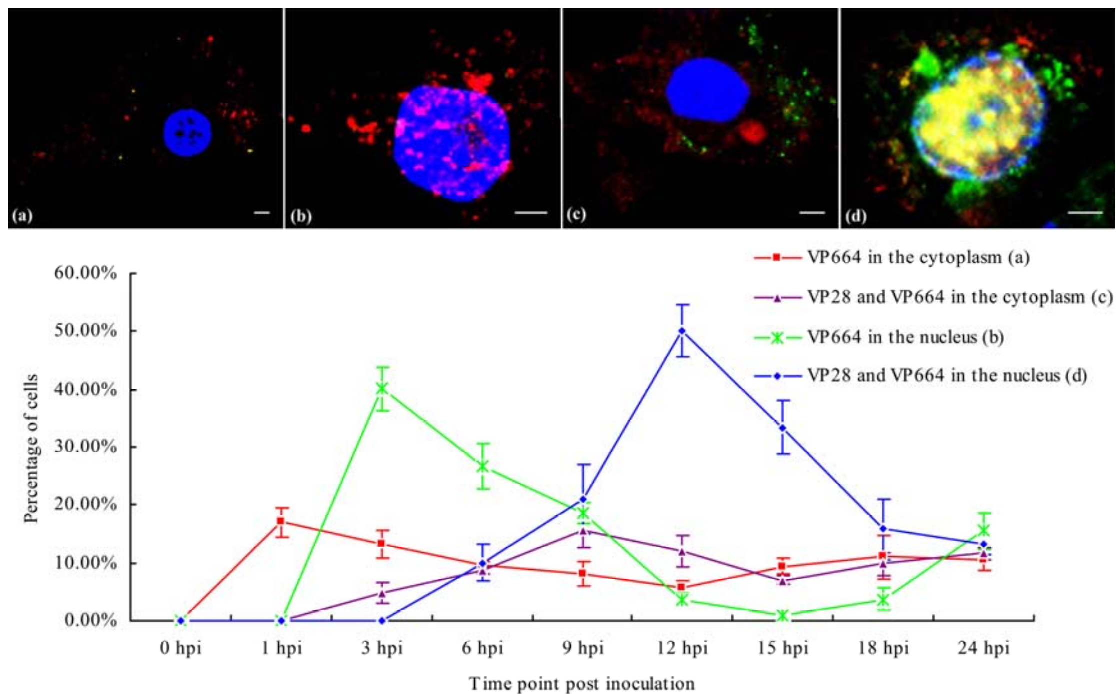

Fig. 6 The kinetics of WSSV infection in the secondary cell cultures from 0 to 24 hr post inoculation. (a): Overlay pictures showed that WSSV nucleocapsid protein VP664 synthesized in cytoplasm; (b): Overlay pictures showed that WSSV nucleocapsid protein VP664 synthesized in cytoplasm and transported to the nucleus; (c): Overlay pictures showed that WSSV nucleocapsid protein VP664 and envelope protein VP28 synthesized in cytoplasm; (d): Overlay pictures showed that WSSV nucleocapsid protein VP664 and envelope protein VP28 synthesized in cytoplasm and transported to the nucleus. Blue: Hoechst 33342 staining for nuclei; Green: w29 & goat anti-mouse IgG FITC - staining for WSSV envelope; Red: WSSV419 & goat anti-rabbit Alexa Fluor 594 - staining for WSSV nucleocapsid; Yellow: co-localization (scale bar = 3 µm).

Quantification of intra- and extracellular WSSV DNA load

The qPCR test showed that the WSSV DNA started to be synthesized in the cells from 6 hpi (6.20 log_{10}/ml) and steadily increased up to 7.14 log_{10}/ml at 24 hpi. WSSV DNA started to be released in the supernatant between 9 and 12 hpi (5.22 to 7.10 log_{10}/ml), and remained at that level up till 18 hpi (7.37 log_{10}/ml). Between 18 and 24 hpi a large increase of the extracellular DNA load from 7.37 to 8.87 log_{10}/ml was observed (Fig. 7(a)).

WSSV production and release

Titration of the supernatant collected from WSSV-inoculated secondary cell cultures from 0 to 24 h post 1-h-inoculation at 27 °C showed that the titer of supernatant increased from 12 hpi (3.42 $\log_{10}$ SID_{50}/ml) and reached a maximum at 18 hpi (4.33 $\log_{10}$ SID_{50}/ml (Fig. 7(b)).

