CLONING, EXPRESSION AND POLYCLONAL ANTIBODIES PRODUCTION AGAINST RECOMBINANT VP19 ENVELOPE AND VP26 CAPSID PROTEINS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN BLACK TIGER SHRIMP (*Penaeus monodon*)

PHIROMSAK PHATTANAPAIJITKUN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(TROPICAL MEDICINE)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005

ISBN 974-04-6102-6

This thesis

Entitled

CLONING, EXPRESSION AND POLYCLONAL ANTIBODIES PRODUCTION AGAINST RECOMBINANT VP19 ENVELOPE AND VP26 CAPSID PROTEINS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN BLACK TIGER SHRIMP (*Penaeus monodon*)

.....
Mr. Phiromsak Phattanapaijitkun
Candidate

.....
Assoc. Prof. Nitaya Thammapalerd,
B.Sc., M.Sc.(Biochemistry)
Major-Advisor

.....
Lect. Somchai Awakairt,
M.Sc.(Biological Chemistry)
Co-Advisor

.....
Assoc. Prof. Paisarn Sithigorngul,
Ph.D.(Zoology)
Co-Advisor

.....
Dr. Siwaporn Longyant,
Ph.D.(Marine Science)
Co-Advisor

.....
Dr. Parin Chaivisuthangkura,
Ph.D.(Molecular and Cell Biology)
Co-Advisor

.....
Assoc. Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies

.....
Assoc. Prof. Songsak Petmitr, Ph.D.
Chair
Master of Science Programme in
Tropical medicine
Faculty of Tropical Medicine

This thesis

Entitled

CLONING, EXPRESSION AND POLYCLONAL ANTIBODIES PRODUCTION AGAINST RECOMBINANT VP19 ENVELOPE AND VP26 CAPSID PROTEINS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN BLACK TIGER SHRIMP (*Penaeus monodon*)

was submitted to Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Tropical Medicine)
on
12 May, 2005

……..…….
Mr. Phiromsak Phattanapaijitkun
Candidate

……
Lect. Somchai Awakairt,
M.Sc.(Biological Chemistry)
Member

……..
Assoc.Prof.Nitaya Thammapalerd,
B.Sc., M.Sc.(Biochemistry)
Chair

..……..
Dr. Siwaporn Longyant,
Ph.D.(Marine Science)
Member

……
Assoc.Prof.Paisarn Sithigorngul,
Ph.D.(Zoology)
Member

……
Assoc.Prof.Weerawan Sithigorngul,
Ph.D.(Zoology)
Member

……..
Dr. Parin Chaivisuthangkura,
Ph.D.(Molecular and Cell Biology)
Member

…….
Assoc.Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

…….
Assoc. Prof. Pratap Singhasivanon,
M.B.B.S., D.T.M. & H.(Bangkok),
M.P.H., Dr.P.H.(Epidemiology)
Dean
Faculty of Tropical Medicine
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and deep appreciation to my advisor Associate Professor Nitaya Thammapalerd, for her kindness, guidance and valuable instruction throughout the course of this study. My special appreciation is expressed to my co-advisors, Associate Professor Dr. Paisarn Sithigorngul, Dr. Parin Chaivisuthangkura, Dr. Siwaporn Longyant and Lecturer Somchai Awakairt for their worthy suggestion and discussion.

I would like to express my great thanks to Associate Professor Dr. Weerawan Sithigorngul and Assistant Professor Thareerat Kalambaheti for their valuable advice.

I would like to thank Department of Biology, Faculty of Science, Srinakharinwirot University, and Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University for providing the facilities and all instruments for my research work.

Special thanks are given to Dr. Wannipa Phianphak, Dr. Phochit Winotaphan, Dr. Nanthika Panchan, Mr. Sombat Rukpratarnporn, Ms. Suneerat Sattaman, Ms. Wilawan Ragsatham and all members of monoclonal antibodies Lab., SWU for their uncountable helps during moment of my worrisome, depression and cheerfulness.

My appreciation is extended to all other members in the Department of Biology, especially the members in rooms 1114 - 3 and 1103, for their friendships and their helps during my study. All the help I have received will be long remembered.

Finally, great appreciation is specially expressed to my family for giving me the warmest life with their love.

Phiromsak Phattanapaijitkun

CLONING, EXPRESSION AND POLYCLONAL ANTIBODIES PRODUCTION AGAINST RECOMBINANT VP19 ENVELOPE AND VP26 CAPSID PROTEINS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN BLACK TIGER SHRIMP, *PENAEUS MONODON*

PHIROMSAK PHATTANAPAIJITKUN 4537238 TMTM/M

M.Sc. (TROPICAL MEDICINE)

THESIS ADVISORS: NITAYA THAMMAPALERD, M.Sc., PAISARN SITHIGORNGUL, Ph.D., PARIN CHAIVISUTHANGKURA, Ph.D., SIWAPORN LONGYANT, Ph.D., SOMCHAI AWAKAIRT, M.Sc.

ABSTRACT

White spot syndrome virus (WSSV) is the causative agent of white spot syndrome in black tiger shrimp (*P. monodon*) cultured worldwide, including Thailand. This virus was first reported in the 1980s, but major epidemics in Thailand were reported in the 1990s. WSSV transmission can occur both horizontally and vertically. Early detection of WSSV in infected *P. monodon* is an effective way to control WSSV epidemics in shrimp culture. The objective of this study was to produce polyclonal antibodies (PAbs) to detect white spot syndrome virus. Structural gene of envelope protein VP19 was cloned into expression vector (pMAL-C2) and expressed in *E. coli* (BL21) as a fusion protein, MBP-VP19 with a M.W. of 55.5 kDa. For WSSV capsid protein VP26, fusion protein with 6-histidine tag, 6 x His- VP 26, was used to generate polyclonal antibodies. The bacterial expression system allowed the production of 10 mg of purified recombinant proteins per liter of bacterial culture. Both recombinant proteins were used to immunize Swiss albino mice to produce polyclonal antibody. The WSSV-specific polyclonal antibodies were characterized by Western blot and immunohistochemistry and detected WSSV in both native and denatured forms. The MBP-VP19 and 6xhis-VP 26 proteins obtained from this project are useful for future vaccine development to prevent WSSV infection in shrimp farms. The recombinant protein will be used to produce monoclonal antibodies (MAbs) and used together with previous WSSV VP28 MAb to confirm and increase detection sensitivity in various immunoassays, including a simple immuno-based test kit (strip- test) and ELISA.

KEY WORDS; WHITE SPOT SYNDROME VIRUS / RECOMBINANT PROTEINS, VP19, VP26 / PABS-MABS / IMMUNOHISTOCHEMISTRY / PENAEUS MONODON

69 P. ISBN 974-04-6102-6

การโคลน การแสดงออกและการผลิตโพลีโคลนอลแอนติบอดีต่อโปรตีนส่วนเปลือกVP19และโปรตีนแคปซิด VP26ของไวรัสตัวแดงดวงขาวในกุ้งกุลาดำ (CLONING, EXPRESSION AND POLYCLONAL ANTIBODIES PRODUCTION AGAINST RECOMBINANT VP19 ENVELOPE AND VP26 CAPSID PROTEINS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN BLACK TIGER SHRIMP, *PENAEUS MONODON*

ภิรมย์ศักดิ์ พัฒนไพจิตต์กุล 4537238 TMTM/M

วท.ม. (อายุรศาสตร์เขตร้อน)

คณะกรรมการควบคุมวิทยานิพนธ์: นิตยา ธรรมพาเลิศ, M.Sc., ไพศาล สิทธิกรกุล, Ph.D.,

ปรินทร์ ชัยวิสุทธางกูร, Ph.D., ศิวาพร ลงยันต์, Ph.D., สมชัย อวเกียรติ, M.Sc.

บทคัดย่อ

ไวรัสตัวแดงดวงขาว (WSSV) เป็นสาเหตุหลักของโรคตัวแดงดวงขาวในกุ้งกุลาดำ *(P. monodon*) ซึ่งพบการแพร่กระจายของโรคดังกล่าวในฟาร์มเพาะเลี้ยงกุ้งทั่วโลก ไวรัสชนิดนี้ถูกค้นพบในปี 1980 และพบการแพร่ระบาดอย่างรุนแรงในประเทศไทยในปี 1990 โดยสามารถติดต่อได้หลายช่องทางทั้งจากพ่อแม่พันธุ์และ สัตว์ที่ติดเชื้อสู่กุ้งโดยตรง การวินิจฉัยการติดเชื้อไวรัสตัวแดงดวงขาวในช่วงเริ่มแรกของการเพาะเลี้ยงเป็นวิธีการป้องกันการแพร่ระบาดของไวรัสชนิดนี้อย่างได้ผล การศึกษานี้ได้ทำการเพิ่มปริมาณยีนโครงสร้างของโปรตีนส่วนเปลือก (VP19) ของไวรัสตัวแดงดวงขาวโดยนำมาโคลนเข้าสู่ expression vector pMAL-C2 และนำเข้าสู่ *E. coli* สายพันธุ์ BL21 และสามารถสร้างโปรตีน MBP-VP19 ขนาด 55.5 kDa สำหรับยีนของโปรตีนส่วนแคปซิด (VP26) ของไวรัสชนิดนี้ที่อยู่ในรูปของ 6xHis-VP26 ได้ถูกนำมาใช้ผลิตโพลีโคลนอลแอนติบอดีเช่นกัน การเตรียม recombinant protein โดยอาศัยแบคทีเรีย *E. coli* สามารถผลิตโปรตีนปริมาณ 10 mg / l โปรตีนทั้งสองนี้เมื่อนำมาทำให้บริสุทธิ์ด้วยการตัดและสกัดโปรตีนจากแถบของ SDS-PAGE พบว่ามีความบริสุทธิ์สูง ซึ่งเมื่อนำไปฉีดให้หนูขาวสำหรับการผลิตโพลีโคลนอลแอนติบอดีได้ โดยทำการตรวจสอบแอนติบอดีที่จำเพาะต่อโปรตีนของไวรัสด้วยวิธี Western blot และ immunohistochemistry โดยโพลีโคลนอลแอนติบอดีที่ได้นี้สามารถตรวจหาเชื้อไวรัสตัวแดงดวงขาวทั้งในรูปธรรมชาติและรูปที่เสียสภาพ นอกจากนี้ยังคาดว่าจะสามารถใช้ MBP-VP19 และ 6xHis-VP 26 ที่ผลิตได้ไปทดสอบความเป็นวัคซีนในการป้องกันการติดเชื้อไวรัสตัวแดงดวงขาว และในอนาคตสามารถนำ recombinant proteinsไปผลิตโมโนโคลนอลแอนติบอดี ที่เพิ่มความไวในการตรวจสอบไวรัส ควบคู่กับโมโนโคลนอลแอนติบอดีต่อโปรตีนส่วน VP28 ที่มีอยู่แล้วโดยใช้วิธีทางอิมมิวโนวิทยาที่หลากหลายเช่น strip test หรือ ELISA ได้

69 หน้า. ISBN 974-04-6102-6

LIST OF CONTENTS

LIST OF CONTENTS (cont.)

LIST OF CONTENTS (cont.)

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

Abbreviations or symbols	Terms
amp	Ampicillin
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
?C	Degree celcius
Ct	Connective tissue
DAB	Diaminobenzidine
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay
et al	and others
FCS	Fetal calf serum
g	Gram
GAM-HRP-conjugate	Goat anti mouse-horseradish peroxidase-conjugate
hr	Hour
H	Heart
Hc	Haematopoietic tissue
H&E	Haematoxylin & Eosin
Hp	Hepatopancreas
Ig	Immonoglobulin
IFA	Immunofluorescence Antibody assay
IHC	Immunohistochemistry
IPTG	isopropyl-?-thiogalactoside
KAc	Potassium acetate
Kan	Kanamycin
Kb	Kilobase

LIST OF ABBREVIATIONS (cont.)

kDa	Kilo Dalton
M	Muscle
MAb	Monoclonal antibody
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
nm	Nanometre
O.D.	Optical density
P_1^+	10 % calf serum in PBS
PAGE	Polyacrylamide gel electrophoresis
PAb	Polyclonal antibody
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	picogram
PMSF	phenyl methyl sulfonyl fluoride
PL	Post larvae
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
rpm	round per minute
Sc	subcuticular epitherium
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) aminomethane
μg	microgram (10^{-6}gram)
μl	microlitre (10^{-6}L)
μm	Micrometre
UV	Ultraviolet
VP	Viral protein
WB	Western blot
WSS	White spot syndrome

LIST OF ABBREVIATIONS (cont.)

WSSV	White spot syndrome virus
YHV	Yellow head virus

CHAPTER I
INTRODUCTION

1.1 General background

After the first discovery of white spot syndrome virus (WSSV) in Asia in the early 1990s, WSSV has become the epizootic disease in the shrimp growing country. The disease has led to severe mortalities of various species of cultured *Penaeus* shrimps including *Penaeus monodon*, *P. japonicus*, *P. chinensis*, *P. vannamei*, *P. penicillatus*, *P. indicus* and *P. stylirostris* all over the world (Inouye *et al*., 1994; Takahashi *et al.*, 1994; Chou *et al.*, 1995; Flegel *et al.*, 1997). WSSV is an enveloped, ellipsoid, large, double-stranded DNA virus (Figure 1). The genome size is 305 Kb with approximate 185 open reading frames (Yang *et al*., 2001; van Hulten *et al*., 2001a). WSSV has found in most shrimp farming area all over the world (Wang *et al*., 1995; Wongteerasupaya *et al.*, 1995; *Lo et al.*, 1996b), and it has a wide host range among crustaceans (Lo *et al.*, 1996a; Lo and Kou., 1998). The virus is transmitted by both horizontally (Chang *et al.*, 1996; Chou *et al.*, 1998) and vertically (Lo *et al.*, 1997). White spot syndrome (WSS) has become widespread and caused economically damaged disease of cultured shrimp in Thailand. White spots in the exoskeleton and epidermis are the most commonly clinical signs of WSS of infected shrimps (Figure 2). However, the presence of white spot might not found in the infected shrimps. For instance, under non-stressful condition those infected shrimps harboring white spot syndrome virus may survive indefinitely. However, the shrimps may also appear lethargic and their color change to pink or reddish brown, they will gather around the edges of ponds at the surface during the day. Then, there are rapid reductions in food consumption and a high mortality rate in the shrimp population can be expected within a few hours to a few days after the onset of the signs. WSSV can cause up to 100 % mortality with correspondingly devastating economic impact (Lo *et al.*, 1996). WSSV is extremely virulent virus, which infected various target tissues (Lo *et al.*, 1996, 1999). The known hosts of WSSV include *P. monodon*, *P. penicillatus* and *P.*

japonicus (Chou *et al.*, 1995). The histopathological studies have demonstrated that the causative agent WSSV most frequently attacks the cuticular epidermis, where its presence is evident in degenerated cells with hypertrophic nuclei (Wang *et al.*, 1995). WSSV has also been shown to cause disease, high mortality and latent infections not only in shrimp, but also in crabs and other arthropods (Lo *et al.*, 1996b, 1997, Lo and Kou, 1998). WSSV targets several of captured *P. monodon* brooders, including the reproductive organs. The WSSV particles were detected in follicle cells, oogonia and developing oocytes of the ovary and in connective tissues. Investigation of WSSV infection using 2-step polymerase chain reaction (PCR) revealed that some positive brooder produced larval batches that gave both 2-step WSSV PCR positive and negative test results with different samples (Lo *et al.*, 1997).

The size of the WSSV genome of different isolates are various: 305,107 bp (GenBank accession No: AF 332093), 292,967 bp (GenBank accession No: AF 369029) and 307,287 bp (GenBank accession No: AF 440570) for viruses isolated from China, Thailand and Taiwan, respectively. The size differences are mostly due to several small insertions and one large (~12 Kb) deletion (Chen *et al*., 2002). The WSSV consists of an enveloped nucleocapsid containing a circular double-stranded DNA genome. The enveloped viruses are symmetrical particle, ellipsoid to bacilliform in shape, and measure about 120-150 nm in diameter and by 270-290 nm in length. Most notable is a tail like appendage at one end. The isolated nucleocapsids measure 65-70 nm in diameter and 300-350 nm in length and have a cross batched appearance. The WSSV contain five major proteins and unknown numbers of minor polypeptides. The structural proteins VP28 and VP19 of WSSV are located at the envelope, while VP26, VP24 and VP15 are located at the nucleocapsid (van Hulten *et al.*, 2001a). The studies of the N-terminal amino acid sequences revealed that the open reading frames (ORFs) encoding VP28, VP26 and VP24 proteins are located in the WSSV genome (van Hulten *et al.*, 2001 a, b). The VP28 protein plays the key role in the systemic infection of shrimp (van Hulten *et al.*, 2001b). Another WSSV structural protein had been reported (Tsai *et al*., 2004). Enveloped viruses of vertebrates and invertebrates contain glycoproteins in their viral envelopes, which often play important role in the interaction between virus and host, such as attachment to receptor and fusion with cell membranes (Tyler, 1999 van Regenmortel; *et al.*,

2000). In this study, VP19 and VP26 encoding genes was cloned into the pMAL-C2 and pQE30 expression vectors and expressed as the recombinant proteins rVP19, rVP19-HPL and rVP26 (Sambrook and Russel, 2001). Polyclonal antibodies against VP19 and VP26 proteins were produced and used for detection of WSSV infection in shrimp in accordingly.

1.2 Significance of the research

White spot syndrome virus (WSSV) is the causative agent of white spot syndrome, the disease found in most shrimp farming areas worldwide, where it causes huge economic losses to the shrimp farming industries. WSSV is able to infect a large number of crustaceans including crabs and crayfish. The early-stage detection of the WSSV infection is the effective way to prevent the spread of the virus.

Nowadays, WSSV detection is being relied on the highly sensitive PCR method (Rout *et al.*, 2005). However, the PCR technique is cost-expensive and required experienced personnel. Also the contamination problem in PCR reaction has been encountered.

Therefore, the development of polyclonal (PAbs) antibodies and monoclonal antibodies (MAbs) against recombinant proteins, especially glycoproteins of WSSV, would be the alternative choices of WSSV detection. These PAbs and MAbs were used to develop the simple immuno-based test kit for WSSV detection with high specificity and high sensitivity at low cost in the near future.

1.3 Specific objectives

1. To clone and express the recombinant VP19 envelope and VP26 capsid proteins of WSSV.
2. To produce polyclonal antibodies against envelope and capsid proteins of white spot syndrome virus (WSSV) in black tiger shrimp (*Penaeus monodon*).

