PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *Vibrio harveyi*

Miss Wannipa Phianphak

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2004

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การเตรียมและลักษณะสมบัติของโมโนโคลนอลแอนติบอดีที่จำเพาะ
ต่อแบคทีเรียที่ก่อให้เกิดโรคเรืองแสง *Vibrio harveyi*

นางสาววรรนิภา เพี้ยนภักตร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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Thesis Title	Preparation and characterization of monoclonal antibodies against *Vibrio harveyi*
By	Miss Wannipa Phianphak
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Sirirat Rengpipat, Ph.D.
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(Assistant Professor Supat Chareonpornwattana, Ph.D.)

วรรนิภา เพี้ยนภักตร์ : การเตรียมและลักษณะสมบัติของโมโนโคลนอลแอนติบอดีที่จำเพาะต่อแบคทีเรียที่ก่อให้เกิดโรคเรืองแสง *Vibrio harveyi*. (PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *Vibrio harveyi*) อ. ที่ปรึกษา : รศ.ดร.ศิริรัตน์ เร่งพิพัฒน์, อ.ที่ปรึกษาร่วม : รศ.ดร.ไพศาล สิทธิกรกุล, 133 หน้า. ISBN 974-17-6095-7.

การตรวจสอบพยาธิสภาพของ *Vibrio harveyi* แบคทีเรียก่อโรคเรืองแสงในกุ้งกุลาดำ *Penaeus monodon* จำนวน 4 ไอโซเลท โดยการแช่กุ้งระยะวัยรุ่น ในถังบรรจุน้ำทะเลและ *V. harveyi* ปริมาณประมาณ 10^7 เซลล์ต่อมิลลิลิตร พบว่าเวลาที่ทำให้จำนวนกุ้งตาย 50 เปอร์เซ็นต์ โดย *V. harveyi* 639 (39 ชั่วโมง) และ *V. harveyi* 1526 (43 ชั่วโมง) สั้นกว่าผลที่เกิดจาก *V. harveyi* VG (49 ชั่วโมง) และ *V. harveyi* VG1 (50 ชั่วโมง) *V. harveyi* 639 และ 1526 สามารถให้แอคติวิตีในการผลิตสารเฮโมไลซินและสลายโมเลกุลสารอินทรีย์ที่สูงกว่า ในงานวิจัยนี้จึงใช้ *V. harveyi* 639 และ 1526 ในการผลิตโมโนโคลนอลแอนติบอดี เพื่อใช้วินิจฉัยแบคทีเรียบนพื้นฐานของอิมมูโนวิทยา โมโนโคลนอลแอนติบอดีที่เตรียมได้มี 31 ชนิด แบ่งออกเป็น 9 กลุ่มตามความจำเพาะต่อโปรตีนต่างๆ ในช่วง 8-95 กิโลดาลตัน แอนติบอดีส่วนใหญ่มีความจำเพาะต่อ *V. harveyi* โดยไม่แสดงปฏิกิริยาข้ามต่อ *Vibrio* spp. และแบคทีเรียแกรมลบที่นำมาทดสอบ นอกจากนี้โมโนโคลนอลแอนติบอดีบางชนิดสามารถนำมาตรวจหา *V. harveyi* ด้วยความไวสูงในระดับ $5X10^4$ เซลล์ต่อมิลลิลิตร (50 เซลล์ต่อจุด) โดยวิธี dot blot และโมโนโคลนอลแอนติบอดีบางชนิดสามารถตรวจติดตามการติดเชื้อ *V. harveyi* ในเนื้อเยื่อกุ้งกุลาดำด้วยวิธี immunohistochemistry ส่วนโมโนโคลนอลแอนติบอดีอีก 3 ชนิดที่เตรียมได้ แสดงปฏิกิริยาข้ามต่อ *Aeromonas hydrophila*, *Salmonella* Typhimurium และ *Escherichia coli* สิ่งที่น่าสังเกตคือ สามารถใช้โมโนโคลนอลแอนติบอดีอีก 3 ชนิดระบุความแตกต่างระหว่าง *V. harveyi* 1526 ออกจาก *V. harveyi* 639, VG และ VG1 โดยวิธี Western blot และสามารถแยกความแตกต่างของ *V. harveyi* ทั้ง 4 ไอโซเลทออกจาก *V. harveyi* อื่นๆ อีก 18 ไอโซเลท ด้วยวิธี dot blot ดังนั้นการศึกษาครั้งนี้ทำให้ได้วิธีทางอิมมูโนวิทยา ที่ไม่เพียงแต่วินิจฉัยเฉพาะ *V. harveyi* เท่านั้น แต่ยังใช้วินิจฉัยซีโรไทป์ของ *V. harveyi* ได้อีกด้วย และคาดว่าจะสามารถนำโมโนโคลนอลแอนติบอดีที่เตรียมได้ไปเตรียมชุดตรวจสำเร็จรูปที่มีความไวเพื่อใช้วินิจฉัย *V. harveyi* ต่อไป

ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา...2547..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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The pathogenicities of four isolates of *Vibrio harveyi*, a bacterium that cause luminescent disease in *Penaeus monodon* were investigated by immersion method. *P. monodon* post larva were reared in sea water containing ~10^7 CFU ml^{-1} of bacteria. The 50% mortality rates of *V. harveyi* 639 and 1526 were shorter (39 and 43 h, respectively) than those of the other two isolates, VG and VG1 (49 and 50 h, respectively) due to the higher hemolytic and degradative enzymatic activities in *V. harveyi* 639 and 1526. Therefore, *V. harveyi* 639 and 1526 were used to produce monoclonal antibodies in order to provide an immunological tool for identification of the bacteria. Thirty-one monoclonal antibodies were obtained and can be divided into 9 groups according to their specificities to various proteins range from 8-95 kDa. Most of the antibodies were specific to *V. harveyi* without cross-reactivity to other *Vibrio* spp. and several closely related Gram-negative bacteria tested in this study. Some antibodies could be used to detect *V. harveyi* with high sensitivity up to 5 X 10^4 CFU ml^{-1} (50 cells/spot) by dot blot assay and some antibodies could be used to detect *V. harveyi* infection in tissues by immunohistochemistry. Three monoclonal antibodies that showed slight cross-reactivity to *Aeromonas hydrophila*, *Salmonella* Typhimurium and *Escherichia coli* were also isolated. Surprisingly, three specific monoclonal antibodies could be used to differentiate between *V. harveyi* 1526 and 639, VG, VG1 by Western blotting and could be used to differentiate *V. harveyi* between these four isolates and other 18 isolates by dot blot assay. Therefore, this study provided an immunological tool for specific identification not only to *V. harveyi* but also to differentiate some serotypes among this bacteria as well. Further development of highly sensitive assay and convenient kit using these monoclonal antibodies for detection of *V. harveyi* are in progress.

Student's signature…….
Field of study…Biotechnology…. Advisor's signature……
Academic year...2004…… Co-advisor's signature……...

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LIST OF ABBREVIATIONS

CFU	=	Colony forming unit
^{O}C	=	Degree celcius
DAB	=	Diaminobenzidine
ELISA	=	Enzyme-linked immunosorbent assay
DAB	=	Diaminobenzidine
DMSO	=	Dimethylsulfoxide
FCS	=	Fetal calf serum
g	=	Gram
GAM-HRP	=	Goat anti mouse-horseradish peroxidase conjugated
GAR-HRP	=	Goat anti rabbit-horseradish peroxidase conjugated
h	=	Hour
H	=	Hemocytes
Hp	=	Hepatopancreas
Ig	=	Immunoglobulin
IFA	=	Immunofluorescence assay
IHC	=	Immunohistochemistry
kDa	=	Kilo dalton
L	=	Lumen of lymphoid tubule
M	=	Muscle
MAb	=	Monoclonal antibody
mg	=	Milligram
min	=	Minute
ml	=	Millilitre
mm	=	Millimetre

LIST OF ABBREVIATIONS (Cont.)

μl	=	Microlitre
μm	=	Micrometre
nm	=	Nanometre
OD	=	Optical density
OPD	=	o-phenylenediamine dihydrochloride
PAGE	=	Polyacrylamide gel electrophoresis
PAb	=	Polyclonal antibody
PBS	=	Phosphate buffer saline
RAM-IgG	=	Rabbit anti mouse-IgG
S	=	Sperm
SDS	=	Sodium dodecyl sulphate
TCBS	=	Thiosulfate citrate bile salt
TSA	=	Tryptic soy agar
Rf	=	Running front
RIA	=	Radioimmunoassay
WB	=	Western blot

CHAPTER I

INTRODUCTION

Vibrio harveyi is a major cause of luminous vibriosis in marine fish, shellfish and lobster worldwide (Egidius 1987; Austin and Austin, 1993; Diggles et al., 2000). It is commonly isolated from various habitats, i.e., warm marine waters, surface of marine animals, light organ of certain marine fish and cephalopods and intestine of aquatic animal (Baumann, Baumann and Mandel, 1971; Ramesh and Venugopalan, 1987 ; 1989). *V. harveyi* has emerged as a serious pathogen of penaeids, especially in hatcheries or farms in South America, Australia and South-East Asia (Pizzutto and Hirst, 1995; Lavilla-Pitogo et al., 1990; Jiravanichpaisal and Miyazaki, 1994; Liu, Lee and Chen, 1996a ; Liu, Lee, Yii et al., 1996b). *V. harveyi*, a Gram-negative bacterium, is the motile rods with bipolar flagella, catalase-positive and produced green colonies on TCBS agar. It is a facultative anaerobe, obligate fermentative and sensitive to the vibriostatic agent O/129 (Pizzutto and Hirst, 1995). In addition, it failed to utilize inositol, sorbitol and melibiose but it was all lysine decarboxylase and gelatinase positive (Liu, Lee, Yii et al., 1996b). High levels of organic matter, salinity range 10-60 ppt, water temperature range 25-32 ^{O}C, water pH 5-9, as well as dissolved oxygen range 0.5-7.8 mg/l enhanced the bacterial growth. Slow growth was observed at low salinity (5 ppt), high alkaline water (pH 9.5) and no growth was noted at acidic water (pH 3). No effects of different levels of ammonium, nitrite and nitrate on its growth were detected. In contrast, higher level of phosphate (50 ppm) had negatively affect on bacterial growth (Kiriratnikom et al., 2000).

V. harveyi was identified as a marine pathogen and cause the luminescent disease in the black tiger shrimp hatcheries or farms in Thailand (Jiravanichpaisal and

Miyazaki, 1994). Many cases and outbreaks of luminescent disease have been associated with *V. harveyi* infection. The usual method for diagnosis of *V. harveyi* infections in farmed shrimp is based on identification of single colony isolates by traditional, nutritional and biochemical tests (Baumann, Furniss and Lee, 1984; Anderson et al., 1988). However, this process can take several days to complete and it may be too slow for practical management decisions by shrimp farmers (Rojlorsakul et al., 1998). The diagnosis of vibriosis is usually performed by presumptive diagnosis based on gross signs, followed by histological and confirmatory diagnosis (Lightner, 1996). Therefore, the development of reagents and methods to detect *V. harveyi* in shrimp and water environment have recently been the focus of much research. Even though several methods have been developed to isolate, detect and control *V. harveyi* (Liu, Lee, Yii et al., 1996b; Jory 1998; Moriarty 1998; Rengpipat et al., 1998; Kiriratnikom et al., 2000; Alvarez et al., 1998; Alcaide et al., 2001; Zorrilla et al., 2003), there is currently no simple, rapid and accurate technique suitable for routinely use by microbiologists or farmers. A major limiting factor for *V. harveyi* detection has been the lack of high-quality immunodiagnostic reagents. By contrast, MAbs diagnostic techniques can be used to speed up diagnosis to only a few hours, without loss in reliability of identification (Dalsgaard et al., 1996).

The use of MAbs to detect other bacteria has been recently reported (Okrend, Rose and Matner, 1990; Adam et al., 1995; He et al., 1996; Jaradat and Zawistowski, 1996; Saha and Nair, 1997; Charni et al., 2000; Rivera-Betancourt and Keen, 2000; Jung et al., 2001). Munro et al. (2003) produced monoclonal antibodies specific to the exotoxin subunits from *V. harveyi* strain 642. However, the assay (Western blot analysis) was not highly sensitive and could not be easily applied during field examinations. Therefore, the production of MAbs against unique epitope on the

antigens is a logical approach to generate useful immunodiagnostic reagent for sensitive and specific identification of *V. harveyi*. Polyclonal antibodies against the *V. harveyi* antigens were produced by Lee, Liu et al. (1997b) but broad-range detection was observed because of cross-reactivity between species. For other techniques, such as ELISA utilizing monoclonal antibodies was more specific and could be available in an unlimited supply through cell culture techniques (He et al., 1996; Quinlan and Foegeding, 1997).

In the present study, an attempt to produce MAbs specific to *V. harveyi* was performed. These MAbs are species specific and can be used to examine antigenic differences among various strains of luminescent bacteria (*V. harveyi*). In addition, an immunohistochemistry protocol was developed to investigate the location of infection by *V. harveyi* in *Penaeus monodon*.

Objectives:

1. To prepare MAbs specific to luminescent *Vibrio harveyi* in order to identify and differentiate *V. harveyi* from other *Vibrio* spp. and other bacteria.
2. To characterize MAbs specific to *V. harveyi* by dot blot, Western blot and immunohistochemistry.