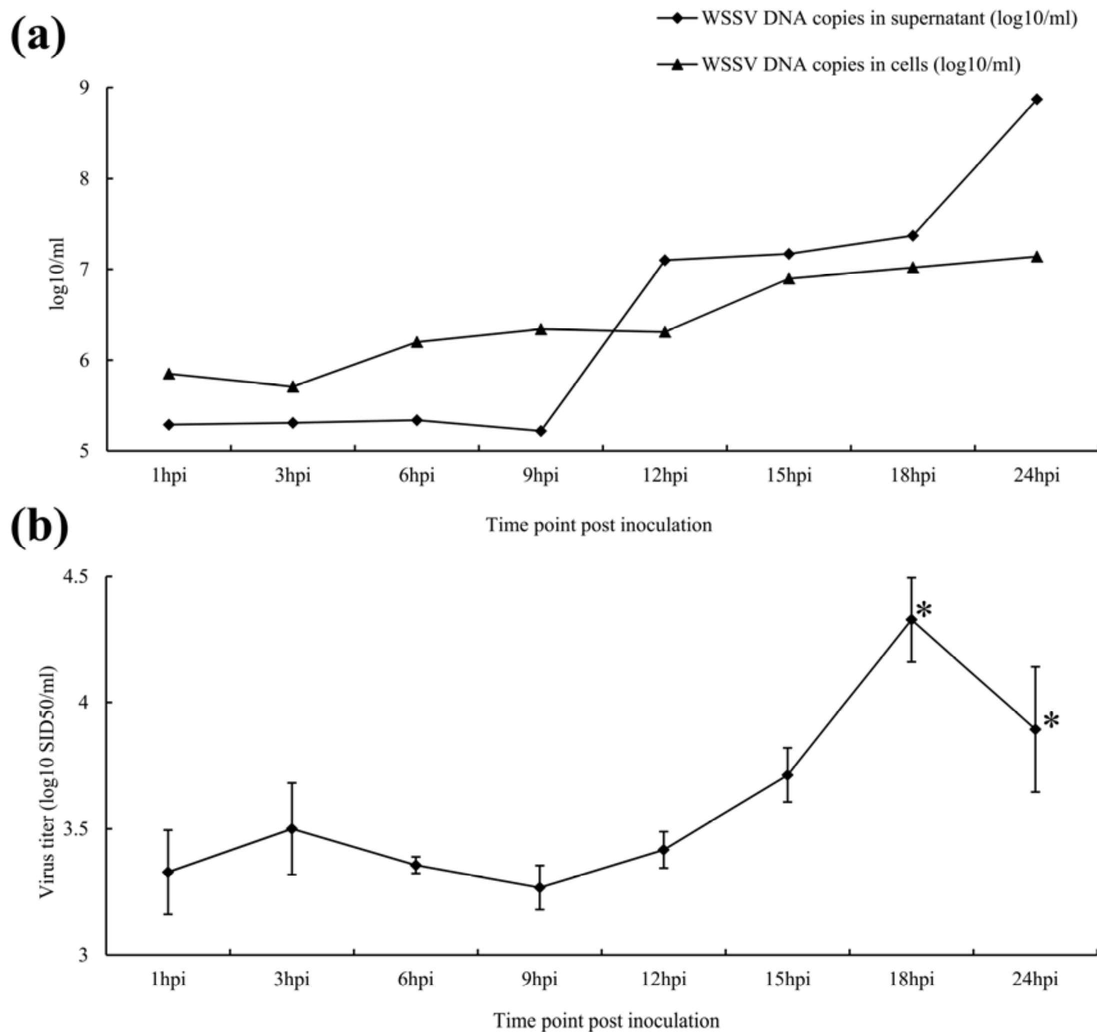

Fig. 7 (a): Real time PCR for the quantification of intra- and extracellular WSSV DNA load. (b): WSSV production in the secondary cell cultures of the lymphoid organ of *P. vannamei*. (Detection limit $\leq 10^{0.8}$ SID_{50}/ml; *: significantly different ($p < 0.05$) in comparison with the data from 1hpi).

Discussion

In the present study, a secondary cell culture from the lymphoid organ of *P. vannamei* was established, which allowed the study of the replication cycle of WSSV in shrimp cells.

Primary cell cultures derived from shrimp lymphoid organ have been reported in many other publications (Assavalapsakul *et al*., 2003; Han *et al*., 2013; Wang *et al*., 2000) and their susceptibility for WSSV has already been demonstrated (Li *et al*., 2014; Tapay *et al*., 1995). However, little was known on the different steps of the replication cycle. Since the cell migration out of lymphoid organ explants is very variable on cell numbers and proportions of cell types in the different wells of primary cell cultures. Therefore, it is impossible to obtain reproducible results. Further, the cells stay at a short distance from the explants and form thick multi-layers. Bringing virus in contact with these cultures will not result in reliable results. In the present study, a solution was found for these problems by using secondary cell cultures. The secondary cell cultures formed a uniform monolayer within 24 h post reseeding, and this monolayer could be maintained for more than 10 days with a viability of 90 %. Moreover, the cell number and cell types in each well were quite uniform, which brings the study of different steps of the WSSV replication cycle in a standardized, reproducible way

The binding of WSSV to the cells gradually increased during 120 min of incubation at 4 °C. After incubation of the secondary cell cultures with WSSV, the number of viruses bound per cell was different between cells. In 27 % of cells, no virus was attached to the cells. In contrast, in 73 % of the cells, 4.84 ± 0.20 virus particles were bound. This difference may be determined by the absence or presence of the receptors. The fact that the secondary cell culture consists of two cell types (fibroblast-like cells and epitheloid cells) may explain this finding. It is very well possible that only one of these two cell types is susceptible for WSSV infection. Furthermore, the virus particles were not equally distributed over the surface of each virus-positive cell, which indicated that the concentration of receptors was variable on different parts of the cell surface.

Following the binding to cell-surface receptors, WSSV particles undergo endocytosis and are present as intact virions (nucleocapsid surrounded by the envelope) into

endosomes. These endocytic events were clearly demonstrated by confocal microscopy. Sritunyalucksana *et al*. (2006) reported that a VP28-binding protein (PmRab7) was found in *P. monodon.* The VP28-binding protein (PmRab7) has a high homology to the small GTP-binding protein Pab7, which represents a family of over 30 proteins that are localized on the surfaces of distinct membrane-enclosed compartments of exocytic and endocytic pathways (Chavrier *et al.*, 1990; Feng *et al*., 1995). Huang *et al.* (2013) already reported that endocytosis was involved in the entry of WSSV into shrimp hemocytes. More work needs to be performed to determine the different viral and cellular proteins involved in this endocytic pathway.

The viruses were found in small endosomes inside the cells from 30 min post inoculation. Later on, between 30 and 60 min post inoculation, large intensely fluorescing spots were observed, suggesting that WSSV virions became accumulated in the cytoplasm during that time period. During virus entry, the virus envelope and nucleocapsid became separated from each other, which notably occurred during the first 60 min after inoculation. The nucleocapsid was released from the virus-endosome and transported to a region close to the nucleus. Degradation of viral particles took place between 60 and 150 min post inoculation.