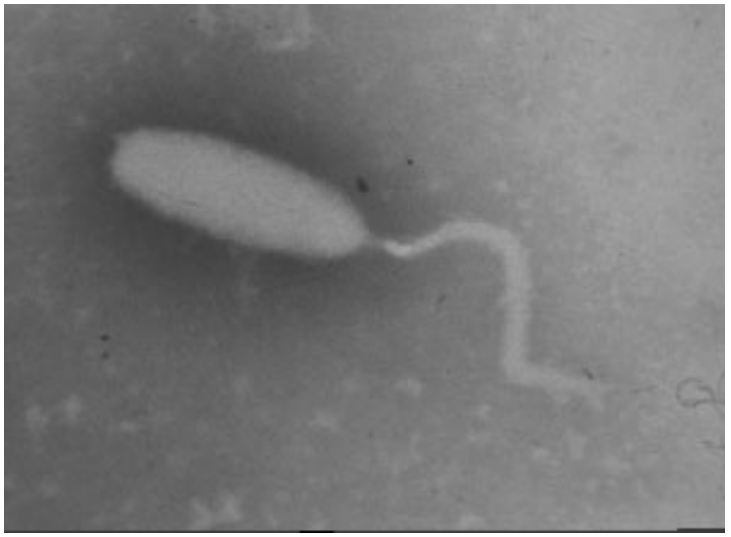


Figure 1. The transmission electron micrograph showing purified WSSV particle (Picture taken from www.plant.wageningen-ur.nl/ news/images/2001-1).

Figure 2. Left: A juvenile *P. monodon* that is displaying the distinctive white spot of white spot syndrome. Right: The black tiger shrimp (*P. monodon* Fabricius, 1978).

CHAPTER II
REVIEW OF THE LITERATURES

2.1 *Penaeus monodon* Fabricius, 1978

2.1.1 Morphology

P. monodon are the largest among species of the *Penaeus* group in the world. The body of this shrimp is slender and typically 2.6 mm. The rostrum is straight or slightly bent upward at the tip, usually having five dorsal spines but lacking ventral spines. The inner lower antennular flagellum is twice lower than the outer upper flagellum. The sixth abdominal segment does not have spinules. The coloration is generally dark colored with carapace and abdomen transversely banded with black and white; rest of body varies from light brown to blue or red; some smaller specimens show a dull red dorsal strip from rostrum to six abdominal segments (Grey *et al.,* 1983). The size is the largest commercially available shrimp, reaching > 330 mm (Dore and Frimodt, 1987) as shown in (Figure 3).

2.1.2 Biology

Habitat of *P. monodon* juveniles occupies shallow estuarine waters sporadically entering rivers whereas adults are usually found in deeper waters. They are trawled over mud or sand bottom to 110 m (Grey *et al.,* 1983). *P. monodon* adult spawning occurs in offshore waters where the larval stages are subsequently found, although the post-larvae can also survive in fresh water. Juveniles are highly efficient osmoregulators and in the general have iso-osmotic medium-hemolymph concentration at 20-30 ppt. Food and feeding habits of *P. monodon* diet preferences includes crustaceans and mollusks (85 % ingested food) and the remaining 15 % consisting of vegetable matter, polychaetes, fish, debris and sand, indicating that the black tiger shrimp is more of a predator rather than a scavenger or detritus feeder (Solis, 1988).

2.1.3 Distribution

The shrimp *P. monodon* is widely distributed throughout Batavia, East coast

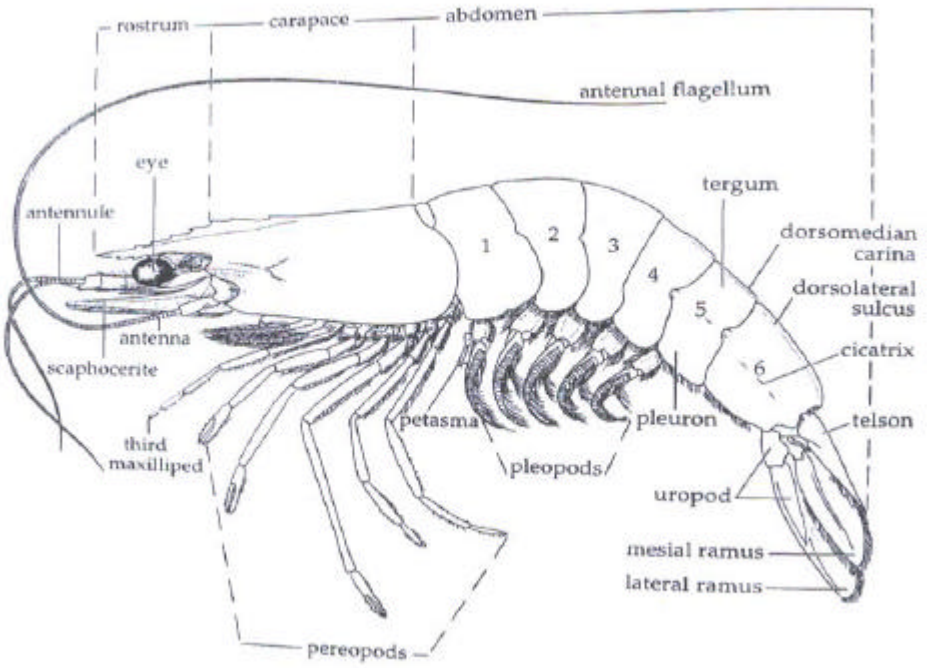


Figure 3. Conventionalized shrimp, illustrating terms used in the description and classification of *Penaeus monodon* (Farfante and Kensley, 1997).

of Africa, Red Sea, Madagascar, Mauritius, India, Srilanka, Malaysia, Singapore, Indonesia, China, Philippines, Hong Kong, Taiwan, Thailand, Japan, Korea, New Guinea, Fiji and Western Australia (GSMFC, 1998).

2.1.4 Aquaculture

In the 1970s, shrimp aquaculture started as an industrial activity and developed rapidly with a huge increase in the number of hatcheries and farms. Shrimp farming provides roughly 30 % of the shrimp supplied to the world market. The activity concern tropical countries in South East Asia, Central and South America (Rosenberry, 1996) and seriously affected by problems linked to environment degradation and to non-infectious and infectious agent (Johnson, 1990; Bachère, 2000; Saulnier *et al.*, 2000).

2.1.5 Disease

Disease is an important factor in reducing shrimp number in natural populations. Natural mortality or death from old age is the potential fate of all shrimp but the toll taken by predation, starvation, infestation, infection and adverse environmental conditions is highly significant. Disease problems are considered important to successful production in shrimp aquaculture. Because high-density, confined rearing is unnatural and produces stress, some shrimp-associated organism will become prominent. Special measures are required to offset their detrimental effects. Diseases are classified as non-infectious and infectious disease (Johnson, 1990).

2.1.5.1 Non-infectious diseases

Non-infectious diseases are often suspected to occur because of environment degradation exacerbated by inappropriate management practices eg. lack of oxygen, poisons, low temperature and salinity extremes (Bachère, 2000).

2.1.5.2 Infectious diseases

The intensification of the *penaeid* shrimp industry and the transfer of aquatic organisms worldwide have accompanied over the last two decades by an increased incidence of infectious pathogens (Saulnier *et al.*, 2000). The causative agent of infectious diseases in shrimp is mainly viruses and bacteria belonging to yellow head virus, white spot syndrome virus and vibrionaceae. These pathologies particularly hamper larval production and lead to profitability problems

due to stock mortalities. They also lead to the over-fishing of wild shrimp larvae and an overexploitation of broodstock. Moreover, the local environment can be contaminated by the discharge of wastewater containing viruses, which can faster the development of viral disease. Finally, the practice of shrimp transfer at national or international levels has contributed to the spread of disease, which is now an epizootic (Bachère, 2000).Viral diseases due mainly to YHV and WSSV species are often associated with low survival rates in hatchery or grow out conditions in shrimp aquaculture (Saulnier *et al.,* 2000).

2.2 White Spot Syndrome Virus (WSSV)

Since 1993, the closely related viruses resembling the characteristics of baculovirus and cause high mortalities in *Penaeus* shrimp have been reported in different parts of Asia and North America. This group of viruses has been described in the literatures using various names, including baculoviral hypodermal and haematopoietic necrosis virus of *P. chinensis* reported in China, bacilliform virus, rod-shaped nuclear virus of *P. japonicus* and *panaeid* rod-shaped DNA virus of *P. japonicus* reported in Japan, systemic ectodermal and mesodermal baculovirus of *P. monodon* reported in Thailand, White-spot syndrome of *P. monodon, P. japonicus* and *P. penicillatus* reported in Taiwan, white-spot baculovirus of *P. japonicus, P. monodon* and *P. penicillatus* reported in Indonesia, Vietnam, Malaysia, India and Chinese baculovirus of *P. japonicus* reported in Hawaii. All of these baculovirus caused the diseases, which are characterized by the presence of white spot on the inner surface of the carapace. For instances, the infected moribund shrimp is characterized by abnormal reddish-brown coloration. The clinical signs of the disease were rapid and cumulative mortality reaching 100 % within 2-7 days. The virus appears to be very similar in size, geographical range and histological signs. Recently, the white spot syndrome group of viruses is called white spot syndrome virus (WSSV) by general consensus. White spot syndrome virus is the causative agent of the shrimp viral disease that affected individuals with white spot on their exoskeleton, this is the principle signs and symptoms of the disease (Chou *et al.*, 1995, Wang *et al.*, 1995). White spot syndrome virus (WSSV) is circular double-stranded DNA genome of 292-967 Kb (van Hulten *et al.,* 2001a). The virus composes of five major proteins,

which are three major nucleocapsid proteins; VP26, VP24 and VP15, and two enveloped proteins VP28 and VP19. The open reading frames (ORFs) encoding VP28, VP26 and VP24 are located in the WSSV genome (van Hulten *et al.,* 2000 a, b). In case of VP19, this protein contains two putative transmembrane domains, which may anchor this protein in the WSSV envelope (van Hulten *et al.,* 2002). WSSV infection was detected by using conventional histopathology (Wongteerasupaya *et al.,* 1995) and electron microscopy (Takahashi *et al.,* 1994). Genome-based diagnosis can be performed using DNA hybridization (Chang *et al.,* 1996, 1998) or Polymerase Chain Reaction (PCR) detection using 1-step or 2-step PCR (Lo *et al.,* 1996 a, b, 1997). The 2-step amplification is 10^3 to 10^4 more sensitive than 1-step amplification. Antigen analysis of the virus has been carrying out using polyclonal rabbit antisera for immunodiagnosis (Nadala *et al.,* 1997, 1998; Cesar *et al.,* 2000). Previously studied indicated that monoclonal antibodies (MAbs) was generated by using whole WSSV particles. After monoclonal antibodies production, four clones of VP28 MAbs can be used to detect the viral infection by dot blot, Western blot, immunohistochemistry and immunofluorescence techniques. However, these MAb cannot be used to differentiate various WSSV strains from China, Thailand, India, Panama and the USA (Texas and South Carolina) (Poulos *et al*., 2001). Recently, the purified WSSV proteins were used to produce three MAbs clones specific to VP28 and VP18 and one clone specific to VP28 only. The specific reaction of the MAbs of WSSV was confirmed by various techniques including immunohistochemistry and immuno dot test. For the limitation of detection of immuno dot test was 500 pg of the viral protein, which similar to 1-step PCR and could be used to detect WSSV in *P. monodon* with and without gross signs of white spot in the cuticle (Anil *et al*., 2002). MAb productions using recombinant proteins of viral envelope (VP28) showed that three clones of MAbs binding to different epitopes were obtained. These MAbs can be used to develop antigen-capture ELISA technique to detect WSSV in haemolymph and lysate from tissue extraction with sensitivity similar to that of PCR (Liu *et al.,* 2002). MAbs against truncated form of recombinant VP28 protein were being used to produce the strip tests for detection of WSSV in shrimp (Chaivisuthangkura *et al*., 2004). The recombinant WSSV envelope proteins of VP19 and VP28 produced by using maltose-binding protein tag (MBP) were purified after expression in bacteria and were used

for studying the adaptive immune response of *P. monodon*. The shrimps were vaccinated by intramuscular injection with the purified proteins and challenged 2 and 25 days after vaccination to assess the onset and duration of protection. The results demonstrated the significantly increase survival rate after shrimp were vaccinated with the MBP-VP19 fusion protein when compared with shrimp injected with only MBP (Witteveldt *et al.*, 2004). In this study, the truncated versions of the VP19 encoding gene containing internal hydrophilic region designated VP19 (HPL) and VP26 were cloned into pQE30 expression vector and the complete VP19 encoding gene was cloned into pMAL-C2 expression vector before transformed into *E. coli* strain M15 (pREP4) and BL-21, respectively. The recombinant proteins were used to produce polyclonal and will be used to produce monoclonal antibodies for detection of VP19 and VP 26 proteins in WSSV infected shrimp. The MAb can be used to develop the WSSV test kit in the future.

2.2.1 Taxonomic classification

The genomic analysis of the viruses revealed that the WSSV was previously classified into the non-segmented, non-occluded circular double-stranded DNA genome of 292-967 Kb (van Hulten *et al.*, 2001a) of the subfamily Nudibaculovirinae of the family Baculoviridae based on the morphology, size, site of assembly, cytopathology and nucleic acid content (Wongteerasupaya *et al.*, 1996; Karnchanaphum *et al.*, 1998). However, analysis of large and small subunit of ribonucleotide reductase, thymidine-thymidylate kinase, protein kinase and phylogeny indicated that WSSV and baculoviridae are not closely related to family Baculoviridae. Recently, the WSSV has been proposed to be a member of family named Nimaviridae (van Hulten *et al.*, 2000), genus Whispovirus (Mikes *et al.*, 2003).

2.2.2 Clinical signs

Infected juvenile and adult shrimps become lethargic, slow swimming, reduce in food consumption and have a loose cuticle with white calcium deposits embedded in it (Takahashi *et al.*, 1994). Pink to reddish discoloration of the body surface and of the appendage is also commonly observed. However, not all shrimps infected with WSSV display white spots in the cuticle. Affected shrimps gather around the edge of pond and the cumulative mortality in infected population may reach 100 % within 2-7 days after infection (Lightner *et al.*, 1996; Wang *et al.*, 1995).

2.2.3 Histopathology

Several cell types are affected by WSSV including the cuticular epithelium over the whole body, connective tissue of some organs, nervous tissues and muscle, lymphoid and hematopoietic tissues (Chang *et al.*, 1996). Since tissues originated either from the embryonic ectoderm or mesoderm, the original name of systemic ectodermal and mesodermal baculovirus was derived (Wongteerasupaya *et al.*, 1995). The virus also severely damages stomach, gills, antennal glands, heart and eyes. During the late stages of infection, these organs are destroyed and several cells are lysed (Chang *et al.*, 1996). The main characteristic feature of the infection is eosinophilic Cowdry A-type in hypertrophic nuclei with marginated basophilic chromatin. This intranuclear inclusion becomes lightly basophilic in the late stages of infection (Wongteerasupaya *et al.*, 1995).

2.2.4 Diagnosis

Several diagnostic tools for white spot syndrome have been developed. Light microscopy of infected cells stained with Haematoxylin and Eosin staining revealed the hypertrophic nuclei with eosinophilic and basophilic inclusions. The infection may confirm by Transmission Electron Microscope (TEM), which shows rod-shape, non-occluded virus in the nuclei of affected cells. Diagnostic DNA probes and published primers are available for *in situ* hybridization and polymerase chain reaction (PCR), respectively (Wongteerasupaya *et al.*, 1996; Lo *et al.*, 1996). Diagnostic PCR is a sensitive tool that is used commonly in Thailand to screen post larvae (PL), broodstock and potential carrier animal. Nested or 2-step PCR has gained popularity over single-step PCR, as it is more sensitive (Lo *et al.*, 1997). RT-PCR has also been used for detection of WSSV (Rout *et al*., 2005). Diagnostic using PAbs against natural virus tested by reversed passive latex agglutination (RPLA) assay were developed for detecting WSSV (Okumura *et al*., 2004).

2.2.5 PCR detection

Polymerase Chain Reaction (PCR) has been introduced in 1985 by Kary Mullis. It is the common method of creating copies of specific fragments of DNA (Figure 4). PCR rapidly amplifies a single DNA molecule into many billions of molecules in a few hours.

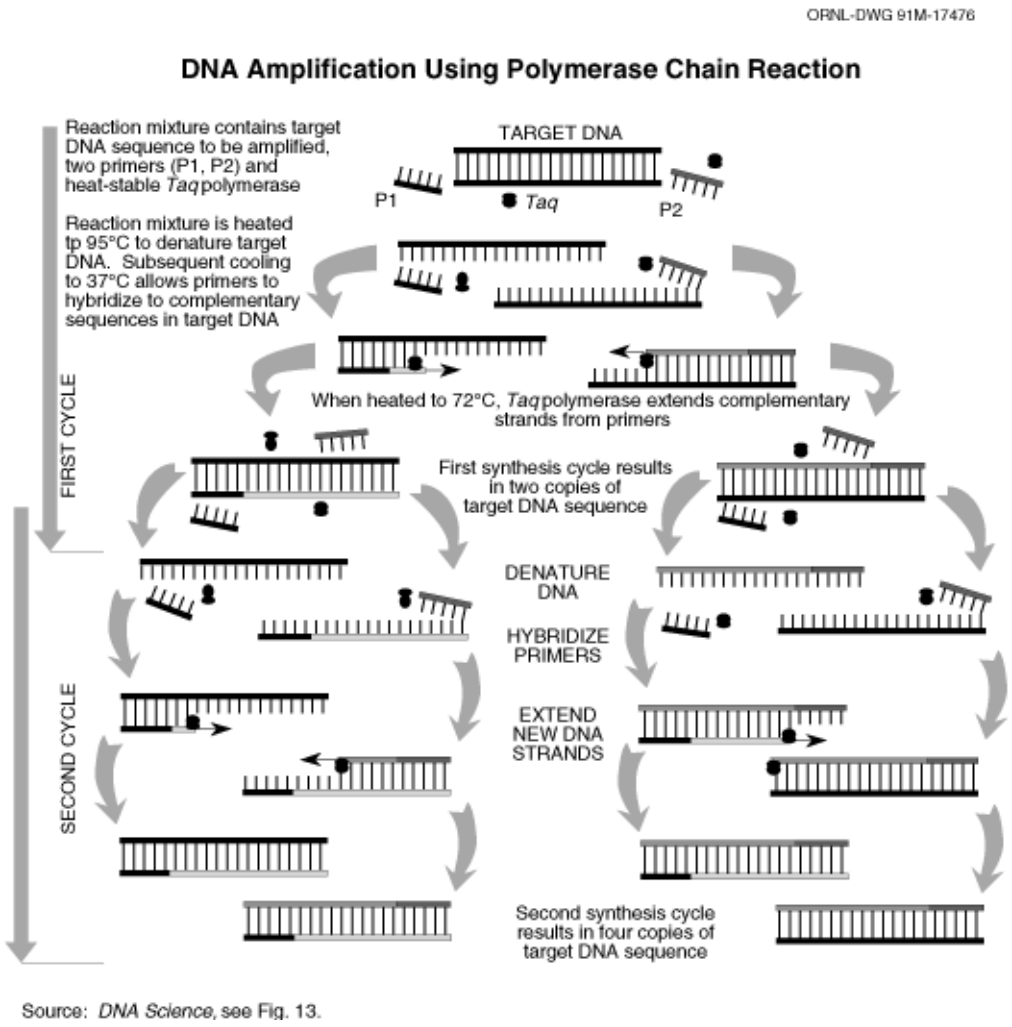


Figure 4. Schematic diagram of the polymerase chain reaction (PCR)
(Picture taken from http://aidshistory.nih.gov/imgarchive/pcr.html).