Benefits from this study:

MAbs specific to luminescent *Vibrio harveyi* can be used to identify *V. harveyi* from other *Vibrio* spp. and other bacteria. Also the MAbs can be utilized to develop strip test, an easy, accurate and rapid technique for detection of *V. harveyi* in hatcheries and farms of cultured *P. monodon.*

CHAPTER II

LITERATURE REVIEW

1. *Penaeus monodon* Fabricius, 1978

1.1 Morphology

The post larvae of *P. monodon* are the largest among species of the *Penaeus* group in the world. The body is slender and typical 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 has spinules. The coloration is generally dark colored with carapace and abdomen transversely banded with black and white; rest of body variable from light brown to blue or red; some smaller specimens show a dull red dorsal strip from rostrum to six abdominal segment (Grey, Dall and Baker, 1983). The size is the largest commercially available shrimp, reaching > 330 mm (Dore and Frimodt, 1987) as shown in Figure 2.1.

Figure 2.1 The black tiger shrimp, *P. monodon* Fabricius, 1978.

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 isosmotic 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).

1.3 Distribution

The shrimp *P. monodon* is widely distributed throughout Batavia, East coast of Africa, Red Sea, Madagascar, Mauritius, Reunion, Pakistan, India, Srilanka, Malaysia, Singapore, Indonesia, China, Philippines, Hong Kong, Taiwan, Thailand, Japan, Korea, New Guinea, Fiji and Western Australia (GSMFC, 1998).

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).

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. Disease may be caused by non-infectious and infectious disease (Johnson, 1990).

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).

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 Vibrionaceae bacteria. These pathologies particularly hamper larval production and lead to profitability problems due to stock mortalities. They also lead to the overfishing of wild shrimp larvae and an overexploitation of broodstock. Moreover, the local environment can be contaminated by the discharge of wastewater containing

antibiotics, which can faster the development of drug-resistant bacteria. Finally, the practice of shrimp transfer at national or international levels has contributed to the spread of disease, which are now an epizootic (Bachère, 2000). Bacterial diseases due mainly to *Vibrio* species are often associated with low survival rates in hatchery or growout conditions in shrimp aquaculture (Saulnier et al., 2000).

2. Vibriosis

Vibriosis has become the economically most important disease in marine culture. General external disease symptoms are hemorrhages and superficial skin lesions, and in most cases there is a general septicemia caused by a bacterial species of the genus *Vibrio* (Inglis, Roberts and Bromage, 1993).

The genus *Vibrio* consists of Gram-negative straight or slightly curved rods, 0.5-0.3 X 1.4-2.6 μm. They are non spore-forming and motile by monotrichous or multitrichous sheathed polar flagella. All are facultative anaerobes and chemo-organotrophs and most are oxidase positive. Most species grow well in media with a sea water base. Sodium ions stimulate the growth of all species and for many are an absolute requirement. They are sensitive to the vibriostatic 2,4 diamino-6,7-diisopropyl pteridine phosphate (O/129). They are common aquatic habitats, particularly in marine and estuarine environments and in association with marine animals. Several species are pathogenic for man as well as marine animals. The mol % G+C of the DNA is 38-51 (Inglis, Roberts and Bromage, 1993).

This genus contains the most significant marine bacterial pathogen. As with *Aeromonas* spp. in fresh water, they are ubiquitous, especially where organic loads are high. Only certain species are pathogenic, and while particular strains within a

species may be highly pathogenic, others may be innocuous or act only as secondary invaders.

The common, generally accepted named of the organism *Vibrio* species disease agent is vibriosis or bacterial disease, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis, red-leg disease, sien dum in Thai which translates to black splint, sea gull syndrome or Sindroma de gaviota in Latin America (Bower, 1997).

The natural abundance of *Vibrio* spp., their ubiquities, multiplication rates and ability to adapt to environment changes in shrimp culture ecosystems increases these preoccupations. *Vibrio* spp. disease as a secondary infection due to opportunistic pathogens and occurring only in immunologically compromised shrimps. Primary causes could encompass other infectious agents, nutritional deficiencies or intoxication, environment and management practices and induced stress (Saulnier et al., 2000).

Disease signs range form localized cuticular lesions, oral and enteric infections to septicemia (Lightner, 1996). Disease outbreaks attributed to other *Vibrio* species such as *Vibrio alginolyticus, V. damsela, V. parahaemolyticus, V. vulnificus* and *V. penaeicida* have been observed in nursery or growout ponds of *Penaeus vannamei, P. monodon, P. japonicus* and *P. stylirostris* in Malaysia (Anderson et al., 1988), Taiwan (Liu et al., 1996a), Philippines (Alapide-Tendencia and Dureza, 1997), Japan (de la Peña et al., 1993) and New Caledonia (Costa et al., 1998; Mermoud et al., 1998).

Larval mortalities associated with the presence of *V. harveyi* have been reported in *Penaeus monodon* and *P. vannamei* in Indonesia (Sunaryanto and Mariam, 1986), Thailand (Jiravanichpaisal and Miyazaki, 1994), India (Karunasagar et al.,

1994), Philippines (Baticados et al., 1990 ; Lavilla-Pitogo et al., 1990), Australia (Pizzutto and Hirst, 1995), Taiwan (Liu et al., 1996a) and Ecuador (Robertson et al., 1998). Impact on the host of *V. harveyi* is anorexia and behavioral changes as well as luminescent disease in the shrimp. Mortality ranges from insignificant to 100%, particularly in post larvae and young juvenile shrimp. There is also concern over the appearance of antibiotic-resistant strains as reported for *V. harveyi* that cause a mass mortality of cultured *P. monodon* larvae in Thailand (Jiravanichpaisal and Miyazaki, 1994). Infection of *Penaeus monodon* with *V. harveyi* is known as luminous bacterial disease.

3. *Vibrio harveyi*

3.1 Characteristic of *Vibrio harveyi*

3.1.1 Morphology, physiology and biochemistry

V. harveyi is a Gram negative straight or slightly curved rods with size about 0.5-0.3 X 1.4-2.6 μm, motile, oxidase and catalase positive, and produced green colonies on TCBS agar (Liu et al., 1996a). They produced cream colored, occasionally translucent, raised, shiny colonies (Alvarez et al., 1998). They are non spore-forming and motile by flagella. The flagellation patterns of *V. harveyi* exhibit mixed flagellation possessing polar and peritrichous flagella. When grown planktonically (liquid medium), the bacteria display polar flagella. The flagellum is in sheathed by the structure, appeared to be an extension of the outer cell membrane. When grown on solid medium, the organisms produce both polar and peritrichous (also called lateral flagella) (McCarter, 2001). They grow well in media with sea water base because sodium ion stimulate the growth. The optimum temperature for growth was 37 ^{o}C while the minimum and maximum temperature was ranging from 22-30 ^{o}C. They could grow in broth medium at pH 6-9 and the optimum % NaCl was

0.5-7.5%. The biochemical characteristics of *V. harveyi* were shown in Table 2.1 (Baumann et al., 1984).

3.1.2 Bioluminescence

Bioluminescence refers to the visible light emission in living organisms that accompanies the oxidation of organic compound (luciferins) mediated by an enzyme catalyst (luciferase). Luminescent organisms, which include bacteria, fungi, fish, insects, algae and squid have been found in marine, freshwater and terrestrial habitats, with bacteria being the most widespread and abundant luminescent organism in nature. The expression of luminescence in many bacteria has been found to be highly dependent on cell density. That is, bacteria found living free in the ocean do not give off light, whereas luminescence is observed from bacteria that are found at high densities, such as in the confined environments of the light organ of squid or shrimp. When *V. harveyi* live freely in seawater, its concentration is less than 10^2 cell ml^{-1}, resulting in no observable luminescence. However at the high cell densities luminescence found in the light organ of certain marine (Baumann et al., 1984).

V. harveyi, whether normal flora or pathogen, are able to emit light of a blue-green color (luminescence) in marine animal. It is called luminous bacteria. The luminescence reaction, which is catalyzed by luciferase, involves the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$) with the liberation of excess free energy in the form of a blue green light at 490 nm.

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + \text{light (490 nm)}$$

Bacterial luciferase is a heterodimer having a molecular weight (MW) of about 80 kDa and consisting of α and β subunits with MW of about 42 and 38 kDa, respectively (Baumann et al., 1984).

Table 2.1 The biochemical characteristics of *V. harveyi* (Baumann et al., 1984)

Biochemical characteristics	***V. harveyi***
Pigmentation: Yellow- orange, Blue-black, Red	-
Arginine hydrolase	-
Oxidase	+
Production of NO_3^- to NO_2^-	+
Luminescence	+
Gas from D-glucose	-
Production of acetoin and / or diacetyl	-
Production of: Amylase	+
Gelatinase	+
Lipase	+
Alginase	d
Chitinase	+
Utilization of : D-Xylose	-
L-Arabinose	d
D-Mannose	+
D-Galactose	d
Sucrose	d
Trehalose	+
Cellobiose	+
Melibiose	-
Lactose	-
Salicin	d
D-Gluconate	+
D-Glucuronate	+
D-Galacturonate	-
Propionate	+
Valerate	-
Heptanoate	+
Glutarate	-
DL-Malate	d
β-Hydroxybutyrate	-
DL-Lactate	+
Citrate	+
Pyruvate	+
D-Mannitol	+
D-Sorbitol	-
Meso-Inositol	-
Ethanol	-
p-Hydroxybenzoate	-

+ = positive, - = negative, d= weakly

Table 2.1 (Cont.)

Biochemical characteristics	***V. harveyi***
Utilization of :	
D-Alanine	+
L-Alanine	d
β-Alanine	-
L-serine	+
L-Leucine	-
L-Glutamate	+
γ-Aminobutyrate	-
δ-Aminovalerate	-
L-Histidine	d
L-Proline	+
L-Tyrosine	+
Putrescine	-
L-Rhamnose	-
D-Ribose	+
D-Glucose	+
D-Fructose	+
Maltose	+
N-Acetylglucosamine	+
Acetate	+
Butyrate	-
Isobutyrate	-
Isovalerate	-
Caproate	d
Caprylate	d
Polargonate	d
Caprate	d
Succinate	+
Fumarate	+
L-Tartrate	-
DL-Glycerate	+
Aconitate	+
Erythritol	-
Glycerol	+
Propanol	d
Phenylacetate	-
Quinate	-
Glycine	d
L-Threonine	+
L-Aspartate	d
L-Arginine	d
L-Ornithine	-
L-Citrulline	d

+ = positive, - = negative, d= weakly

3.2 Life cycle and epidemiology

V. harveyi has been found in seawater, samples close to the larval rearing facilities, infected larvae, surfaces of marine animals and intestine of aquatic animal (Remesh, Nandakumar and Venugopalan, 1986; Lavilla-Pitogo et al., 1990). It is therefore suggested that the route of infection is through the seawater, where *V. harveyi* is a natural inhabitant. Ruby and Morin (1979) reported that it is a normal constituent of the non-pathogenic flora of marine animal. Geographical distribution of *V. harveyi* can occur almost all species of cultures penaeid shrimp and everywhere they are cultivated (Bower, 1997).

Liu et al. (1996a) isolated luminous bacterial strains from the hepatopancreas of moribund prawns *Penaeus japonicus* and *Penaeus monodon* from outbreaked farm in Taiwan during August and December of 1994. These isolated strains were characterized and identified to be *V. harveyi.*

Alvarez et al. (1998) isolated *V. harveyi* as a pathogen of penaeid shrimp and fish from farms and caught in Venezuela waters, have been severely affected by bacterial hemorrhage septicemia, coined vibriosis in Venezuela.

3.3 Impact on host species, clinical signs and pathology

V. harveyi principally infect larval stages of penaeid shrimps. Infected larvae display massive colonization of the appendages and foregut, followed by infection of the midgut, hepatopancreas and a terminal septicemia (Lighter, 1998). Necrosis of the appendages is often seen. Significant mortalities can occur after 48 h when luminescent vibriosis reach an excess of 10^3 CFU ml^{-1} *in vivo* (Lavilla-Pitogo et al., 1990).

Ruangsri et al. (2004) found that the *V. harveyi* concentration of 1.6 X 10^6 – 7.27 X 10^7 CFU ml^{-1} caused 50% juvenile *P. monodon* mortality in ten days by injection. Histological study showed that at and early period of susceptibility, there was swollen tubular lumen, minor cell degeneration of the hepatic tubules and lymphoid organs and aggregation of blood cells around the degenerating cells. During seventh day susceptibility period, there was large scale cell degeneration of hepatopancreas, lymphoid organs, gills and blood forming organs with a great extent of blood cell aggregation and eventually mortality occurred. During 14 days of susceptibility period, there was almost entire degeneration of cells in hepatic tubules and lymphoid organs causing hepatopancreatic tubular necrosis. During this period, juveniles did not accept feed and total mortality occurred.

Robertson et al. (1998) isolated *V. harveyi* from diseased *P. vannamei* was pathogenic in penaeid shrimp larval when used in a bath at 10^5 CFU ml^{-1} for 2 h. Histopathology showed the presence of distinctive melanotic tissue aggregates within the hepatopancreas.

3.4 Pathogenesis

The pathogenesis of *V. harveyi* was poorly understood. Until now there has been no clear explanation why some strains of *V. harveyi* are pathogenic, while others are not (Munro et al., 2003). Although several putative virulence determinants had been identified however they were still poorly characterized. An association with bioluminescence and hemolysin production has been made. Indeed, there are graphic descriptions of luminescent diseased shrimp. Undoubtedly, one of the constraints is that most isolates, even from severe cases of disease, are relatively non-virulent. Therefore meaningful study of pathogenicity with such isolates is questionable.