One hour after inoculation at 27°C, the synthesis of the nucleocapsid protein VP664 was already detected, followed by the synthesis of envelope protein VP28 at 3 hpi. This is in agreement with other studies reported before. Leu *et al*. (2005) reported that the VP664 and VP28 transcripts were detected in small amounts from 2 h post intramuscular injection. The signal of VP664 was detected in most parts of the cytoplasm, which indicates that the nucleocapsid protein VP664 was synthesized in the cytosol. VP664 was detected in the nucleus from 3 hpi. The synthesis of VP664 continued over time. The signal could still be observed in the cytosol when the assembly of WSSV particles in the nucleus already started. The envelope protein VP28 was first detected in the cytoplasm from 3 hpi and in the nucleus from 6 hpi. Li *et al*. (2009) showed that the gene expression of VP28 was first detected at 4 h post infection (samples were collected from primary hemocyte cultures of crayfish). VP28 was not synthesized all over the whole cytoplasm like VP664 but was restricted to certain regions in the cytoplasm, which were close to the nucleus. The lack of antibodies against organelles in shrimp cells made the identification of the synthesis

site impossible. Based on the position of the concentrated signal of VP28, the rough endoplasmic reticulum (rER) might be a good candidate.

The result of quantitative PCR indicated that the WSSV DNA synthesis started from 6 hpi in cells, which indicated that the capsid protein VP664 and envelope protein VP28 both started to be synthesized in the cytoplasm before the DNA genome was synthesized in the nucleus. Consequently, VP664 and VP28 may be considered as immediate-early proteins. This might indicate that VP664 and VP28 have important functions in the virus replication, such as: regulation of cell metabolism and antiviral response, activation of the expression of late genes, the genome replication and intranuclear assembly. However, more experiments should be performed to support this hypothesis. In the supernatant, the quantity of WSSV DNA copies increased between 9 and 12 hpi (from 5.22 log_{10}/ml to 7.10 log_{10}/ml), and stayed at that level at 15 and 18 hpi. At 24 hpi, a strong release of viral DNA was detected. Because the infectious virus titer in the supernatant peaked earlier (at 18 hpi) and was much lower (4.33 log_{10} SID_{50}/ml) than the viral DNA copies, the large number of DNA copies at the end of the experiment is thought to be non-assembled free viral DNA from disrupted cells. This is supported by the observation of cell lysis in the infected monolayer at that time point. This cell lysis is most probably also the reason why a drop was seen in cells that carried VP28 and VP664 in the nucleus. Because only low infectious virus titers are obtained in the supernatant, cells from lymphoid organ can be considered as low productive cells. Another explanation could be the induction of an antiviral state resulting in the block of the virus assembly.

Based on these results, a model of the replication cycle of WSSV in the secondary cell cultures from the lymphoid organ of *P. vannamei* is hypothesized. The WSSV replication cycle starts with the attachment of a WSSV particle (Fig. 8(a)). After the virus particle attaches on the surface of the cellular membrane, the WSSV particle enters the cell via endocytosis (Fig. 8(b)). In the early endosome, the disassembly of the virus particle starts. The WSSV envelope fuses with the endosome membrane and the nucleocapsid becomes released into the cytoplasm. The free nucleocapsid migrates close to the nucleus and injects the viral genome via a nuclear pore inside the nucleus. After this, both envelope and capsid proteins become degraded in the cytoplasm (Fig. 8(c)). Inside the nucleus, transcription starts and the mRNAs of immediate-early genes are produced and subsequently migrate into the cytoplasm, where they become

translated into proteins by free ribosomes. The WSSV major capsid protein VP664 is expressed starting from this time point (1 hpi). The immediate-early proteins activate early and late proteins (Fig. 8(d)). From 3 hpi, the mRNAs for envelope proteins (e.g. the envelope protein, VP28) become transcribed. WSSV major envelope protein VP28 is expressed in the rough endoplasmic reticulum (rER) and becomes transported to the inner nuclear membrane. This inner nuclear membrane proliferates inside the nucleus. From 6 hpi, the virus genome multiplies in the nucleus and the virus capsids become assembled around new viral genomes. The nucleocapsids are formed. Then, the nucleocapsids bud at the inner nuclear membrane with envelope protein VP28 (Fig. 8(e)). The newly formed particles are present in the lumen of the expanded inner nuclear membrane (Fig. 8(f)). Finally, the new WSSV particles are released via cell lysis (Fig. 8(g)).

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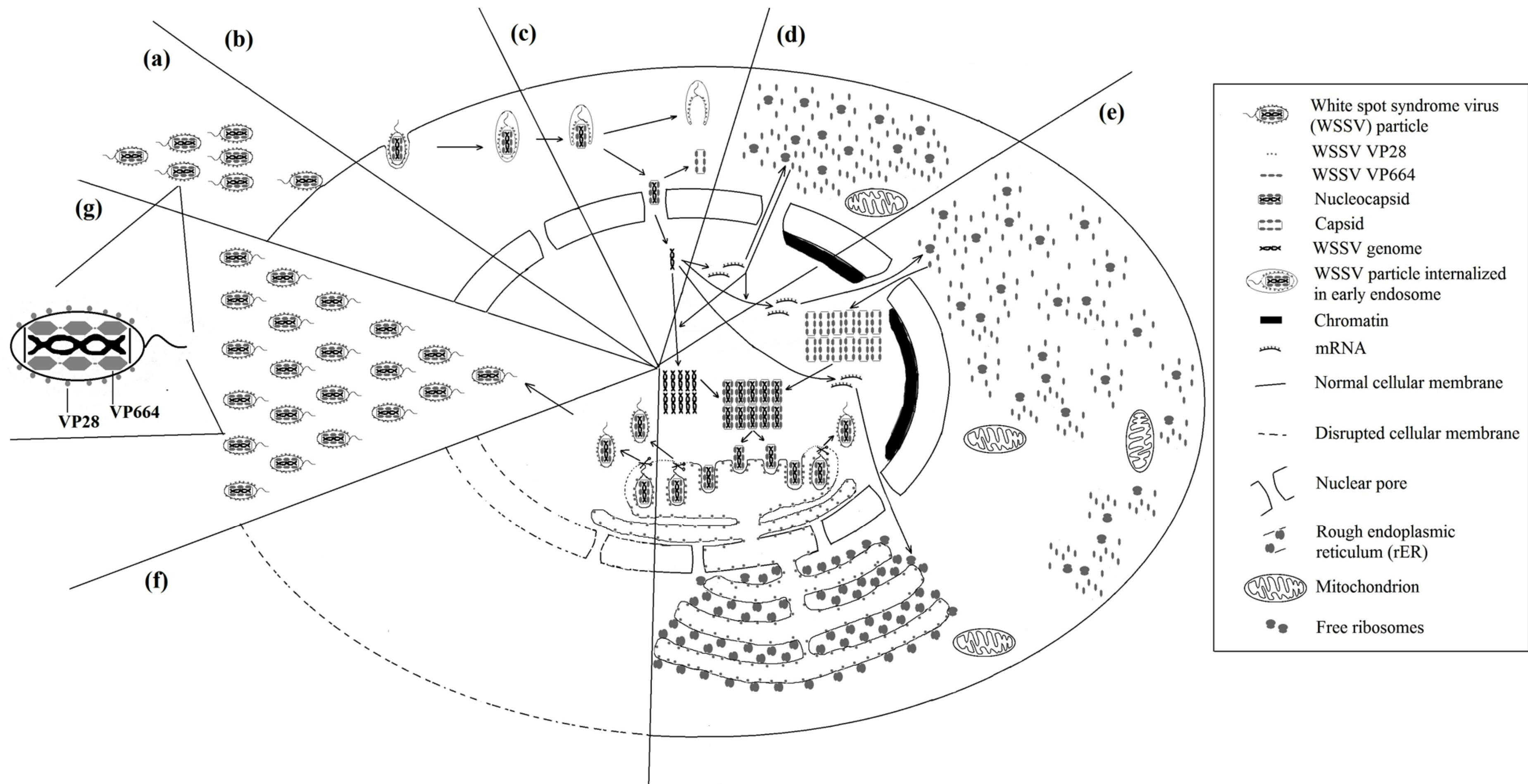