In one application of the technology, small samples of DNA, or femtogram amounts of specific viral nucleic acid, allows this procedure to be the most sensitive technique available for diagnosis of viral infection in shrimp.

2.2.6 Transmission and carriers of white spot syndrome virus

Transmission of WSSV can occur by both the vertical route from broodstock to larvae (Lo *et al.,* 1997), and the horizontal route (Chang *et al.,* 1996; Chou *et al.,* 1998) by means of crustaceans other than penaeids, including crabs, lobsters, *Macrobranchium* spp. and copepods (Lo *et al.,* 1996b; Chou *et al.,* 1996; Sahul Hameed *et al.*, 2003). Evidences obtained from PCR suggest that insect larvae may be the carriers (Maeda *et al.,* 1997). Crustacean carriers that enter prawn ponds may transmit WSSV when they die and are eaten by shrimps. Birds may mechanically transmit the virus between ponds by releasing captured, infected shrimps over neighboring. As WSSV remains infected in seawater for 2-7 days without entering hosts, spread of the disease does not always require the presence of carrier (Flegel *et al.,* 1997).

2.2.7 Monoclonal antibodies

A monoclonal antibody is defined as a uniform homogeneous antibody directed at a single epitope or antigenic determination and produced continuously from one cell clone.

In 1975, Köhler and Milstein first reported a successful method of producing monoclonal antibodies (Finfelman *et al.,* 1988). They fused specific antibody-producing cell with a mutant myeloma cell line to produce a hybridoma, which resulted in immortalization of the specific antibody-producing cell. The technology introduced by Köhler and Milstein has led to an explosive application of monoclonal antibodies in research and clinical diagnostic medicine. The use of monoclonal antibodies in clinical laboratories has been established in numerous assays. The foremost property of a monoclonal antibody is the ability to interact and link with a unique antigen or epitope (Carayanniotis and Barber, 1987).

Hybridoma and monoclonal antibodies offer four advantages, they are derived from one isolated clone and constitute a well-defined reagent, the production process is capable of yielding unlimited quantities of the same homogeneous reagent, they

may be prepared with non-purified antigens, and their affinities and specificities are defined (Hockfield, 1987).

Sithigorngul *et al*. produced monoclonal antibodies against yellow head virus (YHV). Virus extracted from gills of *P. monodon* infected with YHV was injected intraperitonially to Swiss albino mice. After production of monoclonal antibodies of the selected clone, V3-2B that specific to YHV protein gp116 (previously know as 135 kD proteins), was obtained. This MAb can detect virus in both native and SDS-treated forms, which can be used to develop test kit for detecting YHV (Sithigorngul *et al*., 2000).

For the development of WSSV MAb, purified WSSV from haemolymph of *P. monodon* were injected in mice after myeloma fusion and four clones of MAb specific to VP28 was obtained (Poulos *et al*., 2001). These MAbs can detect WSSV by dot blot, Western blot, immunohistochemistry and immunofluorescence, but these MAb cannot differentiate WSSV strains from China, Thailand, India, Panama and the USA (Texas, South Carolina). Three clones of MAbs binding to different epitopes were obtained from recombinant protein of viral enveloped VP28. These MAbs can be used to develop antigen-capture ELISA technique (Liu *et al*., 2002).

Monoclonal antibodies against a truncated viral enveloped protein (VP28) was successfully produced as recombinant fusion protein with 6x histidine tag and applied for the detection of WSSV infection in shrimp (Chaivisuthangkura *et al.*, 2004).

CHAPTER III
MATERIALS AND METHODS

3.1 Preparation of White Spot Syndrome Virus (WSSV)

3.1.1 Shrimp culture

Penaeus monodon shrimps were obtained from farms at Patumthani Province, Thailand and maintained in a re-circulation system at the Department of Biology, Faculty of Science, Srinakharinwirot University (SWU).

3.1.2 WSSV virus stock

The virus isolate used in this study were originated from WSSV infected *P. monodon* from Nakornsrithamarat Province. The WSSV was extracted from gills of WSSV infected shrimps with clinical symptoms and lesion of WSS that appeared on the skin, gill or head tissues. The infected tissues were homogenated with 2x phosphate buffer saline (PBS) pH 7.2, then centrifuged at 3,000 rpm for 30 min. Aliquots of the supernatant were collected and stored at –70 ºC. Approximately 50 ?l of isolated virus was injected intramuscular into *P. monodon* at the fourth or the fifth abdominal segment of the shrimp by using a 26 gauge needle. After 24 and 48 hours, the virus was isolated from fresh haemolymph.

3.1.3 WSSV DNA preparation

Gills from WSSV infected *P. monodon* were homogenized in lysis buffer (50 mM Tris-HCl pH 9.0, 100 mM EDTA, 50 mM NaCl, 2 % SDS; Flegel personal communication). DNA from 200 ?l of the homogenate was prepared using high pure viral nucleic acid kit from Roche Molecular Biochemical (Indianapolis, IN, U.S.A.). The 200 ?l of working solution (binding buffer 250 ?l with Poly A carrier RNA 50 ?l) was added to gill homogenate, then 50 ?l of proteinase K was added and incubated for 10 min at 72?C. The 100 ?l of isopropanol, then the solution was transfered into high pure filter and centrifuged at 8,000 rpm, for 1 min. The 500 ?l of inhibitor removal buffer was added and centrifuged at 8,000 rpm, for 1 min. The filter

was washed twice with 400 ?l of wash buffer by centrifugation at 8,000 rpm, for 1 min, after the second wash the filter was centrifuged at 13,000 rpm for 10 sec in order to remove wash buffer. The 50 ?l of elution buffer was added to the filter to elute DNA by centrifugation at 8,000 rpm, for 1 min. The isolated nucleic acids were stored in micro tube and kept frozen at -20?C.

3.1.4 Amplification of WSSV DNA by polymerase chain reaction (PCR)

The VP19 gene (Figure 5) of WSSV was amplified by using specific primers VP19F and VP19RPSTN with added restriction sites (Table 1, underlined). The hydrophilic region of VP19 gene, VP19-HPL, of WSSV was amplified by using specific primers, VP19HPLF and VP19HPLR with added restriction sites (Table1, underline). Both genes were amplified by Platinum® *Pfx* DNA polymerase (Invitrogen, U.S.A.). The sequences of the primers were shown in table 1. The PCR reaction mixed and PCR profile were shown in table 2 and table 3, respectively. The PCR products were run on 1 % agarose gel at 100 voltages for 1 hour and the DNA were observed under the UV light.

Table 1: Specific primers set for amplification of VP19 and VP19-HPL genes of WSSV

WSSV genes	Restriction sites
VP19 VP19 F (5?-CGGGATCCATGGCCACCACGACTAACAA-3?) VP19RPSTN (5?-TGCACTGCAGTTACTGCCTCCTCTTGG-3?)	*Bam*HI, *Pst*I
VP19-HPL VP19HPLF (5?-CGGGATCCATGGGACCAAAGAAGGAC-3?) VP19HPLR (5?-GGGGTACCATCCCTGGTCCTTGTTC-3?)	*Bam*HI, *Kpn*I

Table 2: PCR reaction mixtures

PCR reaction mixtures	Volume (?l)
WSSV DNA	5.0
10x *Pfx* amplification buffer	5.0
10x PCR enhancer solution	5.0

50 mM $MgSO_4$	1.0 (1 mM)
2.5 mM dNTP	4.0 (200 ?M)
Primer (forward)	1.0 (50 pmol)
Primer (reverse)	1.0 (50 pmol)
Platinum? *Pfx* DNA Polymerase	0.4 (1U)
ddH_2O	22.6
Total volume	50.0

Table 3: PCR conditions for VP19 and VP19-HPL

Conditions	VP19	VP19-HPL
1 Initial denaturation	94?C 2 min	94?C 2 min
2 Denaturation	94 ?C 15 sec	94 ?C 15 sec
3 Annealing	57 ?C 30 sec	57 ?C 30 sec
4 Elongation	68 ?C 45 sec	68 ?C 15 sec
5 Repeat step 2-4	35 cycles	35 cycles
6 Final extention	72 ?C 20 sec	65 ?C 10 sec
7 Cooling	4 ?C	4 ?C

The gel containing PCR product were cut under the UV light for extraction of the DNA by using Minelute gel extraction kit (Qiagen, U.S.A.) as described in the manufactural manual.

3.1.5 Cloning of DNA into pCR Blunt vector

PCR products obtained from amplification by Platinum? *Pfx* DNA Polymerase were blunt ended. Therefore, the pCR? Blunt vector (Zero Blunt? PCR Blunt Cloning kit, Invitrogen, U.S.A.) was used. The ligation mixture is shown in Table 4.

The ligation mixture (Table 4) was incubated overnight at 16?C, then the ligation DNA was added into One shot? TOP 10 cells (50 ?l / tube). The mixture was incubated on ice for 30 min, followed by heating at 42?C for 45 sec, and then incubated on ice for 2 min. The 250 ?l of SOC medium (warm in 37?C) was added

and incubated at 37?C (225 rpm, 1 hr). Spread the TOP 10 cells on LB agar containing 50 µg/ml of Kanamycin and incubated at 37?C for overnight.

Table 4 : Ligation mixture of PCR product and pCR? Blunt vector

Ligation mix	Volume (? l)
1. plasmid pCR? Blunt (25 ng;10 fmol)	1.0
2. WSSV gene (x ng;100 fmol)	x
3. 10x ligation buffer (+ATP)	1.0
4. T4 DNA ligase (4U/? l)	1.0
5. dd H_2O	x
Total volume	10.0

The selected colonies were cultured in 5 ml of LB broth containing Kanamycin and the plasmid preparations were performed. The bacterial pellet was resuspended with 100 ? l of GTE and incubated for 5 min at room temperature. The 200 ? l of the solution containing 1 % SDS and 0.2 N NaOH, mixed gently and incubated on ice for 5 min. The 150 ? l of 5 M KAc was added, mixed and incubated on ice for 7 min, then centrifuged at 3,000 rpm for 5 min. The supernatant was collected and 900 ? l of absolute ethanol was added and incubated at -70?C for 5 min. The mixture was centrifuged at 13,000 rpm for 10 min and the pellet was washed with 70 % cold-ethanol, centrifuged to remove 70 % ethanol and air dried. The pellet plasmid was dissolved with 25 ? l of dH_2O.

The recombinant plasmid VP19-pCR Blunt was restricted with *Bam*HI and *Pst*I and the recombinant plasmid VP19-HPL-pCR-Blunt was restricted with *Bam*HI and *Kpn*I in order to verify the presence of DNA insert.

3.1.6 Cloning of DNA into expression vector

3.6.1 Cloning of VP19 into pMAL-C2 expression vector

The plasmid VP19 pCR-Blunt was restricted with *Bam*HI and *Pst*I. The obtained VP19 gene was elute from the gel and ligated into pMAL-C2 using the molar ratio of insert : vector = 3:1 (NewEngland Biolabs, U.S.A.). The recombinant plasmid VP19-pMAL-C2 was transformed into *E. coli* strain XLI-Blue and plated on LB agar containing 100 µg / ml of Ampicillin.

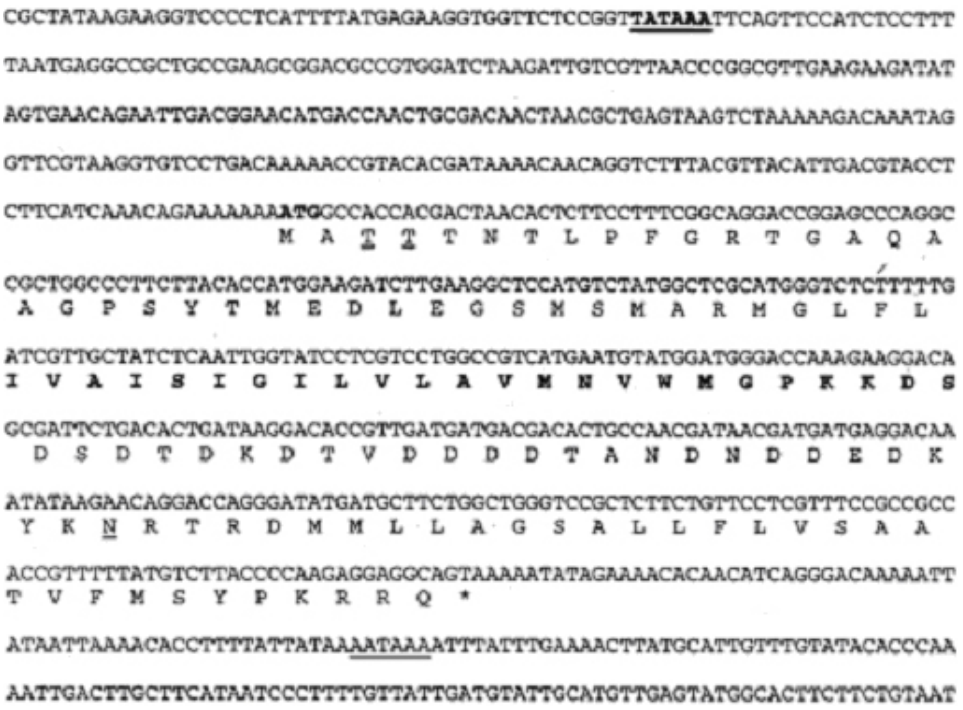


Figure 5. DNA sequence of WSSV VP19 (GenBank accession number AF 369029 ORF 182).

The selected colonies were cultured in 5 ml LB broth containing Ampicillin and the recombinant plasmids were prepared. The presence of the VP19 insert was proven by restriction analysis with *Bam*HI and *Pst*I and the integrity of the open reading frame (ORF) was verified by DNA sequencing. The verified recombinant plasmid was transformed into *E. coli* strain BL21 and plate on LB agar containing 100 µg / ml of Ampicillin for expression experiment further.

3.1.6.2 Cloning of VP19 –HPL into pQE30 expression vector

The plasmid VP19-HPL-pCR-Blunt was restricted with *Bam*HI and *Kpn*I at the internal hydrophilic region, corresponding to amino acid residues 59 to 94 (Figure 6). The obtained VP19-HPL gene was eluted from the gel and ligated into pQE30 (Qiagen, U.S.A.) (Figure 7) using the 3:1 ratio of insert: vector. The plasmid VP19-HPL-pQE30 was transformed into *E. coli* strain XLI-Blue and plated on LB agar containing 100 µg / ml of Ampicillin.

The selected colonies were cultured in 5 ml LB broth containing Ampicillin and the recombinant plasmids were prepared. The presence of the VP19-HPL insert was proven by restriction analysis with *Bam*HI and *Kpn*I and the integrity of the open reading frame (ORF) was verified by DNA sequencing. The verified recombinant plasmid was transformed into *E. coli* strain M15 (pREP4) and plate on LB agar containing 25 µg / ml of Kanamycin and 100 µg / ml of Ampicillin for further expression experiment.

3.1.7 Expression of recombinant proteins

The *E. coli* with recombinant plasmid VP19-pMAL-C2 VP19-HPL-pQE30 and VP26F109-pQE30 (prepared by Dr. Parin Chaivisuthangkura, SWU) were cultured in 5 ml of LB broth containing corresponded antibiotic for overnight. The bacterial culture was transferred into 100 ml LB broth containing corresponded antibiotic and incubated at 37?C with shaking at 200 rpm until the O.D. of 0.5-0.7 was obtained.

The expression of the recombinant proteins was induced by adding 1 mM isopropyl-?-D-thiogalacto-pyranoside (IPTG) and continued the shaking for 4 hr. After centrifugation at 4,000 rpm for 20 min, the bacterial pellet was dissolved in 100 mM NaH_2PO_4, 10 mM Tris-HCl, and 8 M urea pH 8, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and sonicated until the clear lysate was

obtained. The bacterial lysate was separated by SDS-PAGE with 15 % gel. After staining with Coomassie brilliant blue, the recombinant protein were cut out and destained until the gel is clear. The recombinant proteins containing band was collected in dialysis bags and the proteins were eluted with Transblot apparatus (Biorad, U.S.A.) at 70 volts for 6 hr. The proteins solution was dialyze to eliminate SDS and salt before determination of the protein content by Bradford protein assay (Bradford, 1976) then divided into small aliquots and stored at –70^{o}C. For VP19pQE30 (rVP19-HPL) was performed by using MagneHis ^{TM} Protein Purification System (Promega, U.S.A.).

3.1.8 Polyclonal antibody production

Swiss albino mice were intra-peritoneally injected with each of purified recombinant proteins including rVP19, rVP19-HPL and rVP26 (0.1 mg/mouse). Primary injection of recombinant protein was mixed with complete Freund's adjuvant in a 1:1 ratio. Mice were subsequently injected three more times with the proteins mixed with incomplete Freund's adjuvant at 2 week intervals. One week after the fourth injection, mouse antisera were collected then tested against *E. coli* lysate, purified recombinant proteins and gill extract from WSSV infected *P. monodon* by Western blot and tested against head tissues from WSSV infected *P. monodon* by immunohistochemical staining. In order to increase the epitope proteins of recombinant proteins, the rVP19 and rVP19-HPL were conjugated with the native protein bovine serum albumin. BSA was mixed with rVP19 and rVP19-HPL at the ratio of 1:2:2. The conjugated proteins were activated by adding 0.1 % glutaraldehyde in phosphate buffer saline (PBS), overnight and adjusted concentration of glutaraldehyde to 0.2 % before dialysis. Excess reagent was removed using dialysis bag against distilled water. Conjugated proteins were used for immunizing mice as described above.

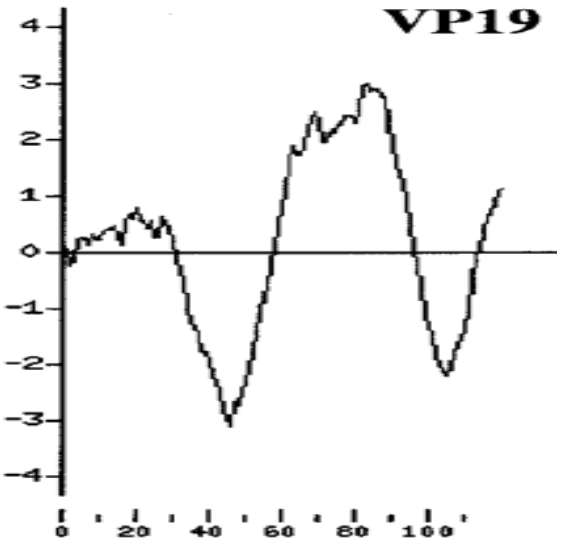


Figure 6. Hydropathic Protein Profile of VP19 (van Hulten *et al.*, 2002).