Generally, attempts to culture the bacteria *in vitro* have led to greatly reduce virulence, with the ability to luminescence being lost. Luminescence is due to the bacteria ability to detect chemicals (autoinducers) released by other bacteria and ability to modify the behavior genetically in accordance with this. This is known as quorum sensing and allows communication between bacteria of the some species. Sensors on the surface of the bacteria pick up autoinducers released by the other bacteria in close proximity and once this release a critical threshold, the genetic expression of the cell is altered and the bacteria begin the luminescence. It has been suggested that pathogenicity and luminescence may be interlinked and controlled by quorum sensing. Therefore if these genes are only switched on under appropriate conditions, this may explain why such cells lose pathogenically in the laboratory. Another clue is that outbreaks of the disease occur predominantly during the rainy season suggesting that environmental factors are involved in the level of pathoginicity expressed by the bacteria, such as lower levels of salinity (Harding, 2000).

Virulence of *V. harveyi* is reported to be associated with the presence of extracellular products (ECPs). The ECPs contain a variety of enzymes, including proteases, glycerophospholipid and a variety of partially characterized toxic activities, including hemolysin and cytotoxins, which allow the bacteria to survive and replicate within the host tissue (Liu et al., 1996a; Lee, Liu et al., 1997b).

Lee, Liu et al. (1997b) demonstrated pathogenicity of the ECPs in passively immunized tiger prawns, *P. monodon* Fabricius. The rabbit antiserum raised against ECPs from a prawn pathogenic isolated of *V. harveyi* conferred protection against subsequent challenge with live bacterial cells.

Montero and Austin (1999) suggested that the lipopolysaccharide characterized from ECPs might constitute the lethal toxin of *V. harveyi* E2 to penaeid shrimp.

Pizzutto (1992) reported that virulent strains of *V. harveyi* could not be separated from avirulent strains on a phylogenetic basis. Subsequently, Pizzutto and Hirst (1995) suggested that the strain specific virulence of *V. harveyi* may be acquired through genetically mobile elements such as plasmid or bacteriophage carrying transposon or insertion sequence. Several toxic factors produced by *V. harveyi* were previously reported by Liu et al. (1996a) as following matters.

3.4.1 Exotoxin

Exotoxin was a protein toxin released from viable bacteria. Harris and Owen (1999) showed that two luminous strains of *V. harveyi* produce proteinaceous exotoxin capable of causing mortality in mice and *P. monodon* at relatively small concentrations. Toxin T1, produce by *V. harveyi* strain 47666-1, had an LD_{50} of 2.1 μg g^{-1} by intra-peritoneal injection on CBA mice and 1.8 μg g^{-1} by intra-muscular injection in juvenile *P. monodon*. This protein comprised 2 subunits of approximately 55 and 45 kDa, giving the native protein a weight of approximately 100 kDa. Partial sequence of T1 that share some homology was found to be toxins or virulence proteins produced by bacterial gut pathogens, *Salmonella* Typhimurium and *Shigella flexneri*. It is possible that toxin T1 also interacts with the midgut microvilli of the larval prawn gut. Toxin T2 produced by *V. harveyi* strain 642 had an LD_{50} of 3.1 μg g^{-1} by intra-peritoneal injection on CBA mice and 2.2 μg g^{-1} by intra-muscular injection in juvenile *P. monodon.* Concentrated protein fraction from *V. harveyi* 642 containing toxin T2 and separated in to 7 major bands (MW~ 58,48,47,46,45,39 and 21 kDa). Toxin T2 seems to be a relatively unique protein given the lack of homology

to the complete N-terminal sequence obtained with other entries in the ANGIS database. The large number of subunits seen in PAGE gels are thought to be derived primarily from T2 suggesting that T2 might be a protoxin and that eukaryotic cell recognition and cleavage might be essential for its action. These toxins are likely to be important virulence factor for these strains.

3.4.2 Proteases

Proteases catalyzed the cleavage of peptide bond of protein substrate. They were responsible for the complex process in the normal physiology of the cell as well as in abnormal pathophysiological conditions (Rao et al., 1998).

3.4.2.1 Physiological function of proteases

Proteases played many physiological and pathological roles in biological system. These roles included protein catabolism, blood coagulation, cell growth and migration. In addition, the proteases also involved in tissue arrangement, inflammation, tumor growth and metastasis, activation of zymogens, releasing of hormones, pharmacologically active peptide and transportation of secretory proteins across membranes. In general, extracellular protease catalyzed the hydrolysis of large protein to smaller molecules for subsequent absorption by the cell whereas intracellular protease played a critical role on the regulation of cellular metabolism. In contrast to the multitude of the roles contemplated for protease, knowledge about the mechanisms by which they performed these functions was very limited (Rao et al., 1998; Beynon and Bond, 1989; Maeda, 1996).

3.4.2.2 Role of microbial proteases in pathogenesis

Many species of pathogenic bacteria produced cell-surface or secreted proteases. The enzymes had high potential to enhance bacterial pathogenesis through degradation of critical host proteins and mimicking the activity of host regulatory proteases that control important zymogen systems. Microbial proteases were not only resistant to human plasma proteases inhibitors, but could also inactivate the plasma protease inhibitors. Therefore, the host proteases were free to be activate or inactivate enzyme (zymogens). Consequently, they would facilitate action of various proteolytic proteases of the host and affected many events. These included the four major protease cascade-regulated zymogram systems: the complement, clotting, fibrionolysis and Hageman factor-prekallikrein-bradykinin systems (Rao et al., 1998; Beynon and Bond, 1989; Matsuka et al., 1999).

Proteases were grossly subdivided into two major groups, exopeptidases and endopeptidases, depending on their sites of action. Exopeptidases cleaved the peptide bond proximal to the amino or carboxy termini of the substrate. Meanwhile, endopeptidases cleaved the peptide bond in the inner regions of the polypeptide. The endopeptidases had widely been studied and reported worldwide. They were subsequently divided into four subgroups based on their catalytic mechanisms: (a) aspartic protease, (b) serine protease, (c) cysteine protease and (d) metalloprotease (Rao et al., 1998; Beynon and Bond, 1989).

(a) Aspartic protease

Aspartic proteases were commonly known as acidic proteases because of the presence of aspartic acid residue on their active sites. These proteases had been grouped into three families: pepsin, retropepsin and enzymes from pararetrovirus. Most of the aspartic protease exhibited maximal activity at low pH

(pH 3 - 4). Their molecular mass was in the range of 30-45 kDa (Rao et al., 1998; Beynon and Bond, 1989).

(b) Serine proteases

Serine proteases were characterized by the presence of a serine group in the active site. The primary structure of the members (chymotrypsin, subtilisin, carboxypeptidase and *Escherichia* D-Ala-D-Ala peptidase A) were totally unrelated, suggesting the there were at least four separate evolutionary origins for serine proteases. Thus serine proteases had a common reaction mechanism consisting of a common catalytic triad of the three amino acids: serine, aspartate and histidine. The serine proteases were generally active at neutral and alkaline pH, with an optimum between pH 7 and pH 11. They had broad substrate specificities. Their molecular mass was ranging from 18-35 kDa (Rao et al., 1998; Beynon and Bond, 1989).

Lee, Yu et al. (1996) purified 33 kDa serine protease obtained from the *Vibrio alginolyticus* Swy originally isolated from hepatopancreas of diseased kuruma prawns (*P. japonicus*) in Taiwan. The effect of this serine protease on the plasma components of *P. japonicus* and *P. monodon* was studied by Lee, Chen et al. (1997a) using crossed immunoelectrophoresis. The result of the coagulogen and unknown component in the penaeid plasma showed an increased migration rate after incubation with a partially purified 33 kDa. In contrast, incubation with protease had no detectable effect on the amount of hemocyanin. These event may play a significant role in the pathogenicity of *V. alginolyticus* in penaeid shrimp.

(c) Metalloproteases

Metalloproteases were defined as a group of endoproteases that required metal ions as a cofactor for their activities. The metalloproteases were found in a wide variety of origins eg. collagenases from higher organisms, hemorrrhagic toxin from snake venom and thermolysin from bacteria. Based on the specificity of their action, metalloproteases could be divided in four groups : (i) neutral, (ii) alkaline, (iii) Myxobacter I and (iv) Myxobacter II. The neutral proteases showed specificity for hydrophobic amino acids while the alkaline protease possessed a very broad specificity. Myxobacter protease I was specific for small amino acid residue on either side of the cleavage band whereas protease II was specific for lysine residue on the amino side of the peptide bond. All of them were inhibited by chelating agent such as EDTA but not by sulfhydryl agent (Rao et al., 1998 ; Beynon and Bond, 1989)

Fukasawa, Nakamura, Kamii et al. (1988a) isolated marine luminous bacteria from seawater at Onjuku, Japan and identified as *V. harveyi* strain FLA-11. This strain can produce proteinase enzyme. The enzyme was most active at pH 8.0 and 55 ^{o}C and stable below 40 ^{o}C. The enzyme activity was completely inhibited by EDTA, orthophenanthrolin and phosphoramidon. Metal ions such as Cu^{2+}, Hg^{2+}, Ni^{2+}, Cd^{2+} and Co^{2+}also inhibited the activity. This result indicated that this enzyme is a metal-chelator-sensitive, alkaline proteinase and had a molecular weigh 80 kDa, comprising a tetramer of 21 kDa subunits.

Fukasawa, Nakamura, Miyahira et al. (1988b) purified two proteinases (I and II) from luminous bacterium *V. harveyi* strain FLN-108 and molecular weight of proteinase I and II were estimated to be 49 kDa and 46 kDa,

comprising a dimer of 23 kDa subunits. These enzyme were most active at pH 8.0 to pH 9.0 and 50 ^{o}C, and stable below 45 ^{o}C. These enzyme activities were inhibited by EDTA and orthophenanthrolin. Prosphoramidon inhibited the activity of proteinase II but not that of proteinase I. Metal ion such as Cu^{2+}, Hg^{2+}, Ni^{2+} strongly inhibited the activities. These results indicate that the proteinases I and II are metal-chelater-sensitive, alkaline proteinases.

(d) Cysteine proteases

Cysteine proteases were found in both prokaryotes and eukaryotes. Apart from the cysteine protease found in animal and plants, the enzymes have also been demonstrated to play important roles in the disease process of some infectious agent such as viruses, bacteria, parasites, in Alzheimer's diseases and in apoptosis (Liu, Lee, Tu et al., 1997). About 20 families of cysteine protease had been recognized. The presence of cysteine and histidine (Cys-His or His-Cys) residues on their active sites differed among the families. The cysteine protease provided their activities in the presence of reducing agent such as HCN or cysteine. Most of cysteine protease functioned at neutral pH but in some case eg. lysosomal protease maximally functioned at acidic pH (Rao et al., 1998 ; Beynon and Bond, 1989).

Liu, Lee, Tu et al. (1997) purified and characterized extracellular protease produced by pathogenic luminous *V. harveyi* strain 820514, originally isolated from diseased tiger prawn (*Penaeus monodon*). This protease is an alkaline protease, heat labile, inhibit by iodoacetamide, iodoacetic acid, N-ethylmaleimide, *p*-chloromercuribenzoate and *p*-chloromercuribenzene-sulfonic acid and showed maximal activities at pH 8 and 50 ^{o}C, having molecular mass of 38 kDa. This protease is the first cysteine protease found in *Vibrio* species.

Lee, Chen and Liu. (1999) reported the effect of cysteine protease on plasma coagulogen observed *in vitro* and *in vivo*. It markedly interfere with hemostasis leading to the occurrence of unclottable hemolymph. These complex events may significantly contribute to the pathogenicity of *V. harveyi* in the prawn.

3.4.3 Hemolysin

Hemolysin is an antibody or one group of bacterial toxins. When complement is also present, it can destroy red blood cells by attacking their cytoplasmic membranes and causing them to lyse (Biotech Resource and Indiana University, 1998). Hemolytic activity has been identified as one of the important virulence properties of the pathogen. The level of hemolysin production has been used often as an indicator of potential virulence (Munro et al., 2003)

The correlation between the pathogenicity of bacteria isolated from prawn hepatopancreas and lysis of prawn hemocytes were also supported (Chang, Liu and Shyu, 2000).

Zhang, Meaden and Austin (2001) found that *V. harveyi* VIB 645, which was the most pathogenic isolate, produced extracellular product with highest titer of hemolytic activity toward fish erythrocytes. It was found to contain two closely related hemolysin genes (designated *vhh A* and *vhh B* ; molecular mass of 47.3 kDa), whereas the majority of strain examined carried only a single hemolysin gene. The hemolytic activity was very high, when the ORF of *vhh B* was cloned in *E. coli* together with the native promoter. It was determined that the high virulence was associated with possession of duplicate hemolysin gene.

3.4.4 Bacteriophage

Bacteriophage is a temperate phage and may have been present within the cell of the pathogen. They have been reported to cause diversity in bacterial

hosts by acting as transposon-like elements. The initial sign of infection was the presence of small eosinophilic inclusion bodies in the region normally occupied by the golgi apparatus. These inclusion bodies enlarged and eventually burst. It is well known that some temperate phage in bacterial hosts play major roles in causing diseased in humans (Cheetham and Katz, 1995; Reidl and Mekalanos, 1995). In marine shrimp however, there is still no confirmed evidence that temperate phage is a causative agent (Pasharawipas et al., 1998). *Vibrio* species, including *V. harveyi* are major hosts of marine bacteriophages (Proctor, 1997).