Fig. 8 A proposed model of replication cycle of white spot syndrome virus (WSSV) in secondary cell cultures from the lymphoid organ of *P. vannamei*.

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Chapter 6

General Discussion

Since the first white spot syndrome (WSS) outbreak in Taiwan in 1992, white spot syndrome virus (WSSV) has seriously threatened the shrimp aquaculture industry and is still causing considerable economic losses worldwide. Although the techniques for detection and diagnosis have been set up based on PCR and histopathology, lack of available techniques for cultivating cells and growing WSSV *in vitro* has hampered virus isolation. In this thesis, primary and secondary cell cultures were developed from the lymphoid organ of *P. vannamei* and tested for their susceptibility to WSSV. Afterwards, the replication cycle of WSSV was successfully unraveled in secondary cell cultures.

Development of the primary and secondary cell cultures from the lymphoid organ of *P. vannamei*.

A well-defined cell culture medium was used in the present thesis consisting of 2x L-15 supplemented with 20% fetal bovine serum, 10% Chen's salt, penicillin (100 IU/ml) & streptomycin (100 µg/ml), gentamycin (50 µg/ml) and fungizone (0.25 µg/ml) (osmolality: 900 mOsm/L; pH: 7.5; temperature: 27 °C). This medium has been shown to be ideal for maintaining shrimp cells (Dantas-Lima *et al.*, 2012; Han *et al.*, 2013; Itami *et al.*, 1999; Jose *et al.*, 2009; Shike *et al.*, 2000). In addition, the molting stage, shrimp organ, coating of culture vessels and culture supplements were further defined for a perfect outcome. The premolting shrimp (D stage, Corteel *et al.*, 2012) provided the best results on cell migration. Compared to other tissues and organs (ovary, muscle, hemocytes, heart, hematopoietic tissue and epidermis tissue) of *P. vannamei*, the lymphoid organ was most suitable for cell migration and survival, which agrees with previous reports (Chen and Kou, 1989; Chen and Wang, 1999; Hsu *et al.*, 1995; Hu *et al.*, 2008; Lang *et al.*, 2002; Mulford *et al.*, 2000; Nadala *et al.*, 1993; Tapay *et al.*, 1997). The culture vessels are generally made of commercial plastic in which the inner surface is treated for a strong attachment of mammalian cells. For shrimp cell culture, proper adherence factors are necessary. Jose *et al.* (2012) have evaluated the efficacy of laminin, fibronectin and poly-L-lysine as attachment factors on the culture efficiency of lymphoid cells and poly-L-lysine gave the best result. In the present thesis (Chapter 3), coating with both gelatin (0.1%) and poly-L-lysine (0.005%) gave good results on cell migration and survival, but gelatin (0.1%) was significantly better than poly-L-lysine (0.005%). Gelatin (0.1%) coating provided

an ideal surface to promote cell migration from the explants. It contains high concentrations of glycine (27.5%) and proline (16.4%) (Eastoe, 1955), which fits very well with the amino acid concentrations in the shrimp body (Vázquez-Ortiz et al., 1995).

Furthermore, in the present thesis, it was demonstrated that supplementing the basic medium with cholesterol (600µg/ml, Chapter 3), L-glutathione (1000µg/ml, Chapter 3) and extract from eyes (10%, Chapter 4) significantly improved cell migration and survival *in vitro*. Cholesterol plays important roles in growth and development of mammals and insects (Clayton, 1964). As major sterol (Gong *et al*., 2000) and precursor of steroid hormones in shrimp, cholesterol can not be synthesized *in vivo* but have to be provided via feed. Dietary source is the only way to obtain cholesterol for shrimp survival, growth and development (Fox *et al*., 1994; Kanazawa *et al*., 1971; Najafabadi *et al*., 1992). L-glutathione (GSH) has been reported to improve mammalian tissue and cell cultures (Ozawa *et al.*, 2006; Wang and Day, 2002) and hemocyte survival of *P. vannamei in vitro* (Dantas-Lima *et al*., 2012), which might be due to the strong anti-oxidative activity. Shrimp muscle extract has been used in shrimp cell cultures to promote the performance of cells *in vitro* (Chen *et al.* 1986; Han *et al.* 2013; Nadala *et al.* 1993). Extracts from different shrimp organs (muscle, eyestalk, ovary and eye) may have similar functions as FBS to supply cells with proteins, amino acids, lipids, growth factors and hormones. However, they may also provide more shrimp-specific factors to shrimp cells. As demonstrated in Chapter 4, the extract from eye (10%) and ovary (3%) enhanced the cell migration and viability significantly. This might be due to certain molecules in these two organs that are more proper for the health of cells from the lymphoid organ. A very large quantity of retinoid (vitamin A) was found in the eyes of euphausiid species but little in the rest of the body (Fisher *et al*., 1952). 11-cis or all-trans retinol were found in most mantis shrimp species (Goldsmith and Cronin 1993). Shiau and Chen (1999) reported that shrimp fed with diets supplemented with 300 retinol equivalent vitamin A/kg had a significantly greater weight gain than the shrimp fed with lower concentration (Shiau and Chen 1999). The functions of retinoid (retinol and other molecular derived from vitamin A) in shrimp may be crucial for the metabolism and essential for normal health, growth, development and reproduction. In mature ovaries, lipids accumulate up to 18% - 41% (in various species) of the total ovarian dry mass (Teshima and