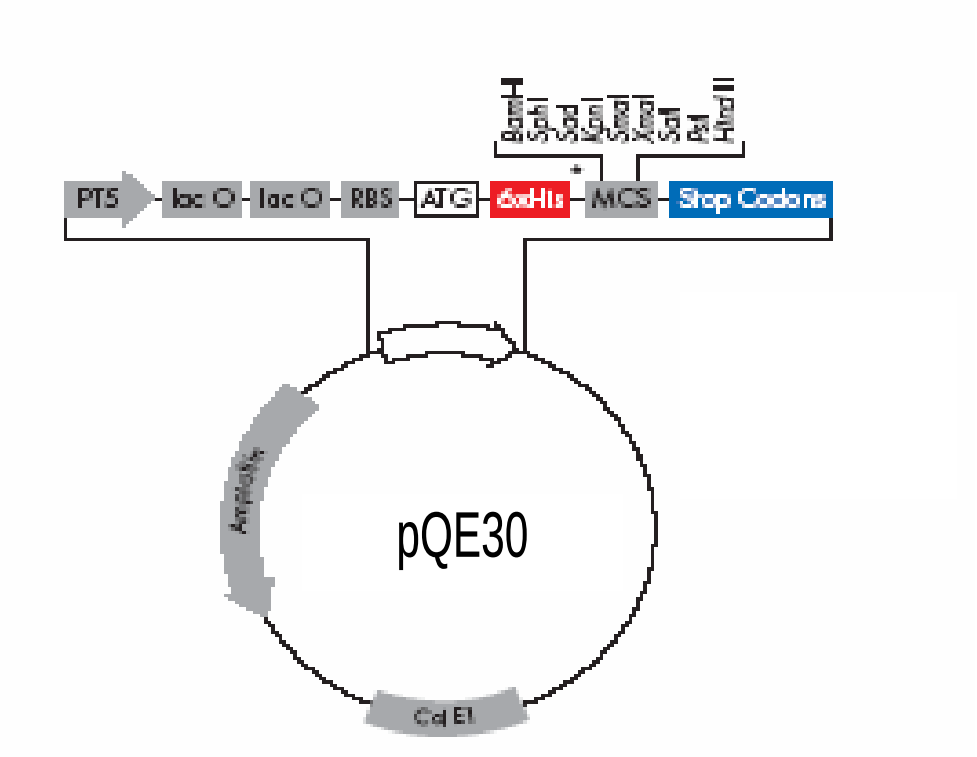


Figure 7. pQE30 vector for direct cloning of PCR products into an expression vector. (Picture taken from A Handbook for high-level expression and purification of 6xHis-tagged proteins, QIAGEN, U.S.A.).

3.1.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS – PAGE) and Western blot analysis

Lysate of *E. coli* without recombinant plasmid, lysate of *E. coli* with empty plasmid, lysate of *E. coli* with recombinant plasmid, purified recombinant proteins and gills extract from WSSV infected *P. monodon* were separated by SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed at 30 V for 6 hr and the gel was stained with Coomassie brilliant blue R250. For Western blot analysis the sample resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a Transblot apparatus (BioRad, U.S.A.) then incubated with each polyclonal antibody and processed. Antigen were seperated and incubated with (1:20 dilution in 5 % Blotto: 5 % nonfat drymilk, 0.1 % Triton-X-100 in PBS) for 8 h. After extensive washing in 0.5 % Blotto, the membranes were incubated in horseradish peroxidase conjugated goat anti-mouse IgG heavy and light chain specific antibody (GAM-HRP; BioRad, U.S.A.) at 1:1000 dilutions for 8 h. The membranes were washed extensively as previously described and incubated in a substrate mixture containing 0.006 % hydrogen peroxide, 0.03 % diaminobenzidine (DAB), 0.05 % cobalt chloride in PBS.

3.1.10 Immunohistochemical staining

Cephalothoraces from *P. monodon* infected with WSSV were cut and fixed in Davidson's fixative solution for 24 h before processing for paraffin sectioning. Serial sections (8 ?m thickness) was prepared and processed for indirect immunoperoxidase staining using various PAbs or MAb against VP28 and GAM-HRP diluted to 1:1000 with 10 % calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03 % DAB and 0.006 % hydrogen peroxide in PBS. Preparations were counter-stained with haematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, cleared in xylene and mounted in permount (Sithigorngul *et al.,* 1999). Positive reactions were visualized as brown coloration against pink and purple colors of H&E. The diagram for immunohistochemical staining was shown in (Figure 8).

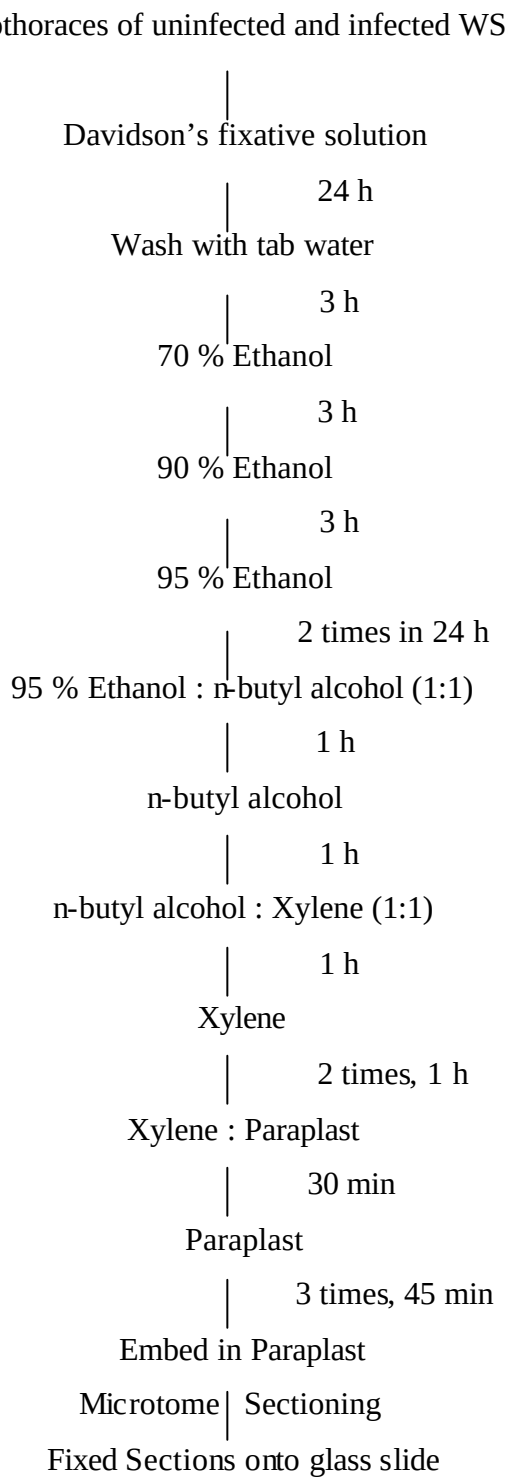


Figure 8. Diagram of Immunohistochemical staining for characterization of polyclonal antibodies (Sithigorngul *et al.*, 1999).

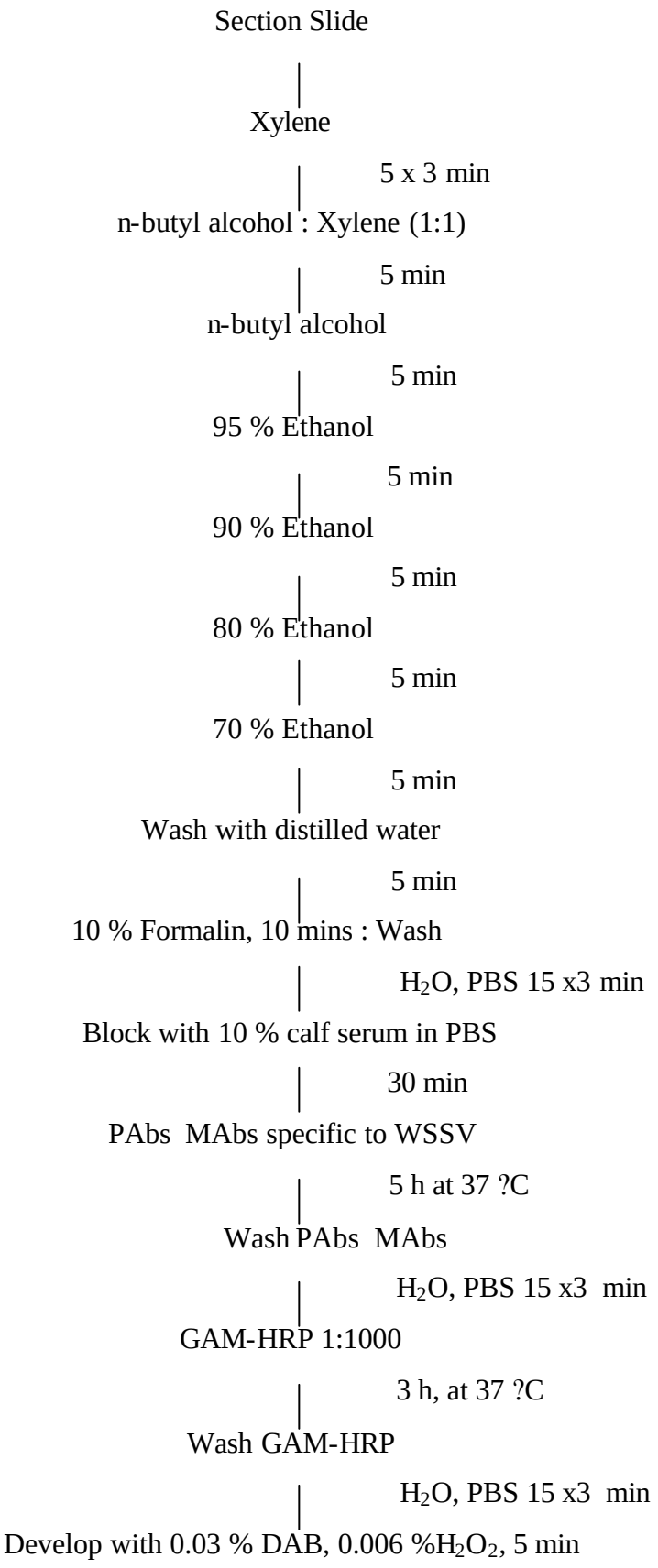


Figure 8. (Cont.).

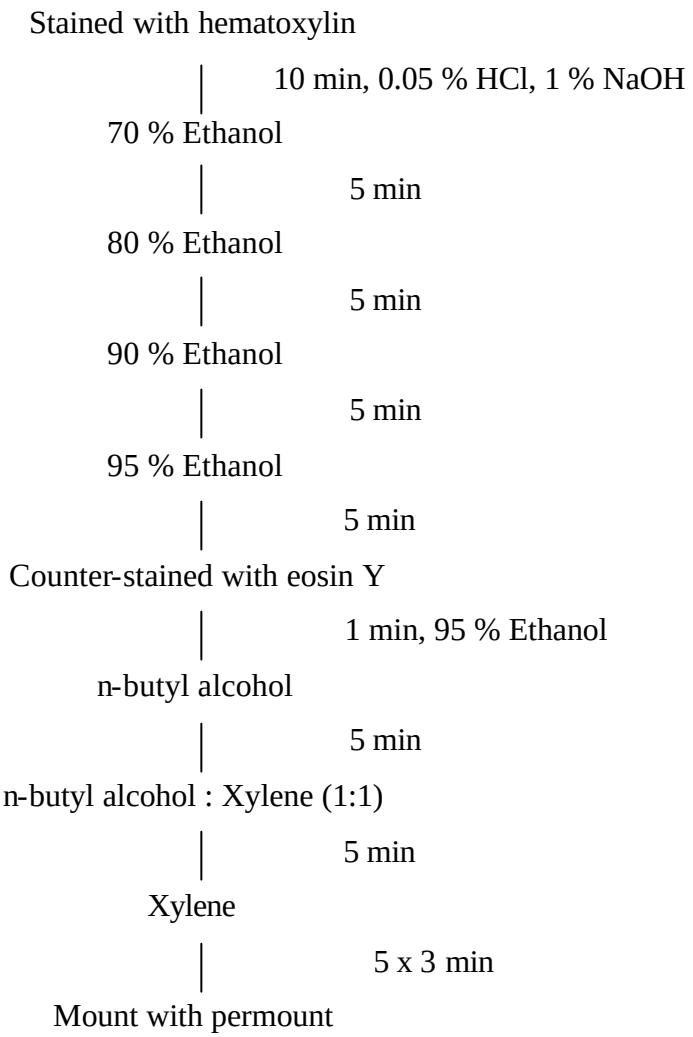


Figure 8. (Cont.).

CHAPTER IV
RESULTS

4.1 Production of WSSV recombinant proteins

4.1.1 PCR amplification of VP19 and VP19-HPL gene of WSSV

The nucleic acid was isolated from gill homogenate of WSSV infected *P. monodon*. The PCR amplification of VP19 and VP19-HPL genes of WSSV were performed using the isolated nucleic acid as the template. The 384 bp PCR product of VP19 (Figure 9) and the 127 bp PCR product of VP19-HPL (Figure 10) were obtained.

4.1.2 Cloning VP19 into pMAL-C2 expression vector

The 384 bp blunt ended PCR product of VP19 was initially cloned into pCR-Blunt vector. The VP19 gene was cut from the recombinant plasmid VP19-pCR-Blunt by using *Bam*HI and *Pst*I. The 384 bp of VP19 gene was obtained (Figure 11). The obtained VP19 gene was cloned into pMAL-C2 expression vector. The presence of VP19 in the pMAL-C2 vector was proven by *Bam*HI and *Pst*I digestion (Figure 12). The integrity of ORF in the expression vector was verify by DNA sequencing and the DNA sequence matched with VP19 gene (GenBank accession number AF 369029 ORF 182) (Figure 12).

4.1.3 Cloning of VP19-HPL into pQE30 expression vector

The 127 bp PCR product of VP19-HPL was initially cloned into pCR-Blunt vector. The presence of VP19-HPL in pCR-Blunt was proven by *Eco*RI digestion. The VP19-HPL gene was cut from the recombinant plasmid VP19-HPL-pCR-Blunt by using *Bam*HI and *Kpn*I (Figure 13). The obtained VP19-HPL gene was cloned into pQE30 vector. The presence of 127 bp of VP19-HPL gene was revealed by *Bam*HI and *Kpn*I digestion (Figure 14). The integrity of ORF in the expression vector was verified by DNA sequencing.

4.1.4 SDS-PAGE and Western blot analysis

4.1.4.1 SDS-PAGE and Western blot of rVP19

After induction, the recombinant fusion protein of VP19 at 55.5 kDa was obtained (Figure 15-1 Lane b and c). The recombinant protein of VP19 in the form of a recombinant fusion protein with maltose binding protein (MBP), tag at the N-terminus. This recombinant protein was used to generate polyclonal antibody. The obtained antisera displayed high immunoreactivity and specificity to recombinant protein by Western blotting (Figure 15-2). The polyclonal antibody also reacted specifically to VP19 protein from the gills of WSSV-infected *P. monodon* (Figure 15 -1 Lane d).

4.1.4.2 SDS-PAGE of rVP19-HPL

After induction, the recombinant fusion protein of VP19-HPL at 4 kDa was obtained (Figure 16). The recombinant protein of VP19-HPL, in the form of fusion protein with 6 residues of histidine tag at the N-terminus. The direct injection of rVP19 (HPL) failed to provoke immune response to VP19 of WSSV due to the small size of antigen (Figure 16). Therefore, this recombinant protein was used to conjugate to rVP19-MBP in order to increase the number epitopes of VP19 on rVP19-MBP for polyclonal antibody production. Polyclonal antibodies against the rVP19-rVP19(HPL) conjugated protein showed stronger reactivity than the antibody against rVP19 alone (Figure 15-2).

4.1.4.3 SDS-PAGE and Western blot of rVP26F109.

After induction, the recombinant fusion protein of VP26F109 at 23 kDa was obtained (Figure 17-1 Lane b and c). The recombinant protein of VP26F109, in the form of fusion protein with 6 residues of histidine tag at the N-terminus. The polyclonal antibody against this recombinant protein also reacted specifically to VP26 protein from the gill homogenate of WSSV-infected *P. monodon* (Figure17-2 Lane d).

4.1.5 Immunohistochemical staining

Tissue of *P. monodon* infected WSSV were treated with polyclonal antibodies against recombinant VP19 and recombinant VP26F109. The positive reactions were visualized at the same locations revealed by monoclonal antibody W29-1A (specific to VP28). This evidence confirms that antisera were specific to WSSV. (Figure 18, 19 and 20).

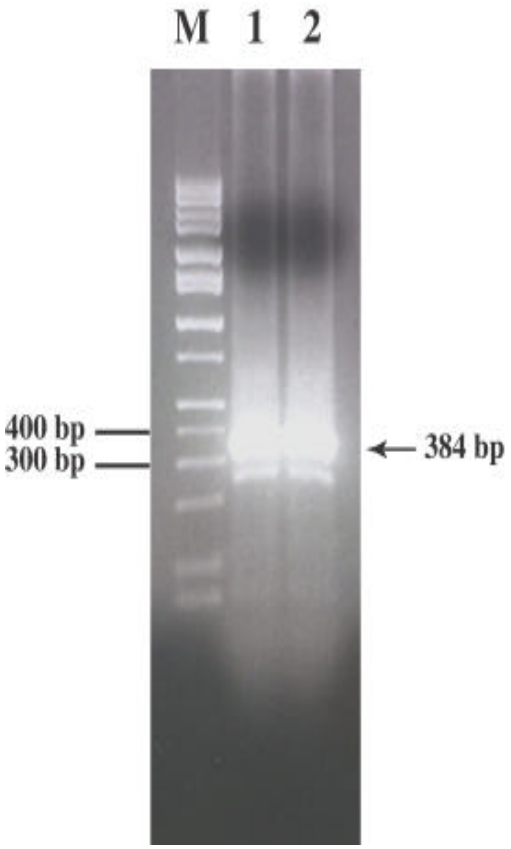


Figure 9. Ethidium bromide staining of 384 bp VP19 PCR product of WSSV on 1.2 % agarose gel.

Lane M = Hi-Lo DNA Marker

Lanes 1 and 2 = 384 bp of VP19 PCR product

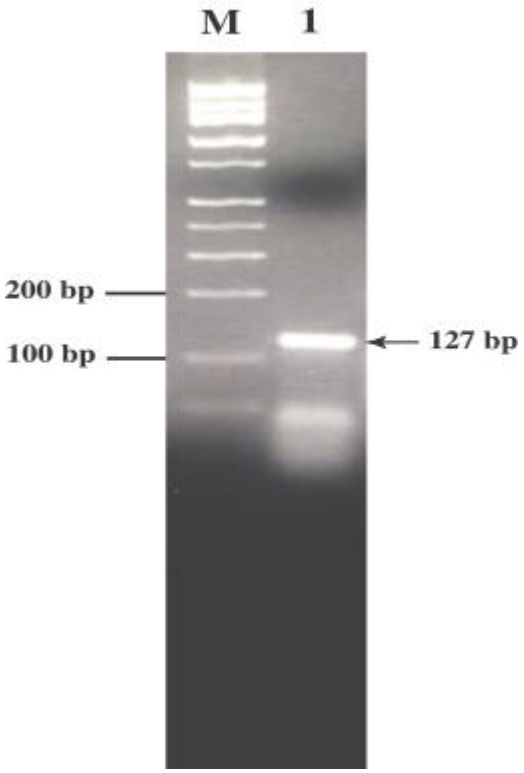


Figure 10. Ethidium bromide staining of 127 bp VP-19-HPL PCR product of WSSV on 1.2 % agarose gel electrophoresis.