Ruangpan et al. (1999) reported that toxicity of *V. harveyi* 1039 to *P. monodon* was mediated by a bacteriophage. The mechanism of the mediation probably is occurred by the transfer of a toxin gene(s) or a gene(s) controlling toxin production. The size and morphology of bacteriophage described is similar to those of lambda phage, T4 phage and μ phage, all of which are DNA viruses.

Oakey and Owen (2000) investigated a possible relationship between virulence and the bacteriophage of *V. harveyi*. The bacteriophage, which has been determined has an icosahedral head, rigid tail and contain double stranded linear DNA, presumptively assigned to the genus *Myovirus*.

In 2003, Austin, Pride and Rhodie studied infection of *V. harveyi* with bacteriophage. It resulted in enhanced hemolytic activity to rainbow trout blood and increased pathogenicity to Atlantic salmon and *Artemia*. It was found that the bacteriophage was clearly exerting a synergistic effect on *V. harveyi*, enhancing the pathogenicity process.

At the same time Munro et al. (2003) demonstrated that the presence of the bacteriophage *V. harveyi myovirus* like (VHML) may confer virulence

to *V. harveyi* strain 642 result in up regulation of hemolysin, an up regulation of protein excretion and significant increase in mortality of larval *P. monodon*.

3.5 Diagnostic techniques

3.5.1 Laboratory diagnosis

Variation in clinical sign and symptom may lead to the misdiagnosis and misrecognition. The definite diagnosis remained on the cultivation to isolate and identify the organism (Liu et al., 1996a; Alvarez et al., 1998; Alcaide et al., 2001; Zorrilla et al., 2003). It was, however time-consuming and false negative could likely occur. The immunological methods for detection of either antibody or antigen were being developed for many instances gearing for early diagnosis of vibriosis. However, none was applied as routine use for *V. harveyi*. Recently, development of polymerase chain reaction (PCR) and sequencing of the 16S rDNA genes for detection of *V. harveyi* infection had been reported (Gauger et al., 2002; Zhang et al., 2001).

3.5.1.1 Gross observation

It was found vary with the type of infection.

3.5.1.1.1 Presumptive diagnosis-level 1 : luminescent larvae in the rearing tank.

3.5.1.1.2 Presumptive diagnosis-level 2 : black or brown cuticular lesions, opacity of musculature, tough and black branching filaments (up to 2 mm wide) in connective tissue of tail segments, black lymphoid organ, melaniztion of appendage tips.

3.5.1.1.3 Presumptive diagnosis-level 3 : affected larvae show heavy bacterial colonization on the cuticle of mouthparts and appendages, on the cuticular lining of oesophagus and component of the stomach. Rounding up and

sloughing of hepatopancratic tubule and midgut epithelial cells into their lumen (little white balls) are also commonly found. In the final stages of the infection, cuticular colonization with systemic infection are observed (Lightner, 1996).

3.5.1.2 Histology

In the histology, significantly necrosis and inflammation especially in the lymphoid organ and frequently, but usually less severe in the gill, heart, hepatopancreas and sometimes other tissues were found. Often there are rod shaped bacteria (usually slightly curved) within the tissues (Bower, 1997).

3.5.1.3 Wet mounts

Direct microscopic examination of hemolymph such provided a presumptive diagnosis when the characteristic of bipolar staining of Gram-negative curved rod was seen (Bower, 1997).

3.2.1.4 Cultivation method

Isolation *V. harveyi* from tissue or hemolymph of moribund samples was performed. The bacteria are Gram-negative rods, motile, oxidase positive, fermentative (O-F medium) and sensitive to the vibriostatic agent O/129. Positive reaction for lysine decarboxylase, growth at 0.5-8% NaCl. Negative reaction for arginine dihydrolase, Voges-Proskauer and utiliztion of D-glucosamine, L-arabinose and citrate variable. The bacteria are not fastidious and will grow on a wide variety of nutrient agar media including the selective media thiosulfate citrate bile salts agar (TCBS) (Lightner, 1998). Colonies of *V. harveyi* varied in morphological features depending on culture media but they often produced cream colored, occasionally translucent, raised and shiny after incubation for 24 hours. The colonial morphology of *V. harveyi* on TCBS was sufficiently characterized (green colonies) to allow presumptive identification (Liu et al., 1996a).

Generally, laboratories required 2-6 days to report the positive identification of this organism from cultured methods. A selective medium such as TCBS agar, eliminates most non-target bacteria in clinical samples but is not satisfactory for environmental samples because many bacteria present in natural water sources can produced colonies on TCBS agar whose appearance is similar to the appearance of *V. harveyi* colonies. Furthermore, the series of biochemical test commonly used to identify *V. harveyi* was originally designed for clinical samples in order to specifically detect pathogenic *Vibrios* (Choopun et al., 2002). Recently, API strips, identified by the database APILAB Plus (BioMérieux, France) had been introduced and replaced a conventional method for routine diagnosis (Alcaide et al., 2001). This strip was based on the biochemical detection and provided time for detection of *V. harveyi* about 2-4 days. However, the result of this strip is identified as *V. vulnificus*. Identification must be continued by testing additional biochemical characteristic as described by Biosca, Oliver and Amaro (1996). Then it was time delayed and false negative could likely occur. Even though these techniques are easily for bacterial detection, there are practical limitations for their widespread commercial application. These limitation include the need for highly trained personnel and the facilities required for analysis; these limitation are particularly significant for field studies involving large numbers of samples and assay costs are expensive for small sample numbers.

3.5.2 Immunological techniques

Immunodiagnosis was particularly useful in the situation when the foci of infection were unknown or inaccessible. Immunological techniques are widely used for diagnosis of many bacterial diseases in human medicine, food, agriculture and aquaculture. In addition to high sensitivity and specificity,

immunological method can be simplified to obtain results quickly at relatively low cost (Sithigorngul et al., 2002).

Antibodies (also known as immunoglobulin) are a class of protein molecules produced by B lymphocytes of the adaptive immune system which act as flexible adapters between the infectious agents and phagocytes (Roitt, Brostoff and Male, 1985). It is produced and appears in the serum when an animal encounters an antigen either by infection or by deliberate injection. The chemical and physio-chemical properties of these highly heterogeneous protein molecules, called polyclonal antibodies. However, their heterogeneity of size, structure, charge and biological activity has made isolation and purification a challenging problem for immunodiagnosis. Immunoglobulins are robust molecules and they can survive in a variety of environmental including heating to 56 ^{o}C and storage at room temperature for extended periods. They can maintain their antibody activity after treatment with high or low pH for shorts periods and even after being in contact with detergent or urea (Steward, 1984).

Antibodies as used in immunological test systems are powerful tools for the specific detection and identification of bacteria. Monoclonal antibodies (MAbs) are important reagents used in biomedical research, in diagnosis of diseases and in treatment of such diseases as infections and cancer. These antibodies are produced by cell line or clones obtained from animal that have been immunized with the substance that is the subject of study. The cell line are produced by fusing B cells from immunized animal with myeloma cell (Köhler and Milstein, 1975). To produce the desired MAb, the cells must be grown in either of two ways : by injection into the peritoneal cavity of a suitably prepared mouse (the *in vivo*, or mouse ascites method) or by *in vitro* tissue cultures.

The general principle of the antibody-producing hybridoma by tissue culture is shown in Figure 2.2 and consist of the fusion of a non-secreting myeloma tumor cell (with approximately 65 chromosomes) and an antibody-producing B-cell (with normal complement of 40 chromosomes). The fused cell retained the immortality of the myeloma cell line and also continue to secrete the antibody produced by the B cell. They posses fewer chromosomes than the sum of the two cells being fused, but have greater than twice the number of chromosomes in normal mouse cells. The fused cell (hybridoma) may then be maintained in tissue culture. This method could be utilized to produce monoclonal antibodies with specificity for any antigen (Steward, 1984).

3.5.2.1 Monoclonal antibodies production

The most monoclonal antibodies used in research, medicine and biotechnology are of mouse origin (Vetterlein, 1989). An outline of the experimental protocol for the production of monoclonal antibodies is shown in Figure 2.3

(I) Immunization

Primary injection of antigen emulsified in complete Freund's adjuvant is injected intraperitoneally (other injection site such as intramuscular hind legs and hind foot-pads are also commonly employed). Secondary injections are given using incomplete Freund's adjuvant at 2-3 week intervals until an antibody response is detected by assay for serum antibodies. To ensure an optimum antigenic response, a final intravenous injection of the antigen in microgram amounts is often given 2-3 days before spleen or lymphoid cells are removed (Steward, 1984).

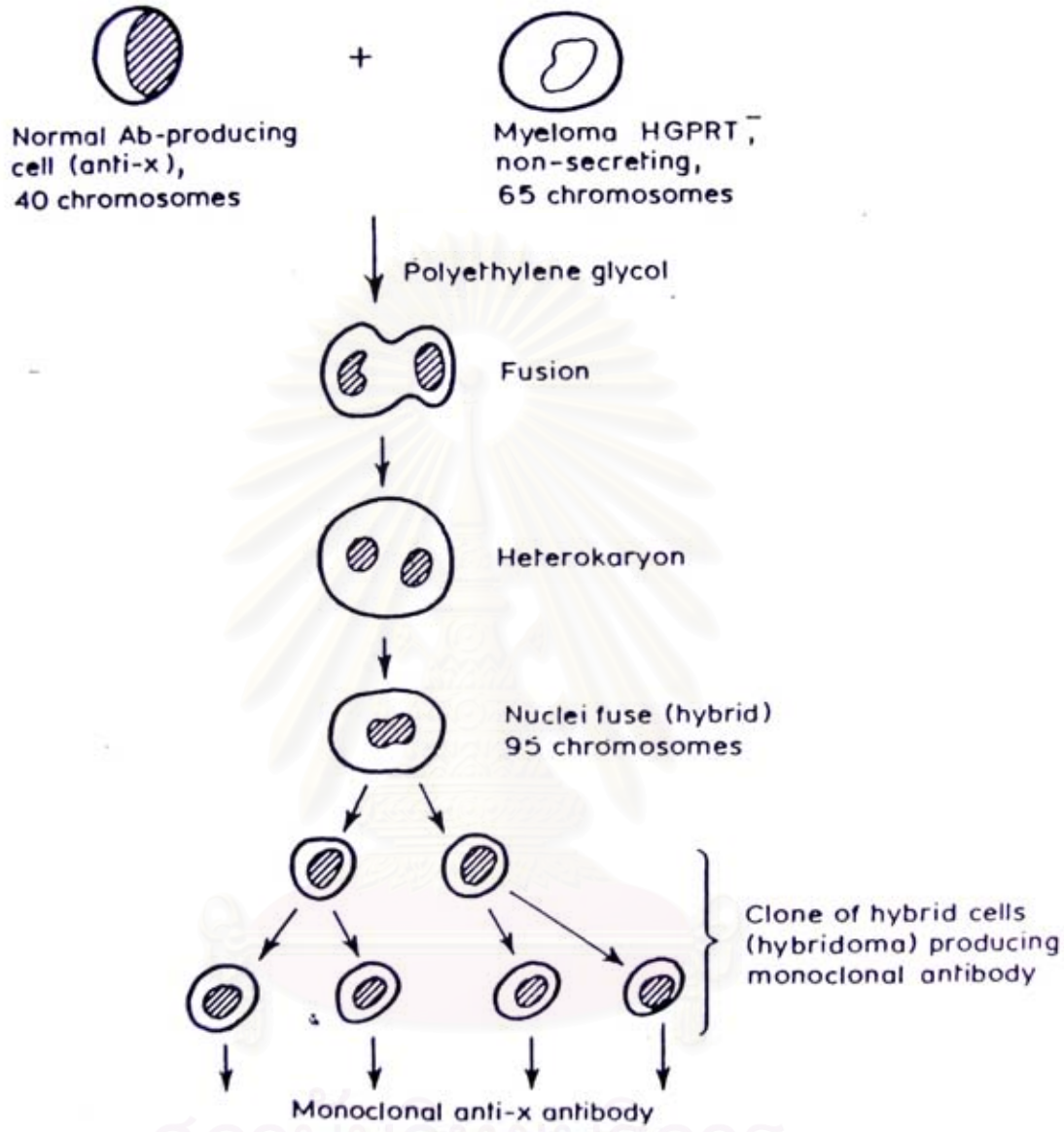

Figure 2.2 The principle of the production of an antibody-producing hybridoma (Steward, 1984).