Kanazawa 1983; Castille and Lawrence 1989) and approximately 30% of the total fatty acids content of both phospholipids and triacylglycerols is made up of polyunsaturated fatty acids in the ovary (Ravid *et al.* 1999). These specific fatty acids support a regular metabolism of shrimp cells *in vitro*.

In the primary cell cultures derived from the lymphoid organ of *P. vannamei*, three cell types were observed: round cells (in the first 24 h post seeding), fibroblast-like cells (after 24 h post seeding) and epitheloid cells (after 48 h post seeding) (Chapter 3). The round cells were reported by Jose *et al*. (2012), Tsing *et al.* (1989) and Wang *et al*. (2000). In our study, the round cells were confirmed to be hemocytes by a granule marker of shrimp haemocytes (van de Braak *et al*., 2001). The fibroblast-like cell and epitheloid cell were also reported before (Itami *et al*., 1999; Wang *et al*., 2000). Wang *et al*. (2000) even separated the cells from the lymphoid organ of *P. monodon* into six different types (fibroblast-like cells, phagocytes, granulocytes, reticular cells, densonuclear cells and adipocytes) by electron microscopy. Due to the lack of reliable markers and functional tests, these cell types could not be identified further.

Several primary cell cultures derived from shrimp lymphoid organ have been proven to be susceptible for WSSV (Han *et al*., 2013; Li *et al*., 2014; Tapay *et al*., 1995). However, since in the primary cell cultures, cell numbers and proportions of certain cell types are variable and these cells usually do not form a uniform monolayer, it is impossible to study virus-cell interactions in a reproducible way. Therefore, we have searched for a solution. An answer was found in the use of secondary cell cultures. The secondary cell cultures formed a perfect uniform cell monolayer with constant cell quantities and cell types, which was ideal for obtaining reproducible results.

In the present thesis, a full standardization finally ensured the successful development of primary and secondary cell cultures from the lymphoid organ of *P. vannamei*:

(i) Molting stage

Since penaeid shrimp have a special molting cycle and the levels of certain proteins and hormones that are involved in cell growth and proliferation are variable in between different stages (Chan *et al*., 1998), it is logical that shrimp should be in a proper molting stage in order to be successful in establishing primary cell cultures and subcultures. During the present PhD work, it was determined that shrimp should be in a premolting stage (D2, Corteel *et al*., 2012) (data not shown).

(ii) Plastic coating

Coating of the culture vessels is a commonly-used method to promote cell attachment and survival in mammal cell cultures. In this thesis, 0.1% gelatin coating on the culture plates successfully stimulated cells to migrate out of the lymphoid organ explants and survive well *in vitro*.

(iii) Medium

The formula of culture medium was successfully determined in this thesis: 2 x L-15, supplemented with 20% FBS, 10% Chen's salt, 10% extract from eye, 600 µg/ml cholesterol, 1000 µg/ml L-glutathione, penicillin (100 IU/ml), streptomycin (100 µg/ml), gentamycin (50 µg/ml) and fungizone (0.25 µg/ml). 10% extract from eye, 600 µg/ml cholesterol and 1000 µg/ml L-glutathione in the culture medium significantly enhance cell migration and survival *in vitro*. With this defined medium, the cells derived from the lymphoid organ successfully form a monolayer within 4 days post seeding. Afterwards, cells continue to migrate out of the explants and finally form multilayers until about 9 days post seeding. At that time, a subculture can be made. The culture medium used for the primary cell culture is also perfect in maintaining the secondary cell cultures for more than 10 days with a viability of 90%.

(iv) Subculturing

The most difficult task for making a secondary cell culture and eventually establishing a cell line from shrimp or other crustaceans is subculturing. In the present thesis, this was successfully done mechanically without the use of enzymes. Previously, some subcultures have been reported (Fan & Wang, 2002; Hsu *et al*., 1995; Hu *et al*., 2008; Tapay *et al.*, 1995). However, the proliferation capacity of these subcultured cells was not verified. Therefore, the authors could not come to sound conclusions. In addition, some of these cells have been shown not to be of shrimp origin but contaminations from micro-organisms, such as protozoa and thraustochytrids (Hsu *et al*., 1995; Rinkevich, 1999). For instance, the crayfish cell line derived from neural ganglia of spiny cheek crayfish *Orconectes limosus* (Neumann *et al.*, 2000) has been proven to be from *Acramoebidae* (Lee *et al*., 2011). Therefore, it is necessary to control cultured cells at the beginning of primary cell cultures. In this thesis, the primary cells were authenticated by sequence alignment of the 1800 bp fragment between the 16S and 12S regions of the mitochondrial rDNA. This authentication has successfully

confirmed that these primary cells were truly from *P. vannamei* (Chapter 3).