Lane M = 100 bp DNA marker

Lane 1 = 127 bp of VP19 (HPL) PCR product

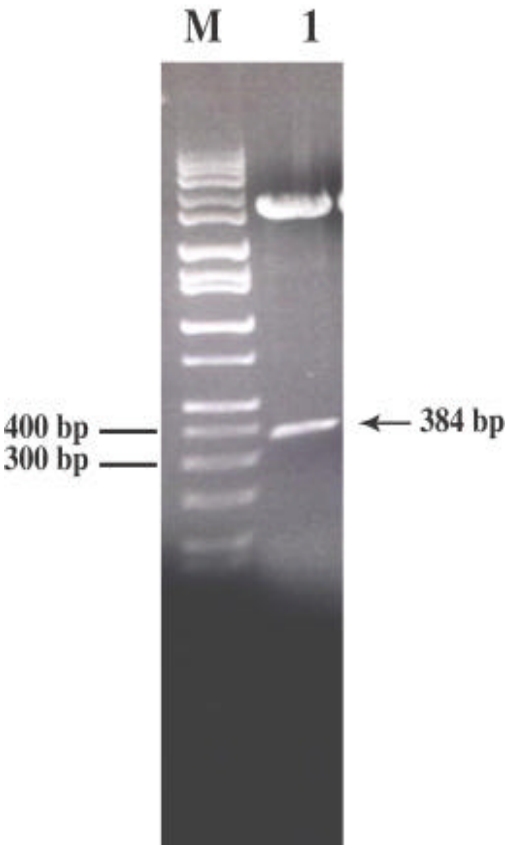


Figure 11. Ethidium bromide stained gel of 384 bp of VP19 gene after digested with *Bam*HI and *Pst*I from pCR-Blunt vector.

Lane M = Hi-Lo DNA Marker

Lane 1 = VP19 gene of WSSV

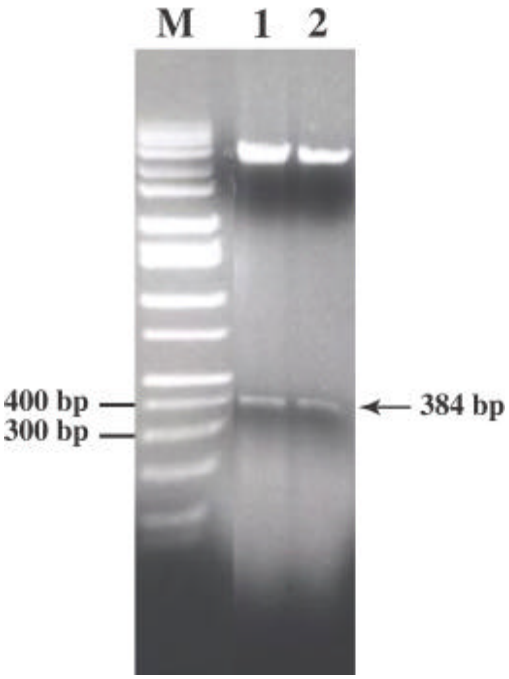


Figure 12. Ethidium bromide stained gel of 384 bp of VP19 gene after digested with *Bam*HI and *Pst*I from pMAL-C2 vector.

Lane M = Hi-Lo DNA Marker

Lanes 1 and 2 = VP19 gene of WSSV

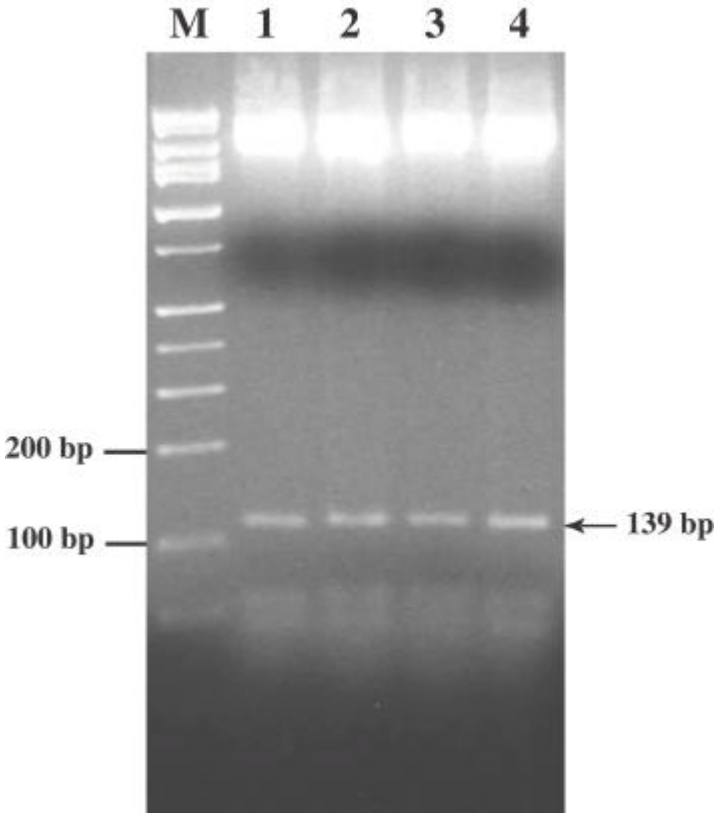


Figure 13. Ethidium bromide staining of 139 bp VP19 (HPL) after digestion by *Eco*RI from pCR-Blunt vector.

Lane M = 100 bp DNA marker

Lanes 1, 2, 3, 4 = 139 bp of VP19 (HPL) digestion from pCR-Blunt vector by *Eco*RI

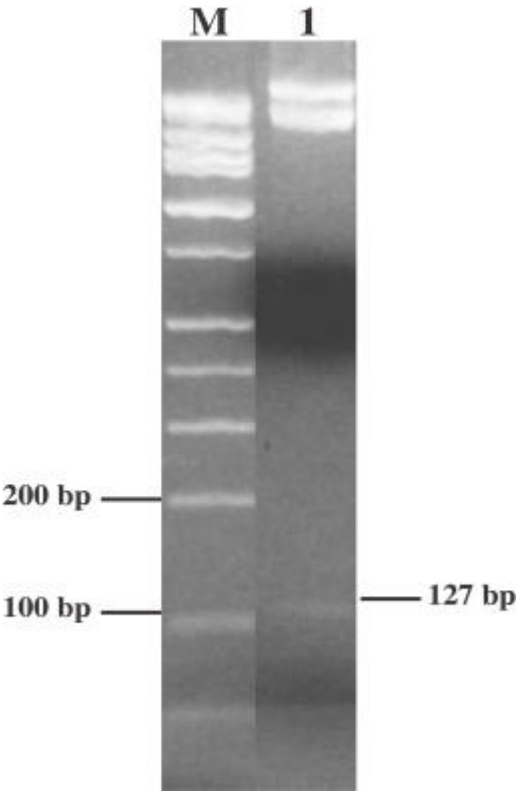


Figure 14. Ethidium bromide staining of 127 bp VP19 (HPL) after digestion with *Bam*HI and *Kpn*I from pQE30 vector.

Lane M = 100 bp DNA marker

Lane 1 = 127 bp of VP19-HPL digestion from pQE30 vector by *Bam*HI and *Kpn*I

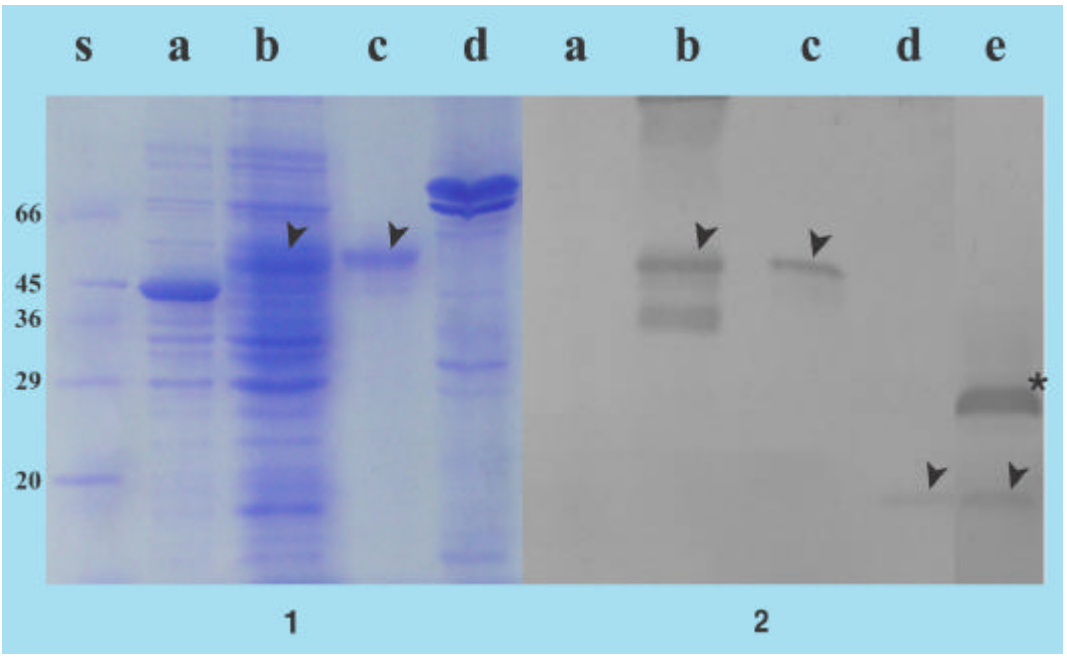


Figure 15. 12 % SDS-PAGE and Western blot of recombinant VP19 (a) lysate of *E. coli* BL21 pMAL-C2 (b) lysate of *E. coli* containing rVP19 (c) purified rVP19 (d) gill homogenate of WSSV-infected *P. monodon* (e) gill homogenate of WSSV-infected *P. monodon* reprobed with VP28 MAb (?) S = Low molecular weight standard proteins, the number denoted kDa. (1) Gel stained with Coomassie brilliant blue (2) Western blot analysis were treated with the anti-rVP19 and rVP19-HPL conjugate antiserum (➤).

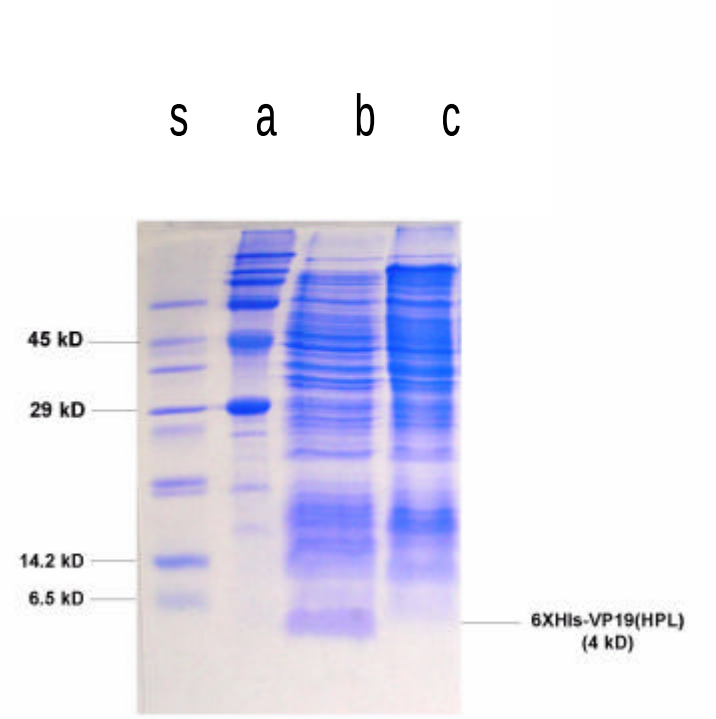


Figure 16. 15 % SDS-PAGE of VP19-HPL (S) Low molecular weight standard (a) High molecular weight standard (b) lysate of *E. coli* containing VP19-HPL-pQE30 plasmid (c) lysate of *E. coli* containing pQE30 plasmid.

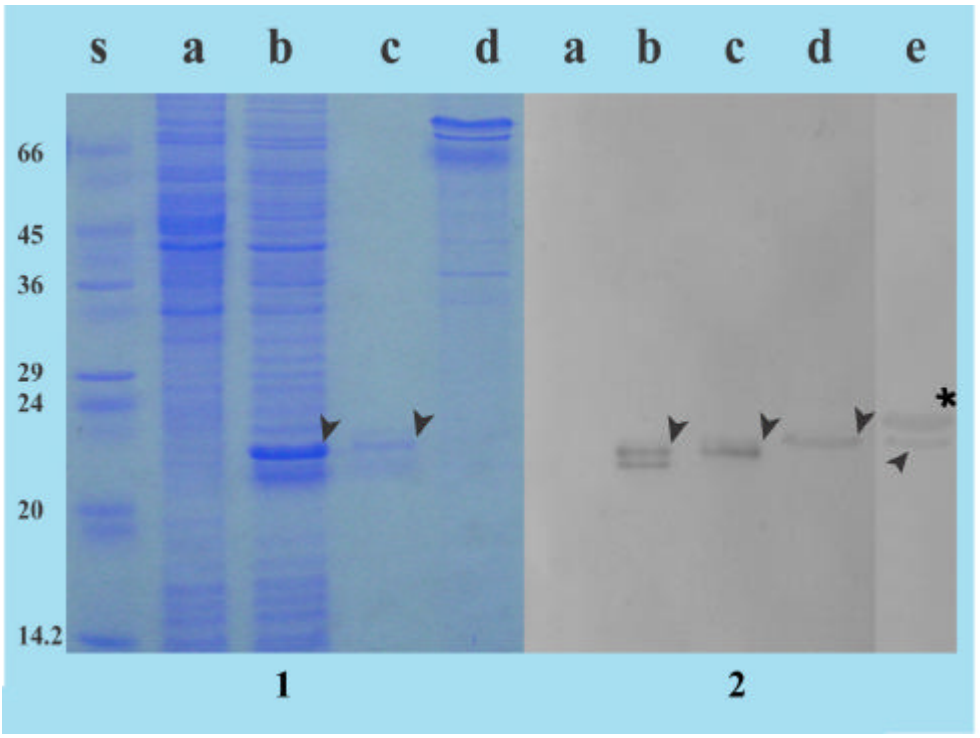


Figure 17. 12 % SDS-PAGE and Western blot of recombinant VP26 (a) lysate of *E. coli* M15 pQE30 (b) lysate of *E. coli* containingVP26pQE30 (c) purified rVP26 (d) gill homogenate of WSSV-infected *P. monodon* (e) gill homogenate of WSSV-infected *P. monodon* reprobed with VP28 MAb (?) S = Low molecular weight standard proteins, the number denoted kDa. (1) Gel stained with Coomassie brilliant blue (2) Western blot analysis were treated with anti-rVP26 antiserum (➤).

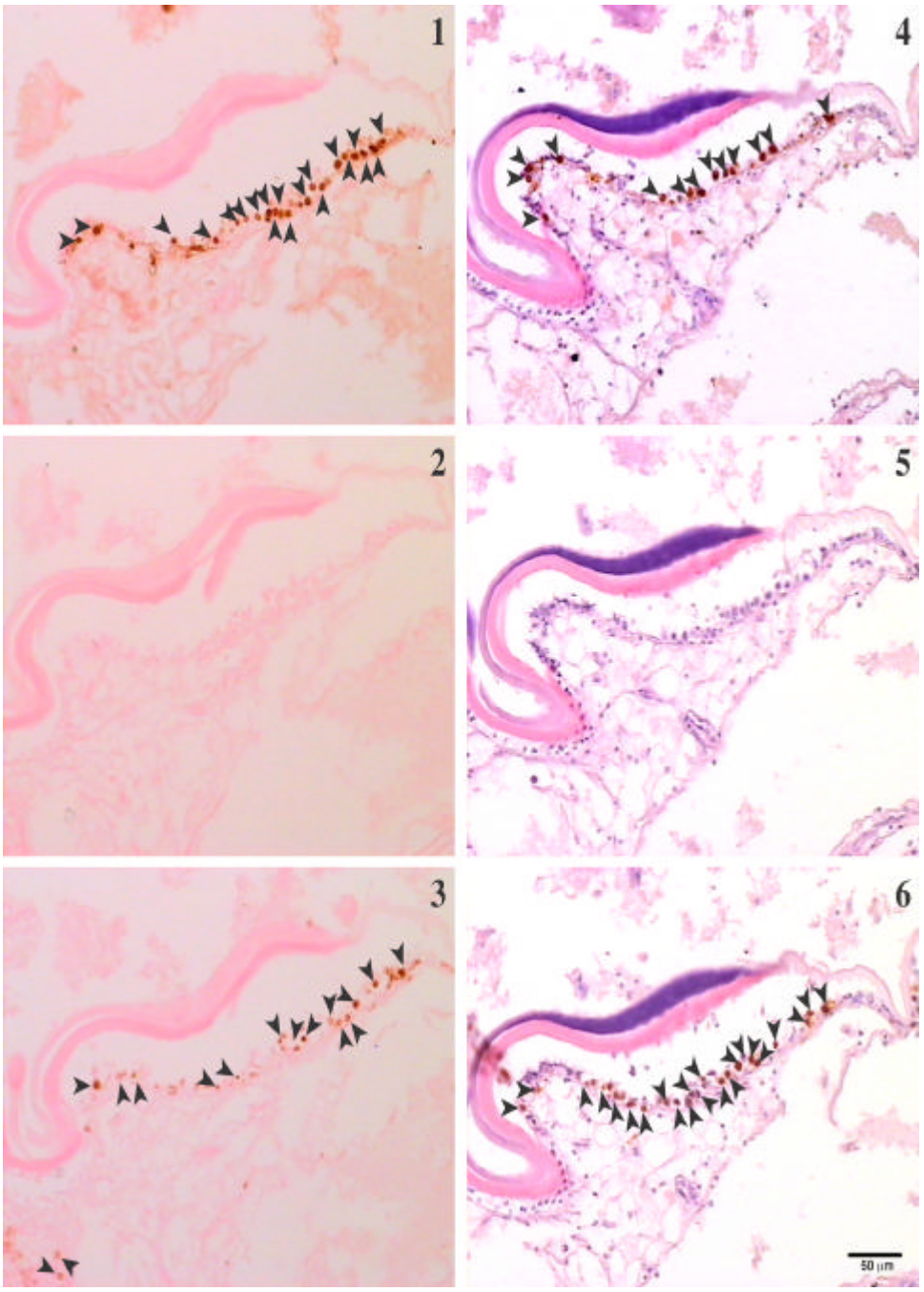


Figure 18. Immunohistochemical staining of *P. monodon* subcuticular epithelium were treated with anti-rVP19 antiserum. The left panel were counter stained with Eosin Y (1, 2 and 3) and in the right panel were counter stained with haematoxylin and eosin Y (4, 5 and 6). Scale bar = 50 µm.