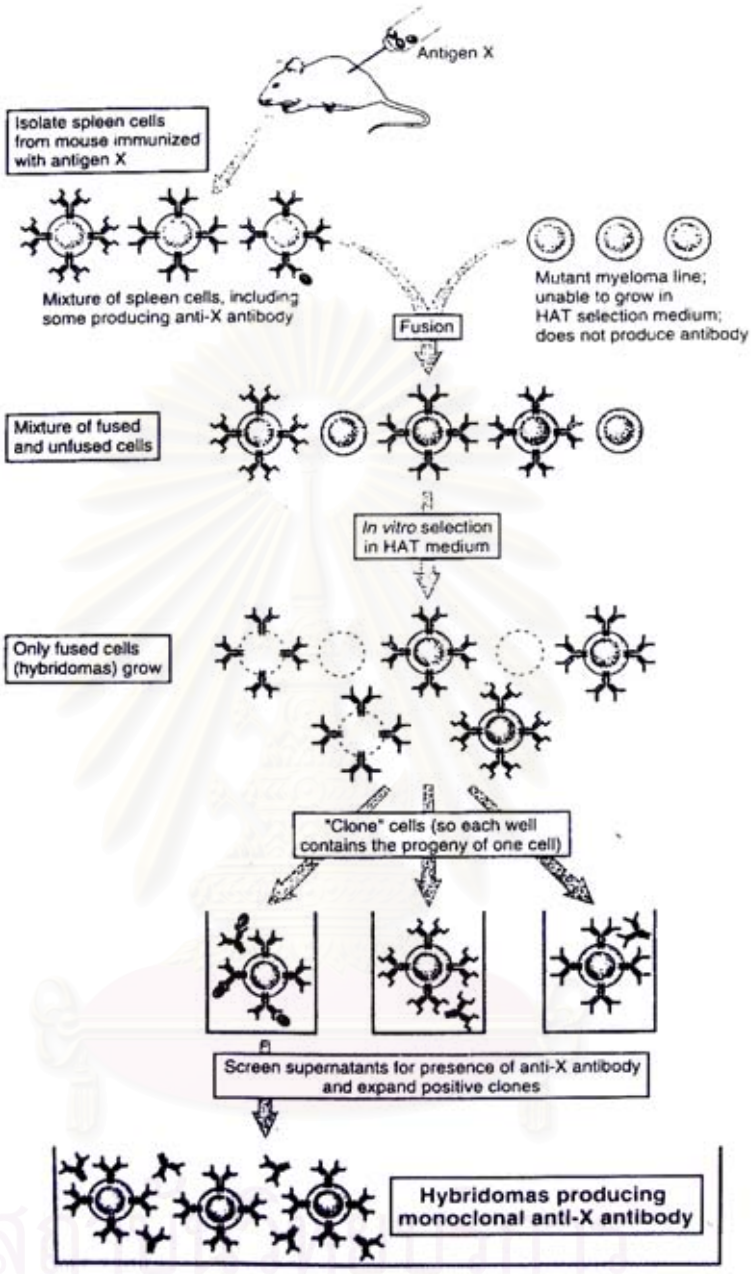

Figure 2.3 Monoclonal antibodies production by somatic hybridization method (Abbas, Lichtman and Pobger, 2000).

(II) Fusion with polyethylene glycol

Myeloma cells lacked the purine salvage enzyme hypoxanthine guanosine phophoribosyl transferase (HGPRT) are selected for the fusion. This selection is achieved by exploiting the fact that $HGPRT^-$ cells are able to grow in Hypoxanthine/ aminopterin/ thymidine (HAT) selective medium. The hybridization of these myeloma cells and the spleen cells (from animal appropriately immunize with the desired antigen) with high concentrations of polyethylene glycol (PEG), normally using 30% PEG. As many as several hundred antigen specific hybridoma cells can be obtained from a single spleen cell fusion. Normally, one hybridoma cell is expected for every 10^4 lymphocytes fused (Steward, 1984).

(III) Selection of hybridoma cells in HAT medium

The fused cells are cultured in a selective media (HAT medium). In this medium, the tumor cells are killed, the normal, non-fused cell die after a short period in culture and the hybridomas survive. The reason for the selective effect of the HAT medium will be briefly described and seen in Figure 2.4. Cells have two ways of producing nucleic acid : (a) *de novo* synthesis pathway and (b) the salvage pathway in which nucleotides from degraded nucleic acids are utilized by a process which requires the enzyme HGPRT. In the presence of aminopterin, *de novo* synthesis is blocked but normal cell can survive by using the salvage pathway and the nucleotides in the HAT medium. However, the $HGPRT^-$ tumor cells die in the presence of aminopterin because they can use neither *de novo* synthesis nor the salvage pathway. The hybridoma cells survive because they have HGPRT derived from genes received from the normal spleen cell parents (Steward, 1984).

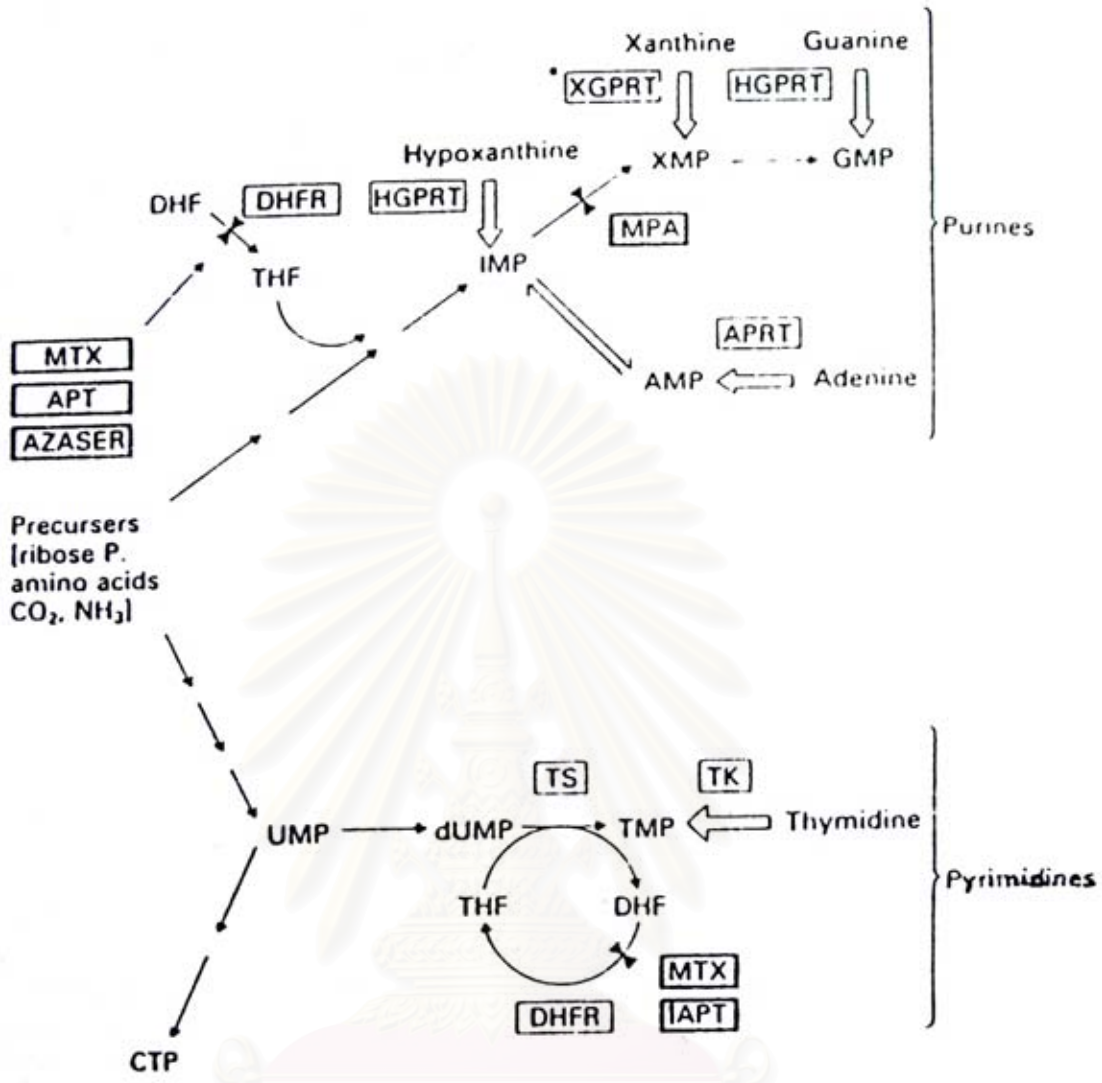

Figure 2.4 Nucleotide synthesis : *De novo* (single) and salvage (open single) pathway; HGPRT = hypoxanthine guanine phosphoribosyl transferase, TK = thymidine kinase, DHFR = dihydrofolate reductase, TS = thymidine synthase. Inhibitor : MTX = methotrexate, APT = aminopterin, AZASER = azaserine, MPA = mycophenolic acid, THF = tetrahydrofolate and DHF = dihydrofolate (Kingsman and Kingsman, 1988).

(IV) Screening of hybridoma

The growing hybridoma are screened for their productions of antibody by appropriate sensitive assay eg. Enzyme-linked immuno sorbent assay (ELISA), Radioimmunoassay (RIA), Dot-blot (DB), Western blot (WB) and Immunohistochemistry (IHC) and the antibody producing hybridoma are cloned (Steward, 1984).

(V) Reclone positive clones

Two widely methods are used to achieve this; cloning in soft agar and cloning by limiting dilution (Steward, 1984).

(a) Cloning in soft agar

High dilutions of the cells are made in agar and appropriate colonies are picked out from the soft agar and grown in culture.

(b) Cloning by limiting dilution

High dilution of the cells are made so that individual cells can be transferred to tissue culture medium in microtitre plates and grown. Wells containing one colony are then grown in culture. Hybridoma can be grown in tissue culture and up to 10 μg/ml of specific antibody may be obtained.

3.5.2.2 Monoclonal antibody structure and diversity

The mouse B cell repertoire is capable of producing over 10 million different antibody molecules with dissociation constants for specific antigen ranging from 10^{-5} to 10^{-10} mol/l (Vetterlein, 1989). Figure 2.5 illustrates the basis structure of a mouse IgG molecule.

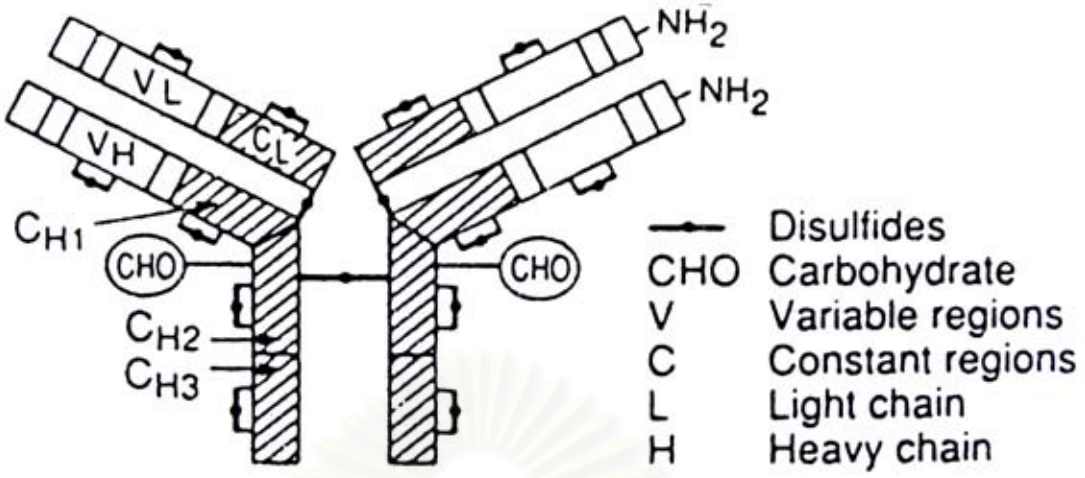

Figure 2.5 The basis structure of a mouse IgG molecule (Vetterlein, 1989).

Most monoclonal antibodies currently employed in biotechnology are of murine origin usually mouse IgG antibodies. The diverse binding specificity of an IgG molecule is a consequence of the unique structure of the IgG molecule and the amino acid contained within two identical binding pockets at the N-terminal end. An IgG molecule is symmetrical and consists of one pair of heavy (H) 55 kDa polypeptides chains and one pair of light (L) 25 kDa polypeptides chains linked by interchain disulfide bonds. The H and L chains are woven into a tight barrel-like structure formed by repeating β-pleated sheets that juxtapose variable and hypervariable stretches of amino acids and fold to form two identical antigen combining sites (Amit et al., 1985).

3.5.2.3 Advantages of monoclonal antibodies (Vetterlein, 1989)

(a) Each cloned B cell produces only one type of antibody of predefined specificity.

(b) Impure antigens can be used to generate monoclonal antibodies.

(c) MAbs can be generated against rare and weakly immunogenic antigen.

(d) MAbs have a predefined isotype and thus variable effector functions.

(e) Reproducible antibody can be made in unlimited amounts.

3.5.2.4 Monoclonal antibodies specific to bacteria

There are many monoclonal antibodies that produced against other pathogenic bacteria in both Gram +ve and Gram –ve bacteria as summarized in Table 2.2.

Table 2.2 Specificities of monoclonal antibodies (MAbs) against pathogenic bacteria reported previously.