Replication cycle of white spot syndrome virus (WSSV)

In previous studies, research was seldom focused on the replication cycle (binding, penetration, disassembly, viral protein expression, virion assembly and release) of WSSV due to the lack of proper cell tools. To better understand this replication cycle and ultimately get an antiviral cure against white spot syndrome, standardized and reproducible techniques are necessary. The secondary cell cultures that were developed in the present thesis made it possible to study the different steps of WSSV replication: binding, penetration, disassembly, viral protein expression, virion assembly and release.

Binding is the first step of a host cell infection. WSS virions bind to the cell membrane using envelope proteins with a cell attachment motif. The envelope protein VP28 was reported before to play a key role in the initial step of WSSV infection (van Hulten *et al.*, 2001). In our study, the attachment of WSSV peaked at 120 min incubation at 4 °C (Chapter 5). The numbers of virus particles on each positive cell varied. This may be explained by the use of the secondary cell cultures which consist of a heterogeneous population of cells. The different cell types may contain different numbers of receptors on the outer surface.

After binding, WSS virions penetrated cells via endocytosis. This endocytic penetration may be mediated by a VP28-binding protein (PmRab7, Sritunyalucksana *et al.*, 2006) that has a high homology to the small GTP-binding protein Pab7, which plays an important role in exocytic and endocytic pathways (Chavrier *et al.*, 1990; Feng *et al.*, 1995). Moreover, endocytosis was reported to be involved in the entry of WSSV into shrimp hemocytes (Huang *et al.*, 2013). Although endocytosis has been proven to be one of the entry pathways of WSSV, the cellular and molecular mechanisms are still not clear. At the same time with the virus penetration, WSSV particles disassembled: the virus envelope and nucleocapsid became separated; the nucleocapsid was transported to the nucleus, most probably to inject the viral genome into the nucleus. Afterwards, the virus envelope and capsid degraded in the following 3 hours.

In the meantime, the expression of viral proteins started. The capsid protein VP664 was synthesized in the cytoplasm from 1 hour post inoculation (hpi) and transported into the nucleus from 3 hpi. Leu *et al*. (2005) reported that the VP664 transcripts were detected in small amounts from 2 hours post intramuscular injection (samples were collected from pleopods of WSSV-infected *P. monodon*). The expression of envelope protein VP28 was first observed in the cytoplasm at 3 hpi and transported into the nucleus from 6 hpi. Li *et al*. (2009) showed that the gene expression of VP28 was first detected at 4 hour post infection (samples were collected from primary hemocyte cultures of crayfish). With a quantitative PCR it is proved that the multiplication of WSSV DNA started from 6 hpi in cells, which indicated that the capsid protein VP664 and envelope protein VP28 both started to be synthesized in the cytoplasm before the DNA genome was multiplied in the nucleus. Then, VP664 may be considered as immediate-early proteins and VP28 as early proteins. VP664 and VP28 may have more important functions in the virus replication.

The assembly of WSSV particles started after viral protein expression. Results in this thesis showed that the assembly, production and release of new infectious virus occurred as early as 12 hpi and continued up to 18 hpi (Chapter 5). New WSS virions became released from the cells via a cell collapse.

A mature WSSV particle contains four structural components: a viral double stranded DNA core, a capsid, a tegument and an outer lipid membrane envelope. This structure resembles well that of alphaherpesviruses (Boehmer&Lehman, 1997). When comparing the replication cycles of WSSV and alphaherpesviruses, many similarities but also some clear differences can be found:

(i) alphaherpesviruses may enter cells via fusion or endocytosis (Campadelli-Fiume *et al*., 1988; Milne *et al*., 2005; Nicola *et al*., 2003; Stackpole, 1969). WSSV enters cells via endocytosis (this thesis; Huang *et al*., 2013). Up till now, no evidence has been found that supports the entry of WSSV by fusion.

(ii) WSSV capsid protein VP664 becomes expressed as early as 1 hour post inoculation (hpi) and reaches already a peak at 3 hpi. The capsid proteins of alphaherpesviruses are expressed starting from 3 hpi and reaching a peak at 5 - 7 hpi (early protein; Boehmer&Lehman, 1997). Therefore, it looks like WSSV capsid proteins should be more considered as immediate early proteins. Consequently, they

most probably have additional functions besides taking part in particle assembly. It is very well possible that they play an essential role in down-regulating the cell metabolism and up-regulating the viral transcription. The huge size of the capsid protein VP664 (6077 a.a.) may be related with the multiple functions of the protein.

(iii) The envelope protein VP28 is detectable as early as 3 hpi and reaches a peak at 6 hpi, which is in line with the expression of early (E) genes of alphaherpesviruses. In contrast, the envelope proteins of alphaherpesviruses are late proteins (Boehmer&Lehman, 1997). The reason why the WSSV envelope proteins are expressed that early is because the final envelopment is taking place at the inner nuclear membrane for WSSV whereas this happens at the trans-Golgi vesicles for alphaherpesviruses, after a primary envelopment/de-envelopment at the inner and outer nuclear membranes (Granzow *et al*., 2001; Siminoff&Menefee, 1966; Skepper *et al*., 2001; Stackpole, 1969).

(iv) WSSV DNA synthesis was detected from 6 to 18 hpi, which is consistent with that of alphaherpesvirus (from 3 to 15 hpi, Boehmer&Lehman, 1997).

In conclusion, in this thesis, primary and secondary cell cultures from the lymphoid organ of *P. vannamei* were successfully developed. The secondary cell cultures were ideal for the study of the replication cycle of WSSV. The full replication cycle (binding, penetration, disassembly, viral protein expression, virion assembly and release) of WSSV was unraveled in the secondary cell cultures. Based on the results presented in this thesis, a model of the replication cycle of WSSV in the secondary cell cultures was presented (Fig. 8, Chapter 5). More work will be focused now on the cellular and molecular aspects of WSSV replication. The secondary cell cultures from the lymphoid organ of *P. vannamei* will allow the screening of molecules for their anti-WSSV activity. In a long term, this may lead to the development of antivirals. In addition, these secondary cell cultures are a good start for the establishment of a shrimp cell line.