1, 4 = treated with anti-rVP19 antiserum 2, 5 = untreated with antiserum

3, 6 = treated with VP28 MAb(W291A)

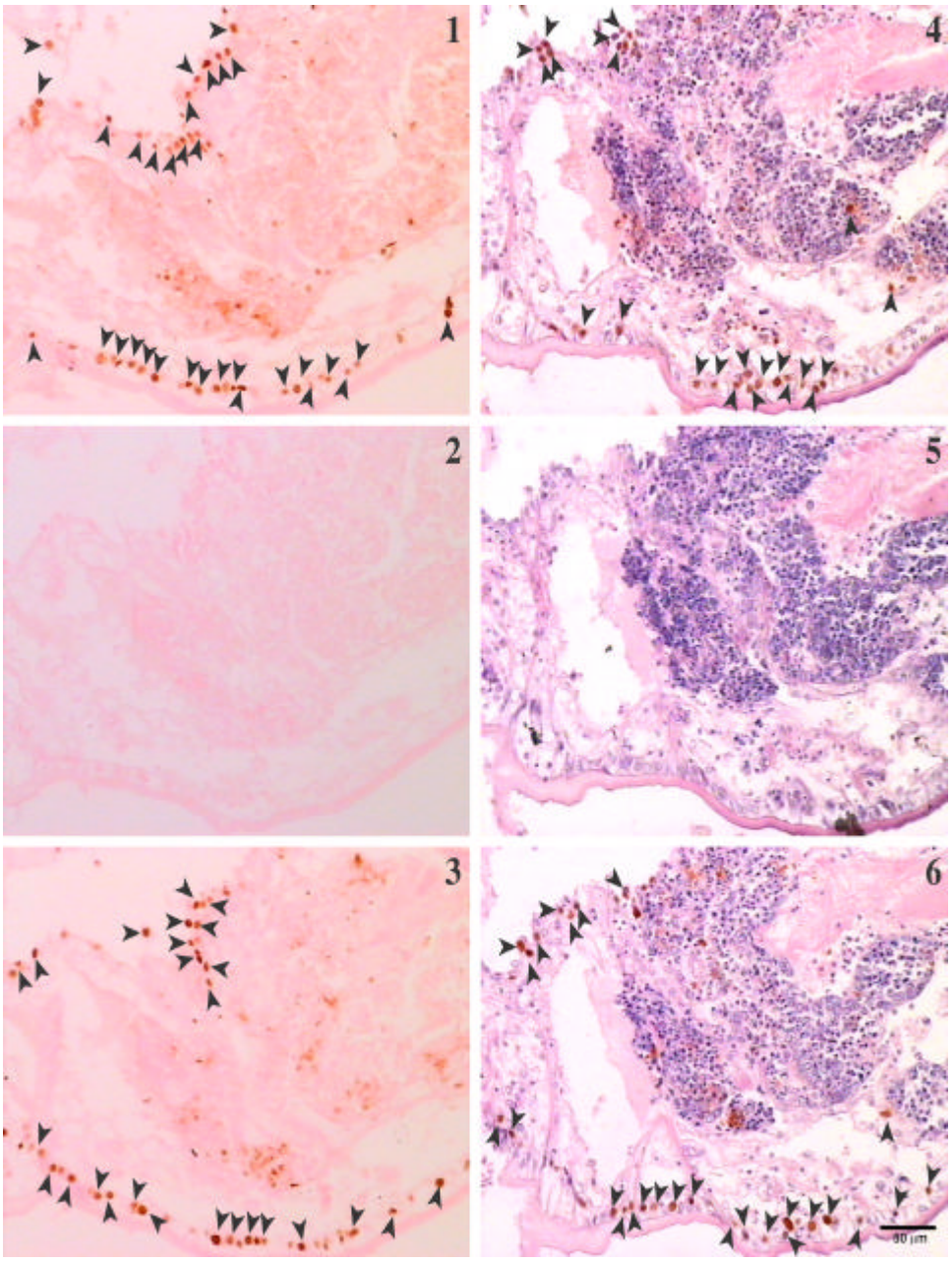


Figure 19. Immunohistochemical staining of *P. monodon* hematopoietic tissue treated with anti-rVP19 and rVP19-HPL conjugate antiserum. Figure in the left panel were counter stained with Eosin Y (1, 2 and 3) and in the right panel were counter stained with haematoxylin and eosin Y (4, 5 and 6). Scale bar = 50 ? m.

1, 4 = treated with anti-rVP19 and rVP19-HPL conjugate antiserum

2, 5 = untreated with antiserum

3, 6 = treated with VP28 MAb (W29-1A)

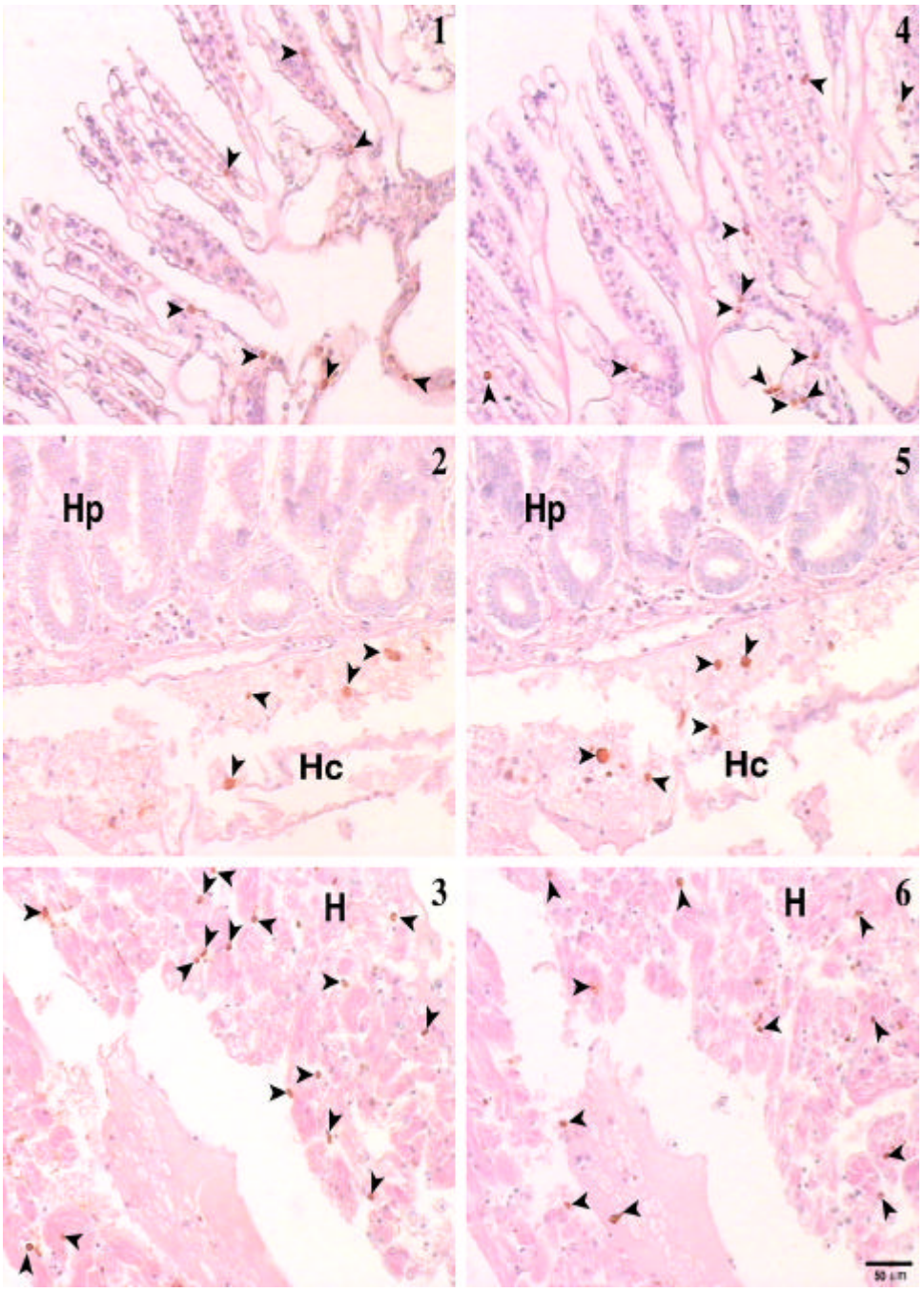


Figure 20. Immunohistochemical staining of *P. monodon* tissues. Figures in the left panel were treated with anti- rVP19 and rVP19-HPL conjugate antiserum and in the right panel were treated with VP28 MAb (W29-1A). All sections were counter stained with haematoxylin and eosin Y. Hepatopancreas (Hp). Scale bar = 50 ? m.

1, 4 = Gill 2, 5 = Haemocoal (Hc) 3, 6 = Heart (H)

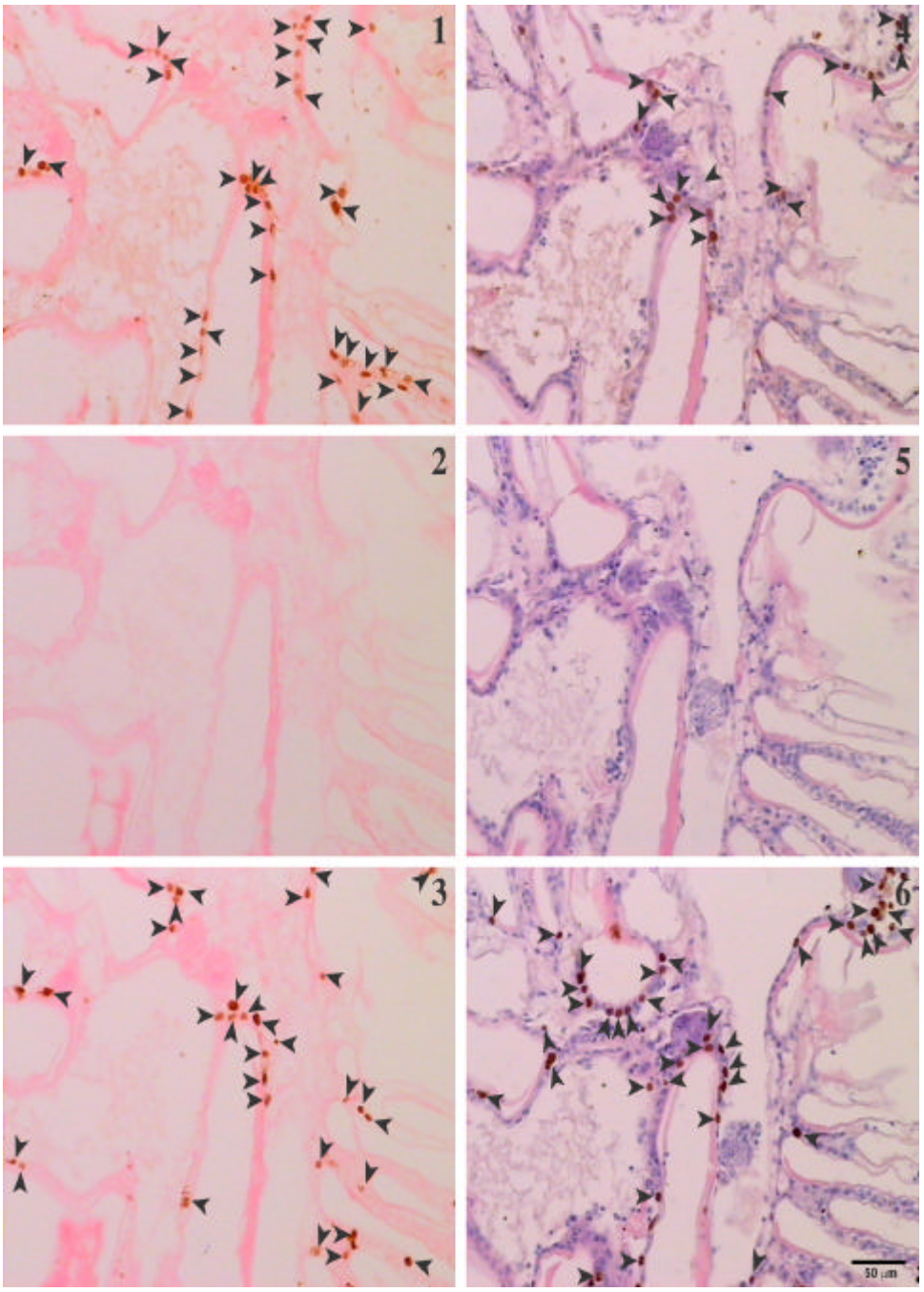


Figure 21. Immunohistochemical staining of gill from *P. monodon*. Treated with anti-rVP26 antiserum. Gills section of left panel were counter stained with Eosin Y (1, 2 and 3) and the right panel were counter stained with haematoxylin and eosin Y (4, 5 and 6). Scale bar = 50 µm.

1, 4 = treated with anti-rVP26 antiserum 2, 5 = untreated with antiserum

3, 6 = treated with VP28 MAb (W29-1A)

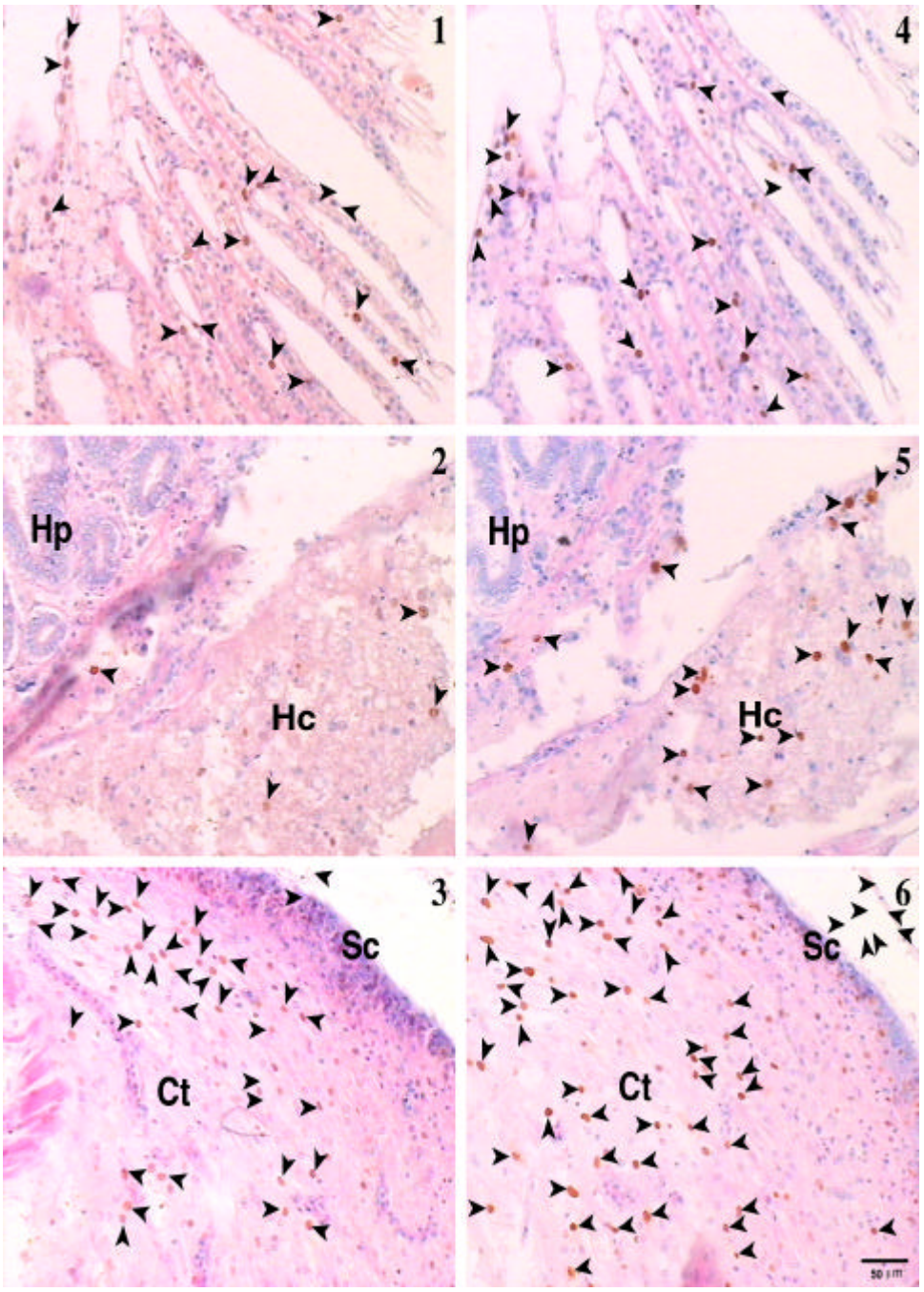


Figure 22. Immunohistochemical staining of *P. monodon* tissues. Figures in the left panel were treated with anti-rVP26 antiserum and in the right panel were treated with VP28 MAb (W29-1A). All sections were counter stained with haematoxylin and eosin Y. Hepatopancreas (HP), subcuticular epithelium (Sc), Scale bar = 50 ? m.

1, 4 = Gill 2, 5 = Haemocoal (Hc) 3, 6 = Connective tissue (Ct)

CHAPTER V
DISCUSSIONS

In this study the expressed recombinant proteins were utilized as antigens in order to generate polyclonal antibodies for detection of WSSV infection in shrimp. The VP19 envelope protein of WSSV was expressed in *E. coli* in the form of recombinant fusion protein at the N-terminus. The bacterial expression system allowed the production of 10 mg of purified recombinant proteins per liter of bacterial culture. The advantage of the expression system used in the present study is that the proteins can be easily obtained in high quantities in pure form. Initially, the VP19 envelope protein failed to express in the form of 6xhistidine tag fusion protein. However, the VP19 was successfully expressed in *E. coli* in the form of recombinant fusion protein with maltose binding protein (MBP) tag at the N-terminus.

Moreover, the hydrophilic region of VP19, VP19-HPL was expressed in the form of 6xhistidine fusion protein. The generation of antibody against this protein was not successful since the antibody did not show immunoreactivity against VP19 envelope protein. Therefore this rVP19-HPL was conjugated with rVP19 in order to increase the epitopes of VP19 for antibody production. Polyclonal antibodies against the conjugated proteins showed stronger immunoreactivity than the antibody against rVP19 or rVP19 (HPL) alone (data not show).

In order to increase the sensitivity for detection of WSSV infection by antibody, the 6xhistidine recombinant fusion protein of truncated VP26 (VP26F109) was also utilized as an antigen for generation of antibody.

The antibodies against rVP19 and rVP26F109 displayed strong immunoreactivities and high specificities to WSSV as shown by Western blot analysis and immunohistochemistry. In Western blot analysis polyclonal antibodies against VP19 recognized the fusion proteins with molecular weight of 55.5 kDa when detected in *E. coli* containing VP19. The specificity of polyclonal antibodies against natural virus was also tested in gill homogenate. Strong immunoreactivity against

VP19 was observed. For VP26F109, the antibody recognized the fusion protein at 23 kDa with the dilution of 1:1000. The immunoreactivity against gill homogenate of WSSV infected *P. monodon* was also confirmed by reprobing with monoclonal antibodies against recombinant VP28 (Chaivisuthangkura *et al.,* 2004). Both polyclonal antibodies against VP19 and VP26 were tested with immunohistochemistry to *P. monodon* tissues at the dilution of 1:4000 and showed strong immunoreactivity to various tissues such as heart, gill, hematopoietic tissue, connective tissue and subcuticular epithelium. The pattern of infection in shrimp tissue was confirmed by VP28 MAb (W29-1A) at dilution of 1:5. From this result, it is clearly shown that polyclonal antibodies against VP19 and VP26 of WSSV can detect WSSV infection in shrimp tissue.