Bacteria ; antigenic specificity	MAbs	Isotype	MW (kDa)	Method of characterization	Reference
Bacillus cereus ; vegetative cells	8D3 9B7	IgG_1 IgM	22 58,62	ELISA, WB	Charni et al., 2000
Bacillus cereus T; spore (exosporium)	B48 B183	IgG_2b IgG_1	76 250	WB	Quinlan and Foegeding, 1997
Clostridium sporogenes PA3679; spore	C33 C225	IgM IgM	ND ND	IHC	Quinlan and Foegeding, 1997
Escherichia coli O26 ; Lipopolysacchride LPS (O-Ag)	12F5	IgM	Ladder pattern of LPS	ELISA, WB	Rivera-Betancourt and Keen, 2000
Escherichia coli O111 ; LPS (O-Ag)	15C4	IgG_3	Ladder pattern of LPS	ELISA, WB	Rivera-Betancourt and Keen, 2000
Escherichia coli ; H7 flagellar Ag	2B7 46E9	ND ND	65 63,65	ELISA, WB	He et al., 1996

ND= non determined, IHC= Immunohistochemistry,

WB= Western blot, ELISA= Enzyme linked immunosorbent assay

Table 2.2 (Cont.1)

Bacteria ; antigenic specificity	MAbs	Isotype	MW (kDa)	Method of characterization	Reference
Campylobacter jejuni ; whole cells	M337 M316 M637	ND ND ND	62 92 31	ELISA, WB	Lu et al., 1997
Photobacterium damselae ssp. *piscicida* ; whole cells	F2B1 1E4 13B10 4D4 F3G12	IgG_2a IgG_2b IgG_1 IgG_2b IgG_2a	22 22 18 Rf Rf	ELISA, WB, IHC	Jung et al., 2001
Comamonas acidovorans PX54 ; whole cells	I4B1 I4C6 I3A1	ND ND ND	Double band of LPS	ELISA, WB, IFA	Faude and Höfle, 1997
Comamonas acidovorans PX62 ; whole cells	I1B2 V3C3	ND ND	ND ND	ELISA, IFA	Faude and Höfle, 1997

ND= non determined, IHC= Immunohistochemistry, IFA= Immunfluorescence assay, WB= Western blot, ELISA= Enzyme linked immunosorbent assay, Rf = Running front

Table 2.2 (Cont.2)

Bacteria ; antigenic specificity	MAbs	Isotype	MW (kDa)	Method of characterization	Reference
Aeromonas hydrophila PU7718; whole cells	III4G8	ND	Ladder pattern of LPS	ELISA, WB, IFA	Faude and Höfle, 1997
Vibrio cholerae non-O1; heat stable enterotoxin	F2	IgG_1	ND	ELISA, amino acid sequence	Takeda et al., 1990
Vibrio cholerae non-O26; Non-membrane-damaging cytotoxin	4B12	IgG_2b	35	ELISA, WB	Saha and Nair, 1997
Vibrio spp. ; flagella (H-Ag)	6 MAbs	ND	52	ELISA, WB	Chen et al., 1992
Vibrio harveyi Strain 642 ; exotoxin subunits	3.3H, 4.8B, 4.9F, 8.10F	ND ND ND ND	58,48, 47,46, 45,39, 21	ELISA, WB	Munro et al., 2003

ND= non determined, IHC= Immunohistochemistry, IFA= Immunfluorescence assay, WB= Western blot, ELISA= Enzyme linked immunosorbent assay, Rf = Running front

CHAPTER III

PATHOGENICITY OF *Vibrio harveyi* CAUSES DISEASE IN PENAEID SHRIMPS

INTRODUCTION

V. harveyi is a Gram-negative, luminous bacterium which is widely distributed in the marine environment. Until now, this organism has been reported as a primary pathogen of cultured penaeid shrimp in the world (Alcaide et al., 2001; Alvarez et al., 1998). However, little is known about the pathogenic mechanisms of *V. harveyi*. Liu et al. (1996a) proposed that proteases, phospholipases, or hemolysins might be important for pathogenically. Cysteine protease has been reported as the major exotoxin to penaeid shrimp (Liu, Lee, Tu et al., 1997). Montero and Austin (1999) suggested that the lipopolysaccharide might constitute the lethal toxin of *V. harveyi* E2 to penaeid shrimp. Zhang et al. (2000) found that the hemolysin activity in the extracellular product was involved in pathogenesis in salmonids. *V. harveyi* VIB 645, which was the most pathogenic isolate, produced extracellular product with the highest titer of hemolytic activity toward Atlantic salmon and rainbow trout erythrocytes. In this study, the pathogenicity of luminous *V. harveyi* in diseased penaeid shrimp was reported.

MATERIALS AND METHODS

Bacterial preparation

Vibrio harveyi strain 639, 1526, VG and VG1 were obtained from CENTEX shrimp, Thailand and stored at –70 ^{O}C in 15% (w/v) glycerol before use. *V. harveyi* were placed in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl and incubated at 30 ^{O}C for 24 h. Pure culture was obtained from all samples. The reisolated strains were Gram-negative rods, motile, oxidase and catalase positive. The isolates were first characterized by API 20 E strips (07584C01-04/2000) and API 50 CHE strips (07945E01-04/2000) (BioMérieux, S.A. France), and identified by the database APILAB Plus (BioMérieux) supplied by the manufacturer.

Pathogenicity

Enzymatic and hemolytic activities of *V. harveyi*

Substrate: 1% sodium caseinate, 0.5% gelatin, 0.2% egg yolk, 1% Tween 80, 0.3% chitin, 2% sheep erythrocytes and 2% hemolymph shrimp and crab were mixed in tryptic soy agar (TSA) supplemented with 2% (w/v) NaCl. Bacteria were spotted onto the center of media, incubated at 37 ^{O}C for 24 h. The diameter of the lytic halo around each well were measured.

Tested shrimp

One-hundred *Penaeus monodon*, PL 15 were maintained in fibreglass tanks supplied with flow-through sea water (20 ppt) at a room temperature. Shrimp were challenged with each strain of *V. harveyi*, at ~ 10^{7} CFU ml^{-1} (Chang et al, 2000) by immersion. Cultured water were monitered by checking pH, temperature, salinity, dissolved oxygen, ammonium, nitrite, nitrate and phosphate (Test kits from Merck, Germany). Infected shrimp with the signs of feeding cessation, slow movement,

luminescence and moribund were recorded. Also, the number of alive shrimp were daily recorded. *V. harveyi* were re-isolated from luminescent shrimp dissected internal organs and reconfirmed the type of strain by biochemical test then collected for future use.

RESULTS

The re-isolated strains are Gram-negative rods, motile, oxidase and catalase positive (Table 3.1) and are able to bioluminescence on agar plate (Figure 3.1). The isolates were firstly tested by API 20 E strips (07584C01-04/2000) (Table 3.2) and API 50CHE strips (07945E01-04/2000) (data did not shown) (BioMérieux, S.A. France), and identified by the database APILAB Plus (BioMérieux) supplied by the manufacturer.

The level of enzymatic activity showed that *V. harveyi* 639 can produce enzymatic and hemolytic activity stronger than those of *V. harveyi* 1526, VG and VG1, respectively (Table 3.3).

P. monodon were challenged by various strains of *V. harveyi* ~ 10^7 CFU ml^{-1}, with water qualities of pH ~ 7.5-8, temperature ~ 25 ^oC, salinity 25 ppt, dissolved oxygen ~ 5 mg/l, ammonium ~ 0.5 mg/l, nitrite ~ 0.5 mg/l, nitrate ~ 0.5 mg/l and phosphate ~ 0.5 mg/l. Time after shrimp were exposed to *V. harveyi* 639, 1526, VG and VG1, and caused 50% mortality were 39, 43, 49 and 50 h, respectively (Figure 3.2).

Table 3.1 Characteristics of different strain of *V. harveyi* 639, 1526, VG and VG1 by conventional tests.

Characteristics	*V. harveyi* 639	*V. harveyi* 1526	*V. harveyi* VG	*V. harveyi* VG1
Colony	Small cream, translucent	Small cream, translucent	Small cream, translucent	Small cream, translucent
Gram's stain and shape	-ve, curved rod	-ve, curved rod	-ve, curved rod	-ve, curved rod
Swarming on TSA+2%NaCl	-	-	-	-
Colony color on TCBS	G	G	G	G
Growth at ^{o}C	22-40	22-40	22-40	22-40
Range of %NaCl (w/v) for growth	0.5-7	0.5-7	0.5-7	0.5-7
pH	7-10	7-10	7-10	7-10
Catalase activity	+	+	+	+
Sensitivity to O/129 (150 µg)	S	S	S	S
Luminescence	+	+	+	+

+ = positive, - = negative, G = green, S = sensitivity to the test

Table 3.2 Biochemical profiles of *V. harveyi* 639, 1526, VG and VG1 by API 20 E (BioMérieux, France)

Biochemical test (API 20 E)	*V. harveyi* 639	*V. harveyi* 1526	*V. harveyi* VG	*V. harveyi* VG1
β-galactosidase(ONPG)	+	+	-	-
Arginine dihydrolase(ADH)	-	-	-	-
Lysine decarboxylase (LDC)	+	+	+	+
Ornithine decarboxylase (ODC)	-	-	-	-
Citrate utilization	-	-	-	-
H_2S production (H_2S)	-	-	-	-
Urease (URE)	-	-	-	-
Trytophane deaminase (TDA)	-	-	-	-
Indole production (IND)	+	+	+	+
Acetoin production (VP)	-	-	-	-
Gelatinase (GEL)	+	+	+	+
Fermentation/Oxidation				
Glucose (GLU)	+	+	+	+
Mannitol (MAN)	+	+	+	+
Inositol (INO)	-	-	-	-
Sorbitol (SOR)	-	-	-	-
Rhamnose (RHA)	-	-	-	-
Sucrose (SAC)	-	-	-	-
Melibiose (MEL)	-	-	-	-
Amygdalin (AMY)	+	+	+	+
Arabinose (ARA)	-	-	-	-
Cytochrome-oxidase (Ox)	+	+	+	+
NO_2 production (NO_2)	+	+	-	+
Reduction to N_2 gas (N_2)	-	-	-	-
Motility on API medium	+	+	+	+
MacConkey medium (McC)	+	+	+	+
Fermentation: under mineral oil (OF-F)	+	+	+	+
Oxidation: exposed to the air (OF-O)	+	+	+	+

+ = positive, - = negative

Table 3.3 Hemolysis and pathogenic characteristics exhibited by various *V. harveyi* isolates.

V. harveyi isolates	Protease against		Phospho-lipase against egg yolk	Lipase against tween 80	Chitinase against chitin	Hemolysis against hemolymph from		
	Casein	Gelatin				sheep	shrimp	crab
639	++	+++	+	+++	+++	++++	++	+
1526	++	++	+	+++	+	++++	+	+
VG	+	++	+	++	+	+++	+	+
VG1	+	++	+	++	+	+++	+	+

Ratio of hydrolysis halo to colony diameter (mm):

+ < 1.0 mm, ++ < 1.5 mm, +++ < 2.0 mm, ++++ < 2.5 mm

Figure 3.1 Luminous bacteria, *V. harveyi* strain 639, grown on tryptic soy agar supplemented with 2% NaCl (w/v).

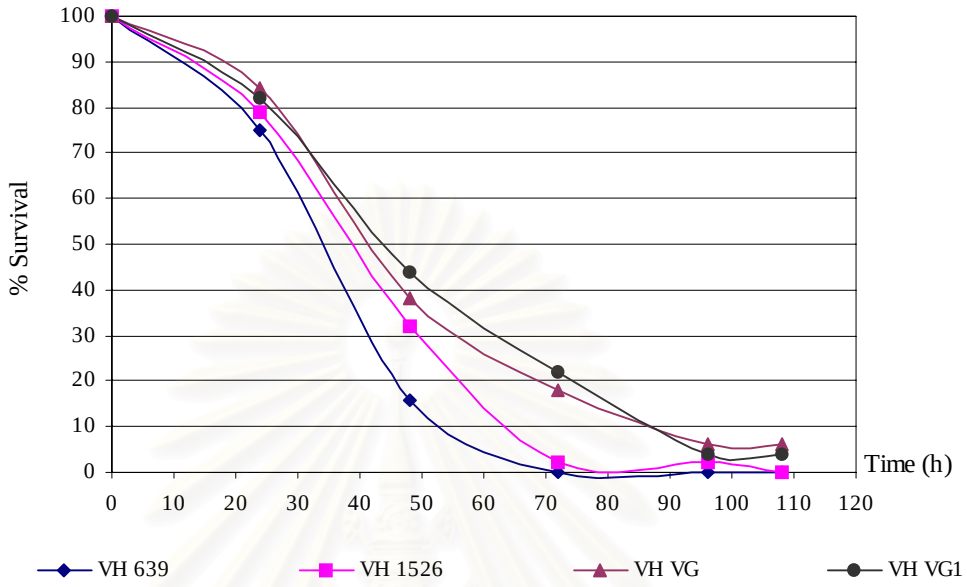

Figure 3.2 Survival (%) of *P. monodon* at various time (h) after being infected by immersion with *V. harveyi* at concentration of ~ 10^7 CFU ml^{-1}; *V. harveyi* 639 (VH 639), *V. harveyi* 1526 (VH 1526), *V. harveyi* VG (VH VG) and *V. harveyi* VG1 (VH VG1).