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Chapter 7

Summary - Samenvatting

Summary

With the rapid expansion of shrimp farming worldwide in the last two decades, viral diseases have caused huge losses in this booming industry. Among these causative viruses, white spot syndrome virus (WSSV) has been one of the most serious viral pathogens that have threatened shrimp production. Reliable diagnostic PCR tests have been established, but most analyses have been done by histopathology and electron microscopy. To develop antivirals in order to control WSS, a complete understanding of the virus replication is essential. Cell cultures are needed to study the virus replication cycle. Since no continuous crustacean cell lines are available for propagation of the virus, it is necessary to set up reproducible cell cultures for studying the replication cycle of WSSV and WSSV-cell interactions.

In chapter 1, a general overview of the current status of aquaculture and *Penaeus vannamei* was provided. Furthermore, a detailed overview of WSSV and shrimp cell cultures was given.

Chapter 2 outlines the aims of this thesis. It was highlighted that a cell culture model for WSSV investigation will be necessary to aid the shrimp aquaculture industry in fighting WSS. Therefore, the present thesis aimed to develop stable cell cultures to serve as a basis for studying the replication cycle of WSSV and the interaction of WSSV with its host cells.

Chapter 3 focuses on the establishment of primary cell cultures from the lymphoid organ of *P. vannamei*. The optimal culture medium formulation (2x L-15, 20% FBS, 10% Chen's salt, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 0.25 µg/ml fungizone), shrimp characteristics (weight: 20 g, molting stage: D2), and culture conditions (temperature: 27 °C, osmolality 900 mOsm/L and pH: 7.5) were determined. Moreover, coating with an extracellular matrix (0.1% gelatin) and addition of medium supplements (cholesterol and L-glutathione) were evaluated and proved to be significantly favorable for cell migration and survival *in vitro*

(cholesterol: 600 μg/ml; L-glutathione: 1000 μg/ml). The cell types (round cells, fibroblast-like cells and epitheloid cells) were preliminarily characterized. Finally, the primary cell cultures were challenged with WSSV and their susceptibility was demonstrated.

Chapter 4 describes the further optimization of the culture medium formulation for the primary cells from the lymphoid organ of *P. vannamei*. Extracts from muscle, brain, ganglia, eyestalk, ovary and eyes were evaluated for their cell migration and survival enhancing properties *in vitro*. Extracts from eyes (10%) and ovary (3%) significantly enhanced the cell migration and survival in the first 6 days post seeding. Extracts from muscle (10%) and eyestalk (10%) also enhanced the cell migration and survival significantly within the first 4 days post seeding. Since eyes are much easier to collect compared to ovaries, the 10% extract from eyes was chosen as supplement, resulting in the following medium formulation: 2x L-15, 20% FBS, 10% eye extract, 10% Chen's salt, 600 μg/ml cholesterol, 1000 μg/ml L-glutathione, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 0.25 μg/ml fungizone.

In chapter 5, secondary cell cultures were used for studying the replication cycle of WSSV, since primary cells were less useful for this purpose. Uniform cell monolayers were formed within 24 h post seeding. The monolayers, consisting of constant cell quantities and cell types, could be maintained for 10 days with a viability of 90%, which was ideal for investigating the replication cycle of WSSV. Therefore, binding, penetration, disassembly, viral protein expression, and virion assembly and release of WSSV were investigated in the secondary cell cultures from the lymphoid organs of *P. vannamei*. Binding of WSSV to the cells reached a maximum (74 ± 3% of cells and 4.84 ± 0.2 virus particles per virus-binding cell) at 120 min at 4 °C. The co-localization of WSSV and early endosomes was observed starting from 30 min post inoculation, which indicated that WSSV entered cells by endocytosis. All cell-bound WSSV virions entered host cells between 0 to 60 min post inoculation and the uncoating of WSSV occurred in the same period. The envelope and nucleocapsid disassembled between 60 and 90 min post inoculation. After 1 h inoculation at 27 °C, the WSSV nucleocapsid protein VP664 and envelope protein VP28 started to be

synthesized in the cytoplasm from 1 to 3 hour post inoculation (hpi) and were transported into nuclei from 3 and 6 hpi, respectively. The percentage of cells that were VP664 and VP28 positive in the nuclei peaked (50 ± 4%) at 12 hpi. With quantitative PCR it is shown that WSSV DNA started to be synthesized from 6 hpi. *In vivo* titration of the supernatants showed that the progeny WSSV became released from 12 hpi and peaked at 18 hpi.

In chapter 6, the main findings of this thesis are discussed. The techniques generated in this thesis were considered to represent a basis for further research. The development of primary and secondary cell cultures from the lymphoid organ of *P. vannamei* provides a good start for the establishment of a shrimp cell line. Furthermore, the insights that were obtained by studying the replication cycle (binding, penetration, disassembly, viral proteins expression, assembly, and release) in the secondary cell cultures are valuable for future WSSV research.

Samenvatting

Tijdens de laatste twee decennia kende de wereldwijde garnalen productie een sterke groei. Helaas ging dit gepaard met de verspreiding van virale infecties bij garnalen, wat leidde tot grote economische verliezen. Een van de belangrijkste virale oorzaken van deze verliezen is het "white spot syndrome virus" (WSSV). Betrouwbare PCR technieken bestaan om WSSV te detecteren, maar de meeste studies beperken zich tot histopathologische en elektronenmicroscopische analyses. Om het WSSV te kunnen bestrijden en de ontwikkeling van antivirale middelen toe te staan, is er nood aan een betere kennis van de virusreplicatie. Celculturen zijn een waardevol middel om de virale replicatie en virus-cel interacties te bestuderen *in vitro*. Omdat er geen continue schaaldiercelculturen bestaan waarin virusreplicatie mogelijk is, is het nodig om celculturen te ontwikkelen waarin de replicatie en de interactie van WSSV met cellen kan worden bestudeerd.

Hoofdstuk 1 geeft een algemene inleiding over de huidige stand van zaken van de kweek van *Penaeus vannamei.* Verder wordt een gedetailleerd overzicht gegeven van het WSSV en het cultiveren van garnalencellen.