Despite the availability of polyclonal antibodies to detect WSS, there is a pressing need for a rapid, simple and cost-effective technique to detect the virus in the field. The shrimp farmer needs a cheap, rapid, and high sensitivity technique for detecting WSS (Cesar *et al.*, 2000).

Development of other types of assay may increase the sensitivity of the antibody-based detection, such as sandwich ELISA that has the detection sensitivity close to that of PCR (Liu *et* al., 2002), or by Western blot analysis. Further development of a very simple convenient test kit for the detection of WSSV such as strip test is being advanced. This technique is intended for use in the field to detect the presence of the virus in infected shrimp.

This study opens the way to produce monoclonal antibodies against WSSV in order to use these monoclonal antibodies to detect WSSV infection by various immunological methods in the future.

CHAPTER VI
CONCLUSIONS

White spot syndrome (WSS) is one of the most serious diseases in *Penaeid* shrimp farming worldwide. It is caused by white spot syndrome virus (WSSV) that has been found in different shrimp species, and also found in various marine and fresh water crustaceans including crayfish, crabs and freshwater *Palaemonid* shrimp (Lo *et al.*, 1996b; Peng *et al.*, 1998 and Chen *et al.*, 2000).

Recently, the various detections of WSSV have been developed, such as PCR and immunologically based diagnostic methods (Inouye *et al.*, 1994; Lo *et al*., 1995; Tapay *et al*., 1999 and Anil *et al.*, 2002). Although the polyclonal antibody against purified WSSV was generated, it cross-reacted with various shrimp proteins (Nadala *et al.*, 2000). Monoclonal antibodies raised against purified WSSV were also produced. Even though these antibodies were capable of detected WSSV infection by dot blot, Western blot, immunohistochemistry and immunofluorescence, they could not distinguish among various WSSV isolates from China, Thailand, India, Panama and the USA (Texas and South Carolina) (Nadala *et al.*, 2000; Poulos *et al.*, 2001).

In previous studies, the monoclonal antibodies against recombinant VP28 proteins have been produce and can detect WSSV infected in early stage with in hours (Chaivisuthangkura *et al.*, 2004).

In the present study, the WSSV proteins (VP19 and VP26) were over-expresses in *E. coli* as fusion proteins in different vectors. In order to avoid the problem of low yield or no expression in *E. coli*, the truncated version of the proteins lacking hydrophobic region was used in VP19-HPL and VP26F109, both truncated proteins were expressed in *E. coli* in the form of 6x histidine tag at the N-terminus. Even with the large hydrophobic region was removed, the truncated recombinant proteins were insoluble when expressed in *E. coli*, yielding approximately 10 mg of recombinant proteins purified from 1 liter of bacteria culture determined using SDS-PAGE.

Mouse antiserum produced by immunization with the recombinant proteins had a high titer and was relatively specific for WSSV proteins.

The specificity of polyclonal antibodies was tested by Western blot analysis and immunohistochemistry with recombinant proteins compared with WSSV infected *P. monodon* gill homogenate. Polyclonal antibody dilution obtained from this studied are 1:1,000 for Western blot and 1:4,000 for immunohistochemical staining.

REFERENCES

Anil TM, Shankar KM, Mohan CV. Monoclonal antibodies enveloped for sensitive detection and comparison of white spot syndrome virus isolates in India. Dis Aquat Org 2002; 51: 67-75.

Bachère E. Introduction shrimp immunity and disease control. Aquaculture 2000; 191: 3-11.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.

Carayanniotis G, Barber BH. Adjuvant-free IgG responses induced with antigen coupled to antibodies against class II MHC. Nature 1987; 327: 59-61.

Cesar EB, Nadala Jr, Loh Philip C. Dot-blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow-head virus of penaeid shrimp. Dis Aquat Org 2000; 84: 175-179.

Chaivisuthangkura P, Tangkhabuanbutra J, Longyant S, Sithigorngul W, Rukpratanporn S, Menasveta P, et al. Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. Science Asia 2004; 30: 359-363.

Chang PS, Lo CF, Wand YC. Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. Dis Aquat Org 1996; 27: 131-139.

Chang PS, Chen HC, Wand YC. Detection of white spot syndrome associated baculovirus WSBV in experimentally infected wild shrimps, crabs and lobsters by insitu hybridization. Aquaculture 1998; 164: 232-242.

Chen LL, Huang CY, Wang CH. Natural and experimental infection of white spot syndrome virus (WSSV) in benthic larvae of mud crab *Scylla serrata.* Dis Aquat Org 2000; 40: 157-161.

Chen LL, Leu JH, Huang CJ, Chou CH, Chen SM, Wang CH, et al. Identification of a nucleocapsid protein (VP35) gene of shrimp white spot syndrome virus and characterization of the motif important for targeting VP35 to the nuclei of transfected insect cells. Virology 2002; 93: 44-53.

Chou HY, Huang CY, Wang CH. Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 1995; 23: 165-173.

Chou HY, Huang C, Kou GH, Durand S, Lightner DV, Nunan LM, et al. Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. Dis Aquat Org. 1996; 27: 59-66.

Chou HY, Huang C, Lo CF. Studies on transmission of white spot syndrome associated baculovirus (WSBV) in *Penaeus monodon* and *P. japonicus* via water borne cantact and oral ingestion. Aquaculture 1998;164: 263-276.

Dore I and Frimodt C. An illustrated guide to shrimp of the world. Huntington, USA: Osprey Books; 1987. p.152-153.

Farfante IP and Kensley B. *Penaeoid* and sergestoid shrimps and prawns of the world: key and diagnoses for the families and genera. Massachusetts: Clearance center; 1997. p.18.

Finkelman F, Katona I, Mosmann T. IFN gamma regulates the isotypes of Ig secreated during in vivo humoral immune responses. J Immunol 1998; 140: 1022-1027.

Flegel TW, Boonyaratpalin S, Withyachamnankul B. Progress in research on yellow-head virus and white spot virus in thailand. In: Flegel TW and MacRae I, editors. Disease in Asian Aquaculture III. Manila: Hish health section, Asian Fisheries Soc; 1997. p. 285-296.

Grey DL, Dall W, Baker A. A guide to the Australian penaeid prawns. Australia: Darwin, Department of Primary Production; 1983. p. 64-65.

Gulf States Marine Fisheries Commission (GSMFC). *Penaeus monodon* (Fabricius, 1978) [online]. Available from :http://nis.gsmfc.org/nis_ factsheeet. __php?toc_id=148, 1998.

Hockfield S. A monoclonal antibody to a unique cerebellar neuron generated by immunosupression and rapid immunization. Science 1987; 337: 67-70.

Inouye K, Miwa S, Oseko N. Mass mortalities of cultured kuruma shrimp, *Panaeus japonicus* in Japan in 1993; electron microscopic evidence of the causative virus. Fish Pathol 1994; 29: 149-158.

Johnson SK. Handbook of shrimp disease in aquaculture. Texas: A&M University, Department of Wildlife and Fisheries Sciences; 1990. p. 1-20.

Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, Boonsaeng V, Panyim S, Tassanakajon A, et al.Experimental transmission of white spot syndrome virus (WSSV) from crab to shrimp *Penaeus monodon*. Dis Aquat Org 1998; 34: 1-7.

Köhler G and Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256: 495-497.

Laemmli UK. Clevage of the structure proteins during the assembly of the head of T4. Nature 1970; 227: 680-685.

Lightner DV. A handbook of pathology and diagnostic procedures for disease of penaeid shrimp. Lousiana, USA: World Aquaculture Society, Baton Rouge; 1996.

Liu W, Wang YT, Tian DS. Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodies (MAbs) specific to an enveloped protein (28 kDa). Dis Aquat Org 2002; 49: 11-18.

Lo CF, Leu JH, Ho CH, Chen CH, Peng YT, Chou CM, et al. Detection of baculovirus with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 1996a; 25: 133-141.

Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL, et al. White spot syndrome baculovirus (WSSV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 1996b; 26: 215-225.

Lo CF, Ho CH, Chen CH. Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 1997; 30: 53-72.

Lo CF, Chang YS, Cheng CT. PCR Monitoring of Cultured Shrimp for white spot syndrome virus (WSSV) infection in growo ut ponds. proceedings to the special session on shrimp biotechnology 5th Asian Fisheries Forum1998: 281-286.

Lo CF and Kou GH. Virus-associated white spot syndrome of shrimp in Taiwan: A review. Fish Pathol 1998; 33(4): 365-371.

Lo CF, Hsu HC , Ho CH. Specific genomic DNA fragment analysis of different geographical isolates of shrimp white spot syndrome associated virus. Dis Aquat Org 1999; 35: 175-185.

Lo CF, Kou GH. Transcription Analysis of the DNA polymerase Gene of Shrimp White Spot Syndrome Virus. Virology 2002; 301: 136-147.

Maeda M, Itami T, Kondo M, Hennig O, Takahashi Y, Hirono I, et al. Characteristics of penaeid rod-shaped DNA virus of kuruma shrimp. In:Inui Y, edtor. New approavhes to viral diseases of aquatic animals. Mie, Japan: Nria International Workshop Proceedings. National Research Institute of Aquaculture; 1997. p. 218-226.

Mikes H, Mennens M, Vlak JM. Transcriptional analysis of the spot syndrome virus major virion protein genes. J Gen Virol 2003; 84: 1517-1523.

Mullis KB, Faloona FA, Scharf S. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. 1986; 51(Pt1): 263-273.

Nadala EC, Tapay LM, Loh PC. Yellow-head virus: a rhabdovirus-like pathogen of penaeid shrimp. Dis Aquat Org 1997; 31: 141-146.

Nadala ECB, Tapay LM, Loh PC. Characterization of a non–occluded baculovirus–like agent pathogenic to penaeid shrimp. Dis Aquat Org 1998; 33: 221-229.

Nadala Jr. ECB, Lo PC. Dot-blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow head virus of Penaeid shrimp. J Virol Methods 2000; 84: 175-179.

Okumura T, Nagai F, Yamamoto S, Yamano K, Oseko N, Inouye K, et al. Detection of white spot syndrome virus from stomach tissue homogenate of the Kuruma shrimp (*Penaeus japonicus*) by reverse passive latex agglutination. J Virol Methods 2004; 119: 11-16.

Peng SE, Lo CF, Ho CH. Detection of white spot baculovirus (WSBV) in giant freshwater prawn, *Macrobranchium rosenbergii*, using polymerase chain reaction. Aquaculture 1998: 164: 253-262.

Poulos BT, Pantoja CR, Bradley-Dunlop D. Development and application of

monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. Dis Aquat Org 2001; 47: 13-23.

Rosenberry B. World shrimp farming. Sandiego: Shrimp News International; 1996. p. 1-3.

Rout N, Citarasu T, Ravindran R. Transcriptional expression profile of a white spot syndrome (WSSV) gene in different organs of infected shrimp. Aquaculture 2005; 245: 31-38.

Sahul Hameed AS, Balasubramanian G, Syed Musthaq S. Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). Dis Aquat Org 2003; 57: 157-161.

Sambrook J and Russel DW. Molecular cloning. 3rd Ed. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor; 2001.

Saulnier D, Haffner P, Goarant C. Experimental infection models for shrimp vibriosis studies : a review. Aquaculture 2000; 191: 133-144.

Sithigorngul P, Panchan N, Vilaivan T. Immunochemical analysis and immunohistochemical localization of crustacean hyperglycemic hormone from the eyestalk of Macrobranchium rosenbergii. Comp Biochem Physiol 1999; 124: 73-80.

Sithigorngul P, Chauychuwong P, Sithigorngul W. Development of a monoclonal antibody specific to yellow head virus (YHV) from *Penaeus monodon.* Dis Aquat Org 2000; 42: 27-34.

Sithigorngul P, Rukpratanporn S, Longyant S. Monoclonal antibodies specific to yellow head virus (YHV) of *Penaeus monodon*. Dis Aquat Org 2002; 49: 71-76.

Solis NB. Biology and Ecology. In: Biology and culture of *Penaeus monodon*, Brackishwater Aquaculture Information System, Philippines: department, Southeast Asian Fisheries Development center, Tigbavan Iloilo; 1988. p. 3-36.

Takahashi Y, Itami T, Kondo M, Maeda M, Fujii R, Tomonaga S, et al. Electron microscopic evidence of bacilliform virus infection in Kuruma shrimp (*Penaeus japonicus*). Fish Pathol 1994; 29:121-125.

Tapay LM, Nadala Jr. ECB, Loh PC. A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. J Virol 1999; 81: 297-302.

Tsai JM, Wang HC, Leu JH, Hsaio HH, Wang AHJ, Kou GH, et al. Genomic and Proteomic Analysis of Thirty-Nine Structural Proteins of Shrimp White Spot Syndrome Virus. J Virol 2004; 78: 11360-11370.

Tyler KL. Animal virus. In: Granof A, Webster RG, editors. Encycopedia of Virology, 2nd ed. San Diego: Academic Press; 1999. p. 1181-118

van Hulten MCW, Tsai MF, Schipper CA. Analysis of a genomic segment of white spot syndrome virus of shrimp containing ribonucleotide reductase genes and repeated regions. J Gen Virol 2000; 81: 307-316.

van Hulten MCW, Westenberg M, Goodall SD. Identification of two major virion protein genes of white spot syndrome virus of shrimp.Virology 2000a; 266: 227-236.

van Hulten MCW,Goldbach RW, Vlak JM. Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. J Gen Virol 2000b; 81: 2525-2529.

van Hulten MCW, Witteveldt J, Peters S, Kloosterboer N, Tarchini R, Fiers M, et al. The White Spot Syndrome Virus DNA Genome Sequence. Virology 2001a; 286: 7-22.

van Hulten MCW, Witteveldt J, Snippe M. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology 2001b; 285(2): 228-233.

van Hulten MCW, Reijns M, Vermeesch AMG. Identification of VP19 and VP15 of white spot syndrome virus (WSSV) and glycosylation status of the WSSV major structural proteins. J Gen Virol 2002; 83: 257-265.

van Regenmortel MHV, Fauquet CM, Bishop DHP, editors. Virus Taxonomy Seventh Report of the International Committee on Taxonomy of Viruses. 1st ed. San Diego: Academic Press. 2000.

Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, et al. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 1995; 23: 139-242.

Witteveldt J, Vlak JM, van Hulten MCW. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. Fish & Shellfish Immunol 2004; 16(5): 571-579.

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, et al. A non-occulded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and cause a high mortality in the black tiger prawn *Penaeus monodon.* Dis Aquat Org 1995; 21: 69-77.

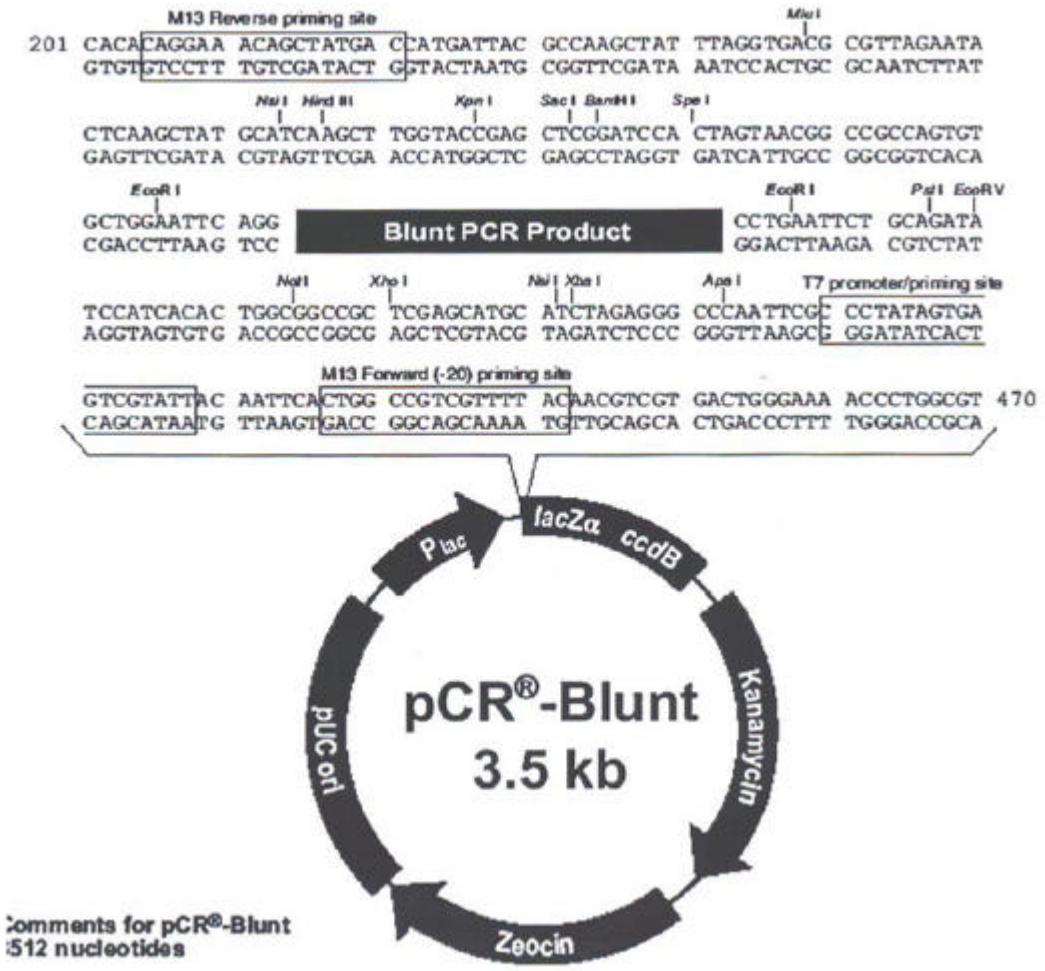
Wongteerasupaya C, Wongwisansri S, Boonsaeng V, Panyim S, Pratanpipat P, Nash GL, et al. DNA fragment of *Penaeus monodon* baculo-virus PmNOBII give positive *in situ* hybridization with viral infections in 6 penaeid shrimp species. Aquaculture 1996; 143: 23-32.

Wongteerasupaya C. Viral characterization and development of specific detection for yellow-head and white spot disease in *Penaeus monodon*, Ph. D. Thesis in Faculty of Biochemistry. Mahidol University, 1996.