DISCUSSION

The re-isolated strains were Gram-negative rods, motile, oxidase and catalase positive (Table 3.1) and showed bioluminescence on agar plate (Figure 3.1). The isolates were firstly tested by API 20 E strips (07584C01-04/2000) (Table 3.2) and API 50 CHE strips (07945E01-04/2000) (data did not shown) (BioMérieux, S.A. France), and identified by the database APILAB Plus (BioMérieux) supplied by the manufacturer as *V. vulnificus*, with a probability of 99.4%. Further identification was achieved by luminescence in shrimp. *V. harveyi* 639 showed luminescence on media as shown in Figure 3.1. In shrimp experiment, they were previously misidentified as *V. vulnificus*. Generally, *V. vulnificus* will not show luminescence on agar. Alcaide et al. (2001) isolated that *V. harveyi* from seahorse, *Hippocampus* sp., and identified by API 20 E strips as *V. vulnificus* with a probability of 95.1%. The identification was confirmed by using colony hybridization, and it was found that this isolate was *V. harveyi* with homology of 99% identification. Thus, *V. vulnificus* identified by API strip test should be reidentified by additional biochemical characteristic testing or using immunological technique. In this study, identification of *V. harveyi* was continued by using biochemical and characteristic tests as mentioned by Baumann et al. (1984). These bacteria failed to utilize inositol, sorbitol, rhamnose, sucrose, melibiose and arabinose. They were all lysine decarbozylase, ornithine decarboxylase and gelatinase positive and sensitive to the vibriostatic O/129. From these results, all *Vibrio* spp. designated as *V. harveyi* from CENTEX shrimp, Thailand were confirmedly identified as *V. harveyi* and resembled the results reported by Liu, Lee, Yii et al. (1996b).

The level of enzymatic activity showed that *V. harveyi* 639 could produce protease, lipase, chitinase and hemolytic activity stronger than those of *V. harveyi* 1526, VG and VG1, respectively (Table 3.3). Extracellular virulence factors such as proteases, phospholipases, or hemolysins have been suggested to play important roles for pathogenicity especially cysteine protease has been reported as the major exotoxin to penaeid shrimp (Liu et al., 1996a; Liu, Lee, Tu et al., 1997). Zhang et al. (2001) reported that *V. harveyi* VIB 645 is very pathogenic towards salmonids and produces extracellular product with high titer of hemolytic activity towards fish erythrocytes.

Time after shrimp being immersed into the conditions with the ~ 10^7 CFU ml^{-1} of *V. harveyi* 639, 1526, VG and VG1, 50% mortality in shrimp were 39, 43, 49 and 50 h, respectively (Figure 3.2). We can conclude that LD_{50} of *V. harveyi* 639 at ~ 10^7 CFU ml^{-1} causing 50% mortality of shrimp within 39 h was the most virulent strain among all *Vibrio harveyi* obtained. However, its virulence was lower than that of *V. harveyi* reported previously by Lavilla-Pitogo et al. (1990). Since only 10^3 CFU ml^{-1} of *V. harveyi* was used to challenge *P. monodon* larvae and caused 100% mortality within 48 h. Chang et al. (2000) suggested that the pathogenicity of bacteria isolated from prawn hepatopancreaes was correlated with lysis of prawn hemocytes. Thus, the ability of producing extracellular products of the highest enzymatic and hemolytic activities to penaeid shrimp might be the factors of being the most pathogenicity found in *V. harveyi* 639 and could cause shrimp death in the shorter time as compared to all *V. harveyi* tested in this study.

CHAPTER IV

PRODUCTION OF MONOCLONAL ANTIBODIES FOR DETECTION OF *Vibrio harveyi* 639

INTRODUCTION

Vibrio harveyi has been identified as a cause of luminescent disease in black tiger shrimp hatcheries or farms in Thailand (Jiravanichpaisal and Miyazaki 1994). Therefore, development of reagents and methods for detection of *V. harveyi* in shrimp and rearing water have been the focus of recent research. Even though several methods have been developed to isolate, detect and control *V. harveyi* (Liu, Lee, Yii et al., 1996b; Jory, 1998; Moriarty, 1998; Rengpipat et al., 1998; Alvarez et al., 1998; Kiriratnikom et al., 2000; Alcaide et al., 2001; Zorrilla et al., 2003), there is currently no simple, rapid and accurate technique suitable for routine use by microbiologists or shrimp farmers. A major limiting factor has been the lack of high-quality immunodiagnostic reagents.

The use of MAbs to detect other bacteria is well-known (Okrend et al., 1990; Adam et al., 1995; He et al., 1996; Jaradat and Zawistowski, 1996; Saha and Nair, 1997; Charni et al., 2000; Rivera-Betancourt and Keen 2000; Jung et al., 2001). Munro et al. (2003) produced monoclonal antibodies specific to an exotoxin from *V. harveyi* strain 642. However, the assay (Western blot analysis) was not highly sensitive and could not be easily applied during field examinations. Polyclonal antibodies against the *V. harveyi* antigens were produced by Lee, Liu et al. (1997b), but cross-reactivity was observed amongst species. Therefore, the production of MAbs against unique antigens or epitopes of *V. harveyi* would be a logical first step

development of specific immunodiagnostic tests. MAb could be produced in an unlimited supply through cell culture techniques (He et al., 1996; Quinlan and Foegeding, 1997).

In the present study, MAbs specific to *V. harveyi* were produced and used to examine antigenic differences among various strains and to locate *V. harveyi* by immunohistochemistry in tissue sections of infected *Penaeus monodon*.

MATERIALS AND METHODS

Antigen preparation and immunization

Bacterial preparation

Vibrio harveyi and other bacteria in this study (Table 4.1) were stored at –70 ^{O}C in 15% glycerol. For revival, stocks of *V. harveyi*, other *Vibrio* isolates and *Photobacterium damselae* were placed in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl. Other bacterial isolates were stored in TSB and incubated at 30 ^{O}C for 24 h. The bacteria were harvested at 3000xg for 20 min at 4 ^{O}C by centrifugation and the pellets were resuspended in sterile 0.15M phosphate buffered saline (PBS) pH 7.2. Absorbance of the suspension at 660 nm was adjusted to 1.0 using PBS. This was equivalent to approximately $2x10^{9}$ CFU ml^{-1} (Lee, Liu et al., 1997b), as determined from a standard curve of bacterial concentration on TSA with 2% NaCl. *Vibrio harveyi* 639 was originally isolated from Thai *Penaeus monodon* farm showing gross signs of luminous vibriosis and was provided as a stock culture from Centex Shrimp, Chalermprakiat Bldg., Faculty of Science, Mahidol University, Bangkok, Thailand.

Tested shrimp

Twenty *Penaeus monodon* PL 30 (30-day post-larvae) were maintained in fiberglass tanks supplied with flow-through sea water (20 ppt) at room temperature. They were injected (50 µl $shrimp^{-1}$) with a suspension of *V. harveyi* 639 at approximately 10^{9} CFU ml^{-1} through the arthrodial membrane of the second walking leg. When the injected shrimp showed signs of feeding cessation, slow movement or death, an organ was aseptically removed for isolation of *V. harveyi* 639. Cephalothoraces from moribund shrimp were also collected and fixed in Davidson's

fixative (Lightner, 1996) in preparation for immunohistochemical studies. Cephalothoraces from non-injected shrimp were prepared in the same manner for the negative control.

Antigen preparation

Vibrio harveyi 639, isolated from *Penaeus monodon* infected with luminous vibriosis from a shrimp farm in Thailand (provided by CENTEX shrimp, Thailand), was cultured as described above. The bacteria were heat killed at 60 ^{O}C for 60 min then cooled to 4 ^{O}C. The pellet was resuspended in an equal volume of PBS. Distilled water and SDS-mercaptoethanol were then added to the bacterial preparation at the ratio of 1:1 (v/v). Both heat killed and SDS-mercaptoethanol treated bacteria were used for immunization.

Immunization

Four Swiss mice were injected intraperitoneally with heat killed and SDS treated *V. harveyi* 639 (50 µl of 10^9 CFU ml^{-1}) mixed with complete Freund's adjuvant in a 1:1 ratio. Mice were subsequently injected 3 more times with the treated bacteria mixed with incomplete Freund's adjuvant at 2 weeks intervals. One week after the fourth injection, mouse antisera were collected and tested against heat killed and SDS treated *V. harveyi* by dot blot assays and Western blot. The best performing mouse was later boosted 3 days before hybridoma production.

Hybridoma production

A cell fusion procedure was adapted from the method developed by Köhler and Milstein (1975; 1976) with modifications described by Mosmann, Bauman and Williamson (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 40 microculture plates (96

wells per plate). After identification of positive cultures by screening methods described in the next section, cells were cloned by the limiting dilution method (Eshhar, 1985), kept in 12% DMSO and stored in liquid nitrogen.

Hybridoma screening

Primary screening by Dot blotting

V. harveyi 639 (~10^9 CFU ml^{-1}) in both heat killed and SDS-mercaptoethanol treated forms were used for screening. *V. harveyi* (1 µl $spot^{-1}$) samples were applied to a nitrocellulose membrane and subsequently baked at 60 ^{O}C for 30 min and then incubated in hybridoma conditioned media from each clone (1:20 dilution in 5% Blotto) for 3 h. After extensive washing in 0.5% Blotto, the membrane was incubated in horseradish peroxidase labelled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; BIO-RAD) antibody at 1:1500 dilution for 3 h. The membrane was then washed for 4 times in Blotto with 10 min intervals and incubated in a substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000). The intensity of the reaction with each MAb was compared by eye and scored relative to the reaction against *V. harveyi* 639. Hybridoma clones that displayed immunoreactivity against *V. harveyi*, but not against other bacteria, were confirmed for bacterial specificity by Western blot and immunohistochemistry before cloning and cryopreservation for further investigation.

Secondary screening by Western blotting

V. harveyi and other bacterial proteins were seperated by 15% SDS-PAGE according to the method described by Laemmli (1970). Bacterial samples were electrophoresed for 6 h at 30 V and gels were stained using Coomassie brilliant blue

R-250. For Western blotting, the samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a Transblot apparatus (BIO-RAD). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 hybridoma conditioned media for 3 h and then processed as described above in the 'Dot-blotting' section. Low molecular standard markers (BIO-RAD) were used as standards. The reactivity of the MAbs with different isolates of *V. harveyi* and other bacterial species were tested by dot-blotting and by Western blot analysis.

Monoclonal antibodies characterization

Dot blotting

Bacteria (~10^9 CFU ml^{-1}) (See in Table 4.1) in heat killed forms were used for screening. *V. harveyi* (1 µl $spot^{-1}$) samples were applied to a nitrocellulose membrane and subsequently baked at 60 ^{O}C for 30 min and then incubated in MAbs (1:200 dilution in 5% Blotto) for 3 h After extensive washing in 0.5% Blotto, the membrane was incubated in horseradish peroxidase labelled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; BIO-RAD) antibody at 1:1500 dilution for 3 h. The membrane was then washed for 4 times in Blotto with 10 min intervals and incubated in a substrate mixture containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS (Sithigorngul et al., 2000). The intensity of the reaction with each MAb was compared by eye and scored relative to the reaction against *V. harveyi* 639.

SDS-PAGE

a) Sample preparation

V. harveyi 639, 1526 and *Aeromonas hydrophila* were denatured and solubilized with 10% SDS and the cell debris was removed by centrifugation at 1,500 g for 5 min. The resultant supernatant was kept at -20°C until use

b) Electrophoresis

Bacteria were separated by 15% SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed at 100 V for 3 h and part of the gel was stained using Coomassie brilliant blue R-250.

Western blot analysis

For Western blot analysis, the samples separated by SDS-PAGE were electroblotted at 50 V for 3 h onto a nitrocellulose membrane using Transblot apparatus (BIO-RAD). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, then treated with MAb supernatant dilute 1:200 for 3 h. After extensive washing in 0.5% Blotto, the membrane was incubated in GAM-HRP (1:1500) for 3 h. The membrane was then washed for 4 times in Blotto with 10 min intervals and incubated in a substrate mixture containing 0.03% DAB, 0.006% H_2O_2, 0.05% $CoCl_2$ in PBS.

Standard proteins ranging from 6.5 to 205 kDa (BIO-RAD) were used as molecular weight markers, and prestained standard molecular weight markers from 31.4 to 126 kDa (BIO-RAD) were also used in blotting experiments.

Immunohistochemistry

Cephalothoraces of uninfected shrimps and *V. harveyi* 639 infected shrimps were cut and fixed in Davidson's fixative solution for 24 h then processed for paraffin sectioning. Serial section (8 µm thickness) were prepared and permeabilized with 1% Triton X-100 in PBS for 30 min at 37 °C and were washed three times in PBS with 5 min intervals, then blocked with $P1^{+}$(10% fetal bovine serum in PBS with 0.1% merthiolate) and stored in a moist chamber at 4°C until use.

The section were processed for indirect immunoperoxidase staining using various MAbs 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% H_2O_2 in PBS. Preparations were counter-stained with hematoxylin and eosin Y (H&E), dehydrated in a graded ethanol series, cleared in xylene and mounted in permount (Sithigorngul et al. 2000; 2002). Positive reaction was visualized as brown coloration against the pink and purple of H&E.

Isotype and subisotype determination

Isotype and subisotype of mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

Sensitivity of MAbs for *Vibrio harveyi* detection by dot blot

Serial dilution of *Vibrio harveyi* 639 ($5X10^3$ – $1X10^6$ CFU ml^{-1}) in PBS was performed and 1 µl of each dilution was spotted onto nitrocellulose membrane then fixed in 10% formalin for 30 min and processed for dot blot using various MAbs as described above. The last dilution revealed by each MAb that showed distinct and clear immunoreactivity was determined.