Hoofdstuk 2 beschrijft de doelstellingen van dit doctoraat. Er werd benadrukt dat een celcultuur model belangrijk is in de strijd tegen WSS. Het doel van deze thesis was dan ook om een stabiele celcultuur te ontwikkelen die de basis zal vormen voor het bestuderen van de replicatiecyclus en de interactie van WSSV met cellen.

Hoofdstuk 3 focust op de ontwikkeling van een primaire celcultuur van cellen uit het lymfoïde orgaan van *P. vannamei.* De formulering van het medium (2x L-15, 20% FBS, 10% Chen's salt, 100 IU/ml penicilline, 100 µg/ml streptomycine, 50 µg/ml gentamycine en 0.25 µg/ml fungizone), de invloed van het moment van isolatie (garnalen met een gewicht van 20 g in vervellingstadium D2) en de cultuur condities (temperatuur: 27 °C, osmolaliteit 900 mOsm/L en pH: 7.5) werden bepaald. Verder werd het effect van een extracellulaire matrix coating (0.1% gelatine) en het

toevoegen van medium supplementen (cholesterol en L-glutathione) geëvalueerd. Deze hadden een positieve invloed op de celmigratie en overleving *in vitro* (cholesterol: 600 µg/ml; L-glutathione: 1000 µg/ml). Er werd ook een preliminaire karakterisatie van de aanwezige celtypes uitgevoerd en de gevoeligheid voor WSSV werd getest.

In hoofdstuk 4 werd de formulering van het medium voor de primaire cultuur van cellen uit het lymfoïde orgaan van *P. vannamei* verder geoptimaliseerd. Er werden extracten gemaakt van spierweefsel, hersenen, ganglia, oogsteel, oog en eierstokken en geëvalueerd of deze extracten de levensduur en migratie konden verbeteren *in vitro*. De extracten gemaakt van het oog (10%) en de eierstokken (3%) gaven een significante verhoging van de celmigratie en overleving gedurende de eerste 6 dagen na het planten. Extracten van het spierweefsel en de oogsteel verbeterden de migratie en overleving ook gedurende de eerste 4 dagen na planten. Omdat de ogen gemakkelijker te verzamelen zijn dan de eierstokken werd gekozen om een 10% oogextract toe te voegen aan het celcultuur medium. Zo werd de uiteindelijke formulering van het medium (2x L-15, 20% FBS, 10% oogextract, 10% Chen's salt, 600 µg/ml cholesterol, 1000 µg/ml L-glutathione, 100 IU/ml penicilline, 100 µg/ml streptomycine, 50 µg/ml gentamycine en 0.25 µg/ml fungizone) bekomen.

In hoofdstuk 5 werden secundaire celculturen gebruikt om de replicatiecyclus van WSSV te bestuderen. De primaire culturen waren namelijk weinig bruikbaar voor deze studies. Een monolaag van cellen werd gevormd 24u na het planten van de cellen. Deze monolaag, met uniforme celverdeling en celtypes, kon gedurende 10 dagen in cultuur gehouden worden met een vitaliteit van 90%. De replicatiecyclus (binding, binnendringen, ontmanteling, expressie van de virale proteïnen, de samenstelling van virions en de vrijstelling van virus) van het WSSV werd bestudeerd in de secundaire celculturen van het lymfoïde orgaan van *P. vannamei*. De binding van WSSV aan de cellen was maximaal (74 ± 3% van de cellen met 4.84 ± 0.2 virus partikels per virusbindende cel) 120 minuten na incubatie bij 4°C. Er werd een co-lokalisatie van WSSV met vroege endosomen vastgesteld 30 minuten na inoculatie, wat aangeeft dat het virus de cellen binnendringt via endocytose. Dubbelkleuringen toonden aan dat de

celgebonden WSSV partikels de cellen binnendringen binnen het uur na inoculatie en dat het ontmantelen van de partikels gelijktijdig gebeurt. De envelop en het nucleokapsied werden afgebroken tussen 60 en 90 minuten na inoculatie. Synthese van het WSSV nucleokapsied proteïne VP664 en het envelop proteïne VP28 begon tussen 1 en 3 u na een inoculatie van 1 u bij 27°C. Deze proteïnen werden naar de kern getransporteerd op respectievelijk 3 en 6 u na inoculatie. Het percentage cellen met VP664 en VP28 in de kern (50 ± 4%) piekte op 12 u na inoculatie. Met de kwantitatieve PCR test bleek dat WSSV DNA begint te worden gesynthetiseerd vanaf 6 hpi. De *in vivo* titratie van het supernatans van deze cellen toonde aan dat nieuwe viruspartikels worden vrijgesteld vanaf 12u, met een piek op 18u na inoculatie.

Een algemene discussie van het verrichte werk kan worden teruggevonden in hoofdstuk 6. De technieken die op punt werden gesteld in deze thesis zullen de basis vormen voor verder onderzoek naar WSSV. De ontwikkeling van primaire en secundaire celculturen van het lymfoïde orgaan van *P. vannamei* zijn een start in het genereren van een garnalencellijn. Verder werd de replicatiecyclus van WSSV in kaart gebracht, waardoor er nu verder onderzoek kan gebeuren naar de verschillende stappen in dit proces.

Curriculum vitae

Wenfeng Li was born on February 4th, 1984 in Yantai City, Shandong Province, P.R. China.

He obtained the degree of Biological Science in 2007 at the Ocean University of China. At the same year, he continued his master studies in the Faculty of Fisheries, Ocean University of China and finished his master thesis entitled "Somatic cell culture of turbot (*Scophthalmus maximus*) and its application in virus research" in 2010.

Subsequently, he obtained a 4-year scholarship from the Chinese Scholarship Council and started his doctoral studies under the supervision of Prof. Dr. Hans Nauwynck at the Labotatory of Virology of the Faculty of Veterinary Medicine in Gent University from October, 2010.

Wenfeng Li is author or co-author of 5 scientific publications. He supervised 1 Master student.

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