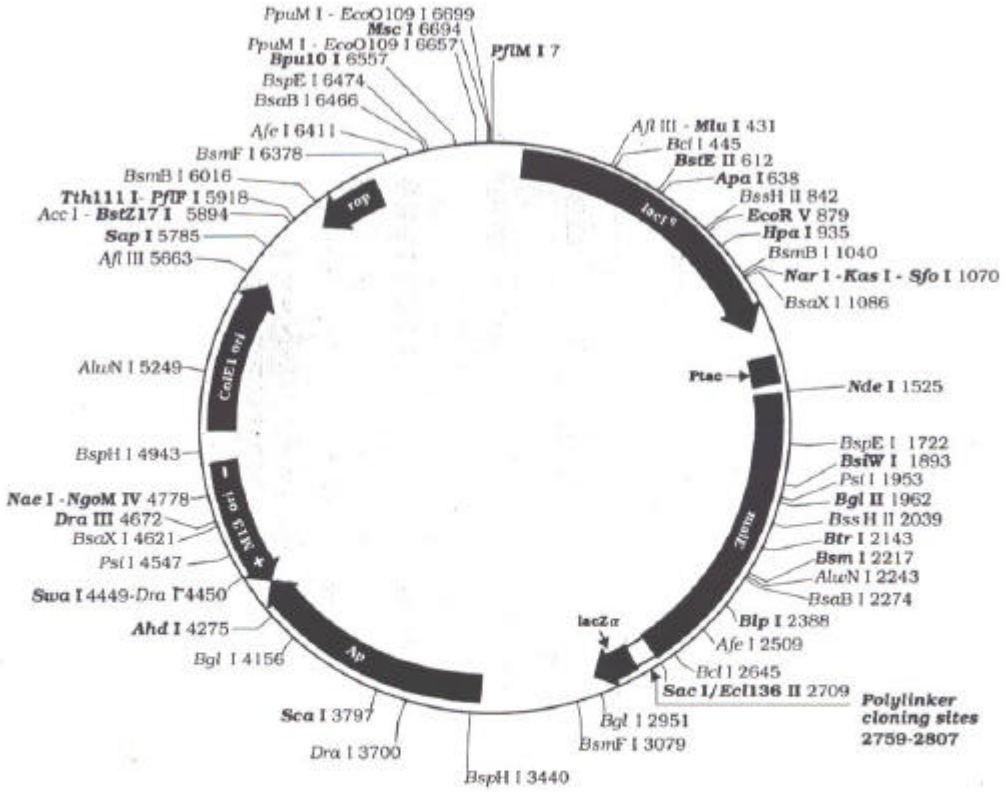
Yang F, He J, Lin X, Li Q, Pan D, Zhang X, et al. Complete genome sequence of the shrimp white spot bacilliform virus. J Virol 2001; 75: 11811-11820.

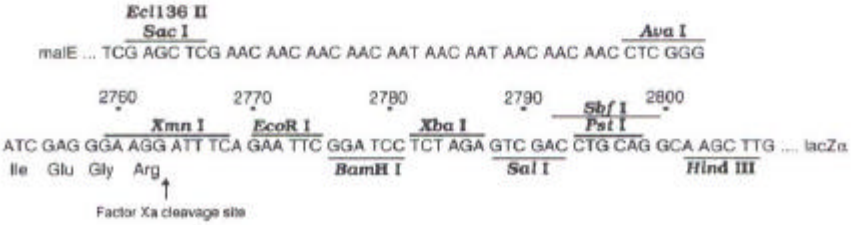
APPENDIX

APPENDIX A



pCR-Blunt plasmid and restriction sites (Invitrogen)





pMAL plasmid map (New England Biolabs)

Strains	Genotypes
1. One shot *E. coli* Top 10	F - *mcrA D (mrr-hsdRMS-mcrBC)* ? *80 lacZ*? *M15* ? *lacX74 deoR recA1 araD139 D(ara-leu)7697 galU glK rpsL (Str* ^{R} *) endA1 nupG*
2.*E. coli* XL1 blue	*recA endA1 gyrA96 thi- hsdR17 supE44 relA1 lac* [F' *proAB lacI* ^{q} *ZM15* Tn10 (Tet ^{r})]
3. *E. coli* M15 (pREP4)	Nal ^{s} Str ^{s} Rif ^{s} *lac ara gal mtl* F *recA* $^{+}$ *uvr* $^{+}$ [pREP4; *lacI, kan* ^{r}]
4. *E. coli* BL21	F $^{-}$ *omp*T hsdS $_{B}$ (r_{B} $^{-}$,m_{B} $^{-}$) dcm gal ? (DE3), pLysS, Cm ^{r}

Plasmids	Genotypes
1. pCR-Blunt (Invitrogen)	P_{tac} , *lacZ*? *-ccdB* fusion gene, Multiple cloning site, T7 promoter, T7, M13 Foreward (-20), M13 Reverse priming sites, Kan ^{r}, *Sh ble* (Zeocin ^{TM} resistance factor) and pUC origin
2. pQE30 (Qiagen)	Amp ^{r}, T5 promoter, six His tag coding sequence, ColE1 and multiple cloning site downstream of T5 Promoter
3.pMAL-C2 (New England Biolabs)	P_{tac}, *lacZ*? *-rrnB* terminator, Amp ^{r}, *M13 ori, pBR322 ori, lacI* ^{q} , malE, MCS, *bla*(Ap ^{R}), *rop* and multiple cloning site

E. coli host strains and plasmid Genotypes

APPENDIX B

1. Lysis buffer (Flegel Pers. Comm., 1988)

(50mM Tris-HCl pH 9.0 100 mM EDTA 50 mM NaCl, 2 % SDS)

Tris-HCl (pH 9.0)	6.055	g
EDTA	37.225	g
NaCl	2.922	g
SDS	20	g
Adjust volume with H_2O to	1	L

2. GTE buffer (50 mM Glucose 10 mM EDTA 25 mM Tris-HCl pH 8.0)

Glucose	0.9	g
EDTA	0.732	g
Tris-HCl (pH 8.0)	0.3	g
Adjust volume with H_2O to	100	ml

3. TE buffer (1 mM EDTA 10 mM Tris-HCl pH 8.0)

EDTA	0.0372	g
Tris-HCl (pH 8.0)	0.1211	g
Adjust volume with H_2O to	100	ml

4. 5M KAc buffer

KAc	14.72	g
Glacial acetic acid	5.75	g
Adjust volume with H_2O to	50	ml

5. 10X TBE buffer

Tris-HCl (pH 8.3)	108	g
Boric acid	55	g
EDTA	9	g
Adjust volume with H_2O to	1	L

6. 100 mM IPTG

IPTG	0.238	g
Adjust volume with H_2O to	10	ml

7. Buffer B (100 mM NaH_2PO_4 10 mM Tris-HCL 8M Urea pH 6.3)

NaH_2PO_4	1.38	g
Tris-HCl (pH 6.3)	0.12	g
Urea	48.05	g
Adjust volume with H_2O to	100	ml

8. 100 m M PMSF

PMSF	17.4	g
Isopropanol (conc.)	1	ml

MEDIA PREPARATION

9. LB broth

Tryptone	5	g
Yeast-extract	2.5	g
NaCl	5	g
Adjust volume with H_2O to	500	ml

10. LB agar

Tryptone	5	g
Yeast-extract	2.5	g
NaCl	5	g
Agar	7	g
Adjust volume with H_2O to	500	ml

BUFFER AND REAGENT PREPARATION

1. Phosphate buffered saline (PBS) 0.15 M pH 7.2

NaCl	8.0	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
or Na_2HPO_4. $7H_2O$	2.15	g
H_2O (distilled water) adjust volume to	1000.0	ml

2. 5 % Blotto solution (Johnson et al., 1984)

Skimmed milk	5.0	g
PBS 0.15 M pH 7.2	100.0	ml
1 % Merthiolate (Sigma)	1.0	ml
Triton X-100 (Sigma)	0.1	ml

3. 1 % Merthiolate

Thimerosal (Sigma)	1.0	g
H_2O (distilled water) adjust volume to	100.0	ml

BUFFER AND SOLUTION FOR SODIUM DODECYL SULFATE POLYCRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT ANALYSIS

1. Stock solution

1.1 Monomer solution (30 %T, 2.7 % C_{Bis})

Acrylamide (BIO-RAD)	58.4	g
Bis (N, N'-methylene-bis-acrylamide, BIO-RAD)	1.6	g
H_2O (distilled water) adjust volume to	200.0	ml

Stored at 4 ?C in dark bottle.

1.2 4 X Running gel buffer (1.5 M tris-Cl pH 8.8)

Tris (hydroxymethyl) aminomethane (BIO-RAD)	36.3	g
H_2O (distilled water) adjust volume to	200.0	ml

Adjusted pH with HCl

1.3 4 X Stacking gel buffer (0.5 M tris-Cl pH 6.8)

Tris	3.0	g
H_2O (distilled water) adjust volume to	50.0	ml
Adjusted pH with HCl		

1.4 10 % SDS

SDS (sodium dodecyl sulfate, BIO-RAD)	50.0	g
H_2O (distilled water) adjust volume to	500.0	ml

1.5 10 % Ammonium persulfate (freshly prepared)

Ammonium persulfate (BIO-RAD)	0.1	g
H_2O (distilled water)	1.0	ml

1.6 Running gel overlay (0.375 M tris-Cl pH 8.8, 0.1 % SDS)

1.5 M Tris (1.2)	25.0	ml
10 % SDS (1.4)	1.0	ml
H_2O (distilled water) adjust to	100.0	ml

1.7 2 X Treatment buffer (0.125 M tris-Cl pH 6.8,4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol)

0.5 M Tris (1.3)	2.5	ml
10 % SDS (1.4)	4.0	ml
Glycerol	2.0	ml
2-Mercaptoethanol	1.0	ml
H_2O (distilled water)	0.5	ml

2. Preparation of separating gel and stacking gel

2.1 Separating gel for SDS-PAGE 15% gel (15 % T 2.7 % C_{BIS})

Monomer solution (1.1)	15.0	ml
1.5 M tris-Cl (1.2)	7.5	ml
10 % SDS (1.4)	0.3	ml
H_2O (distilled water)	6.75	ml
10 % Ammonium persulfate (1.5)	150.0	?l
TEMED	20.0	?l

2.2 Stacking gel for SDS-PAGE 4 % gel (4 % T 2.7 % C_{BIS})

Monomer solution (1.1)	2.66	ml
1.5 M tris-Cl pH 6.8 (1.3)	5.0	ml

10 % SDS (1.4)	0.2	ml
H_2O (distilled water)	12.2	ml
10 % Ammonium persulfate (1.5)	100.0	?l
TEMED	10.0	?l

Table 1E Preparation of separating and stacking gel

	Separating gel	Stacking gel
	15 % T 2.7 % C_{BIS} (for SDS-PAGE)	4 % T 2.7 % C_{BIS} (for SDS-PAGE)
30 % T 2.7 % C_{BIS}	15.0 ml	2.66 ml
1.5 M tris-Cl pH 8.8(1.2)	7.5 ml	-
0.5 M tris-Cl 6.8 (1.3)	-	5.0 ml
10 % SDS	0.3 ml	0.2 ml
H_2O	6.75 ml	12.2 ml
Mixed and deaerated using vacuum pump		
10 % Ammonium persulfate (1.5)	150 ?l	100 ?l
TEMED	20 ?l	10 ?l
Mixed and rapidly poured between the glass plate		

3. Running buffer

3.1 SDS-PAGE Tank buffer (0.025 M tris pH 8.3, 0.192 M glycine, 0.1 % SDS)

Tris	12.0	g
Glycine	57.6	g
10 % SDS (1.4	40.0	ml
H_2O (distilled water)	4000.0	ml

4. Staining and destaining solution

4.1 Staining solution for protein (Coomassie blue)

4.1.1 Stain stock (1 % Coomassie blue R-250)

1 % Coomassie blue R-25	1.0	g
H_2O (distilled water)	100.0	ml

4.1.2 Stain (0.1 % Coomassie blue R-250, 50 % methanol, 10 % acetic acid)

Stain stock (4.1.1)	50.0	ml
Methanol	250.0	ml
Acetic acid	50.0	ml
H_2O (distilled water) adjust to	500.0	ml

4.2 Destaining solution for Coomassie blue

4.2.1 Destain I (50 % methanol, 10 % acetic acid)

Methanol	500.0	ml
Acetic acid	100.0	ml
H_2O (distilled water) adjust to	1000.0	ml

4.2.2 Destain II (5 % methanol, 7 % acetic acid)

Methanol	50.0	ml
Acetic acid	70.0	ml
H_2O (distilled water)	1000.0	ml

Method of gel staining for protein. A gel WAS stained with 0.1 % Comassie blue R-250, 50 % methanl, 10 % acetic acid for 5-6 hrs. The gel was then washed in destian I for 1 hr. with 1-2 changes and followed by destain II until the gel was cleared. After washing in distilled H_2O for a few times, it was dried in a gel air dryer (BIO-RAD)

5. SDS molecular weight markers (Sigma) consist of:

-Myosin, rabbit muscle	205	kDa
-?-Galactosidase, *Escherichia coli*	116	kDa
-Phosphorylase b, rabbit muscle	97	kDa
-Fructose-6-phosphate kinase, rabbit muscle	84	kDa
-Albumin, bovine serum	66	kDa
-Glutamic dehydrogenase, bovine liver	55	kDa
-Ovalbumin, chicken egg	45	kDa
-Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36	kDa
-Carbonic anhydrase, bovine erythrocytes	29	kDa
-Trypsinogen, bovine pancreas	24	kDa
-Trypsin inhibitor, soybean	20	kDa
-a-Lactalbumin, bovine milk	14.2	kDa
-Aprotinin, bovine lung	6.5	kDa

6. Towbin transfer buffer pH 8.8 for Western blot analysis
(25 mM tris, 192 mM glycine, 20 % methanol)

The buffer consisted of the following ingredients:

Tris	3.03	g
Glycine	14.4	g
Methanol	200.0	ml
H_2O (distilled water) adjusted to	1000.0	ml

The buffer was pre-chill before use.

REAGENT AND SOLUTION FOR IMMUNOHISTOCHEMISTRY

1. Slide coated solution

Gelatin	1.0	g
Chrome alum (chromium potassium sulphate)	0.05	g
H_2O (distilled water) adjust volume to	100.0	ml

2. Davidson's fixative

95 % Ethanol	30.0	ml
100 % Formalin	20.0	ml
Glacial acetic acid	10.0	ml
H_2O (distilled water)	30.0	ml

3. Phosphate buffered saline (PBS) 0.15 M, pH 7.2

NaCl	8.0	g
KCl	0.20	g
KH_2PO_4	0.20	g
$Na_2HPO_4.7H_2O$	1.15	g
H_2O (distilled water) adjust volume to	1000.0	ml

4. Calf serum 10 % in PBS(P_1^+)

Calf serum	10.0	ml
PBS	100.0	ml

5. Erlich's acid hematoxylin

Hematoxylin	8.0	g
95 % ethanol	400.0	ml
Aluminium potassium sulphate	8.0	g
Distilled water	400.0	ml
Glycerine	400.0	ml
Glacial acetic acid	400.0	ml

6. 0.2 % Eosin Y in 95 % ethanol

Eosin Y	0.2	g
95 % ethanol	100.0	ml

METHOD OF IMMUNOHISTOCHEMISTRY AND INDIRECT IMMUNO PEROXIDASE ASSAYS

1. Immunnohistochemistry

1.1 Cephalothoraces of uninfected and WSSV infected *P. monodon* were cut.

1.2 Fixed in Davidson's fixative solution 24 h.

1.3 Washed in water 3 h.

1.4 Dehydrated in a graded ethanol series and N-butyl alcohol respectively

1.4.1 70 % ethanol : one change at 3 h.

1.4.2 90 % ethanol : one change at 3 h.

1.4.3 95 % ethanol : two changes at overnight each

1.4.4 95 % ethanol : n-butyl alcohol (1:1) : one change at 1 h.

1.4.5 n-butyl alcohol : one change at 1 h.

1.4.6 n-butyl alcohol : xylene (1:1) : one change at 1 h.

1.4.7 Xylene : two changes at 1 h each.

1.4.8 Xylene : paraplast at 60^{o}C (1:1) : one change at 30 min.

1.4.9 Paraplast at 60^{o}C : three changes at 45 min each.

1.5 Infiltrated tissue by paraplast and embed in the block.

1.6 Cut tissue serial section 8 µm thickness as a ribbon by rotary microtome.

1.7 Transfer section into room temperature water, on the glass slide and transfer slide on the slide warmer 50^{o}C and incubated slide at 50^{o}C overnight to dry section.

1.8 Deparaffinized and rehydrated tissue sections through a xylene and a graded ethanol series.

1.8.1 Xylene : three change at 5 min each.

1.8.2 n-butyl alcohol : one change at 5 min.

1.8.3 95 % ethanol : one change at 5 min.

1.8.4 90 % ethanol : one change at 5 min.

1.8.5 80 % ethanol : one change at 5 min.

1.8.6 70 % ethanol : one change at 5 min.

1.8.7 Distilled water : one change at 5 min.

1.8.8 10 % formalin : one change at 10 min.

1.8.9 Distilled water : five changes at 5 min each.

1.8.10 PBS : three changes at 5 min each.

1.9 Remove slides from slide basket, dry around tissue section with vacuum pump. Tissue must never dry out. Place each slide in a humidity chamber as it is prepared.

2. Indirect immunoperoxidase method

2.1 Blocking

2.1.1 Covered tissue sections with P_1^+ solution.

2.1.2 Incubated slides for 30 min in humidity chamber, room temperature.

2.2 First antibody

2.2.1 Removed P_1^+ solution from section.

2.2.2 Dropped first antibody cover each section (first antibody is PAbs and MAb specific to WSSV).

2.2.3 Incubated at 37^{o}C for 5 h in humidity chamber.

2.2.4 Washed section with distilled water : one change rapidly.

2.2.5 Washed section with PBS : three changes at 10 min each.

2.3 Second antibody

2.3.1 Removed PBS from section.

2.3.2 Dropped second antibody covers each section (goat anti-mouse horseradish peroxidase (GAM-HRP) as second antibody.

2.3.3 Incubated at 37^{o}C for 3 h in humidity chamber.

2.3.4 Washed section with distilled water : one change rapidly.

2.3.5 Washed section with PBS : three changes at 10 min each.

2.4 Peroxidase activity was revealed by incubation with 0.03 % diaminobenzidine tetrahydrochloride and 0.006 % hydrogen peroxide in PBS at 5 min.

2.5 Washed section with water

3. Counter-stained with hematoxylin and eosin Y (H&E)
   3.1 Stained with hematoxylin
   3.2 Dehydrated in a graded ethanol series 70 %, 80 %, 90 % and 95 %: one change at 5 min each.
   3.3 Counter-stained with eosin Y
   3.4 Dehydrated in n-butyl alcohol : one change at 5 min.
   3.5 Dehydrated in n-butyl alcohol : xylene (1:1) : one change at 5 min.
   3.6 Cleaned in xylene : three changes at 5 min each.
   3.7 Mounted in permount as permanent slide
   3.8 Positive reaction was visualized as brown coloration against the pink and purple of H&E by light microscope.

BIOGRAPHY

NAME : Mister Phiromsak Phattanapaijitkul

DATE OF BIRTH : 28 May 1980

PLACE OF BIRTH : Chumporn

INSTITUTIONS ATTENDED : Rangsit University, 1998-2002
Bachelor of Science
(Medical Technology)
: Mahidol University, 2002-2005:
Master of Science
(Tropical Medicine)

HOME ADDRESS : 4/12 Latphrao 18, Rd. Bangkok 10900,
Thailand
Tel: 0-2938-7826
E-mail: phiromsak@yahoo.com