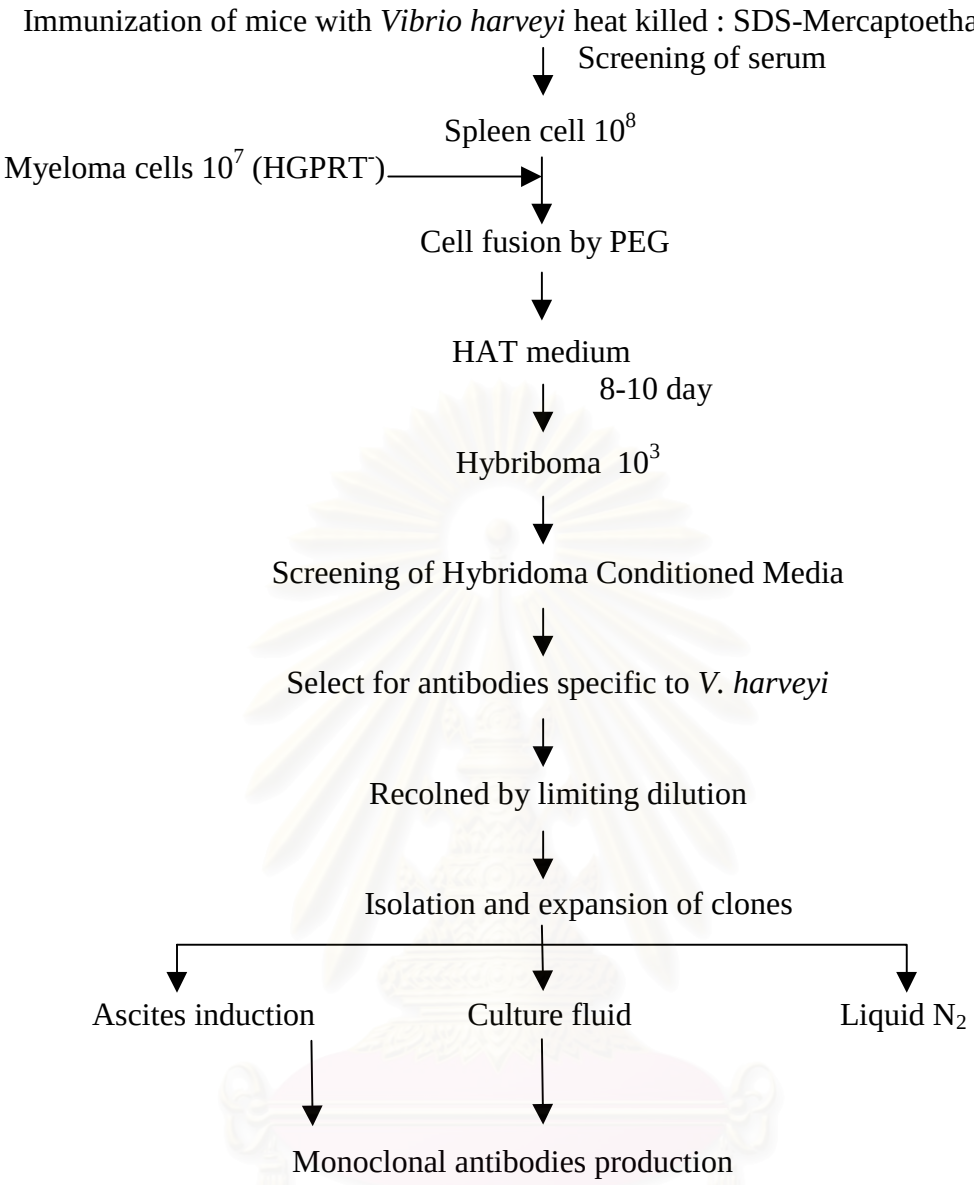

Figure 4.1 Diagram of hybridoma production (Sithigorngul et al., 2000).

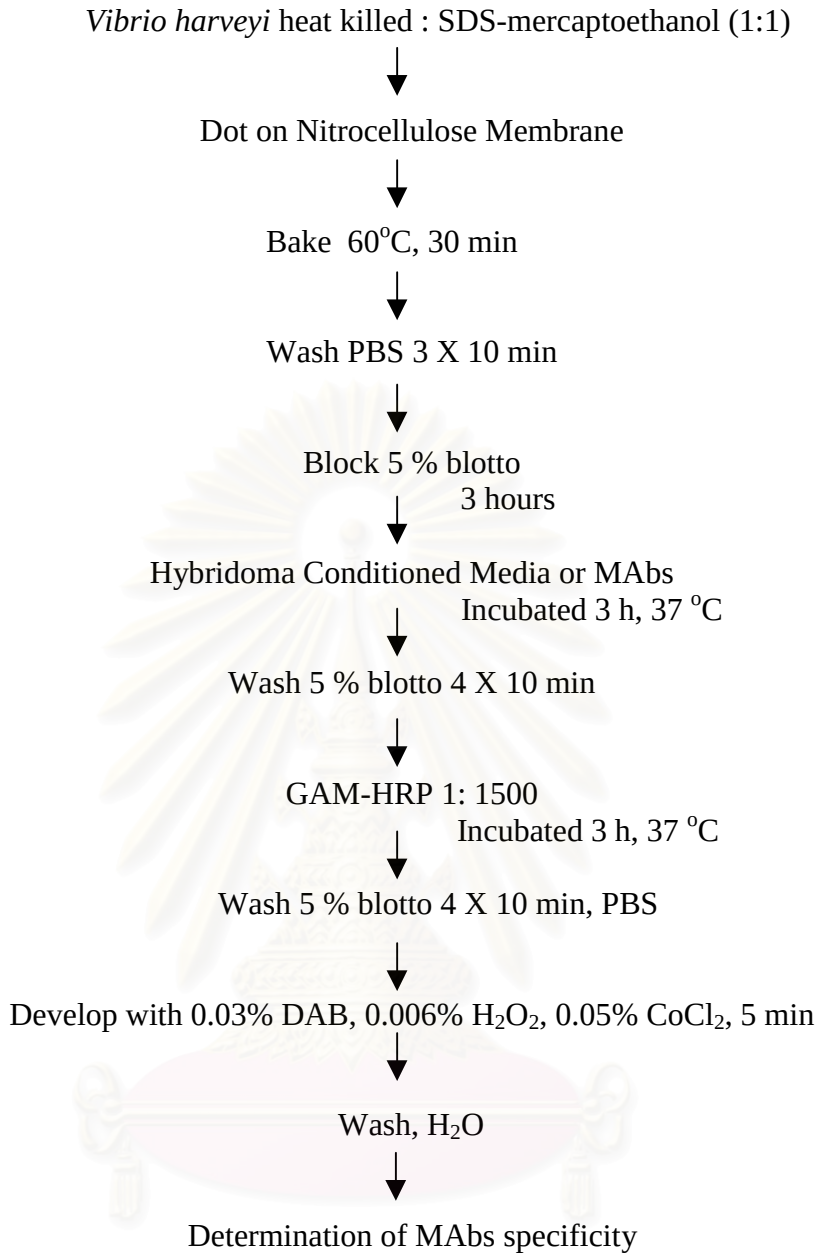

Figure 4.2 Diagram of dot blotting for screening method and characterization of monoclonal antibodies (Sithigorngul et al., 2000).

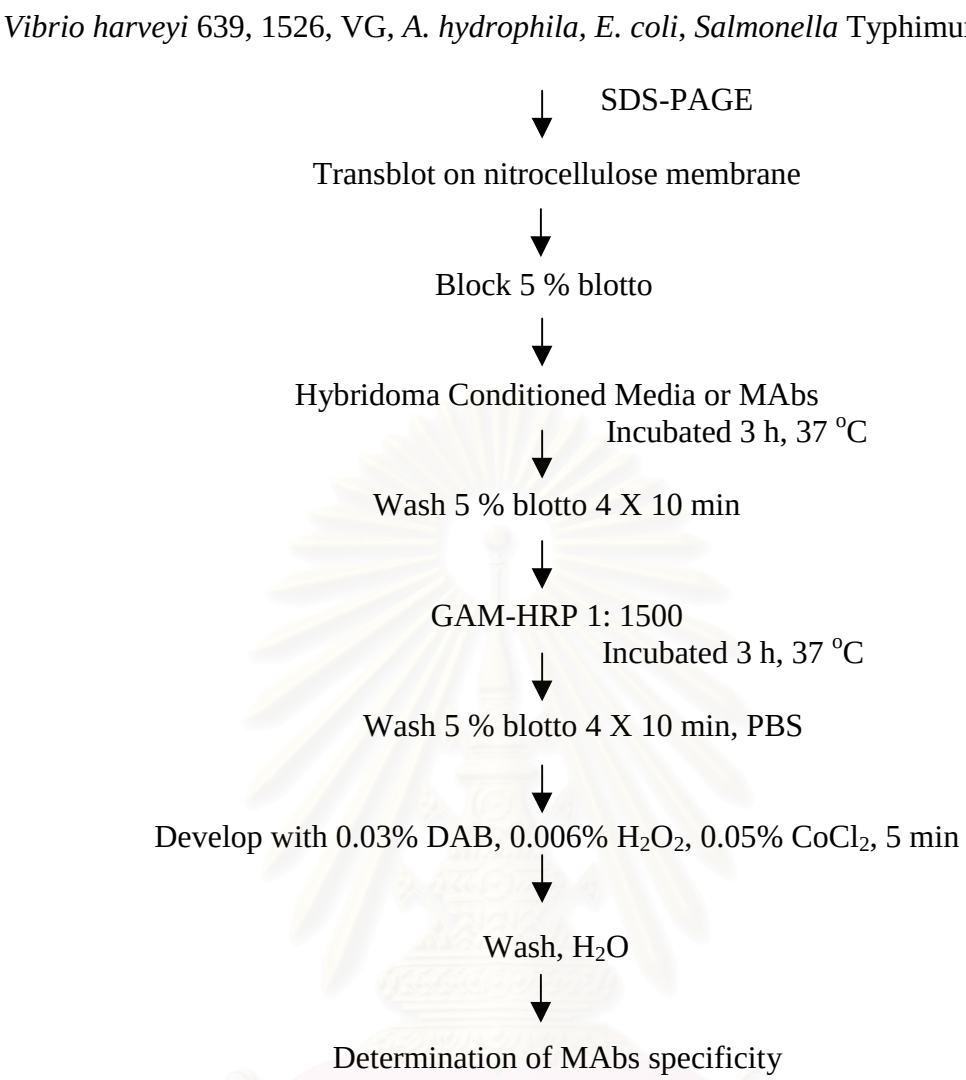

Figure 4.3 Diagram of Western blot analysis for screening method and characterization of monoclonal antibodies (Sithigorngul et al., 2000).

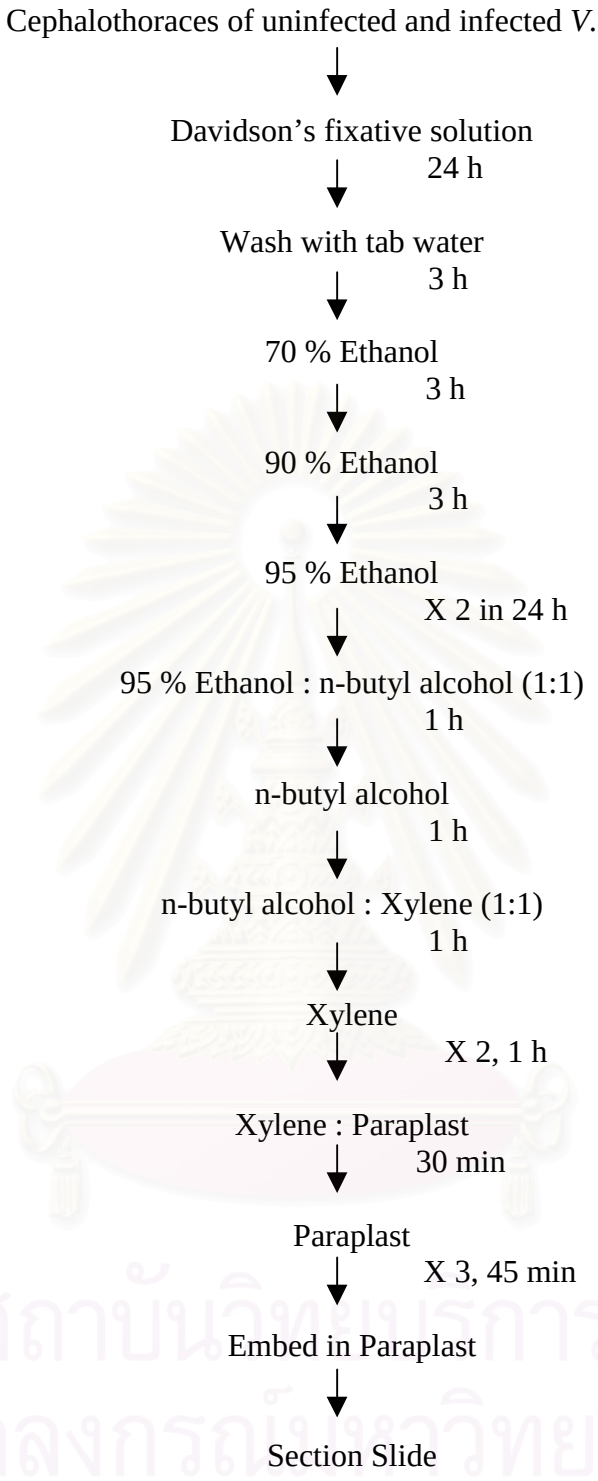

Figure 4.4 Diagram of Immunohistochemistry for characterization of monoclonal antibodies (Sithigorngul et al., 2000).

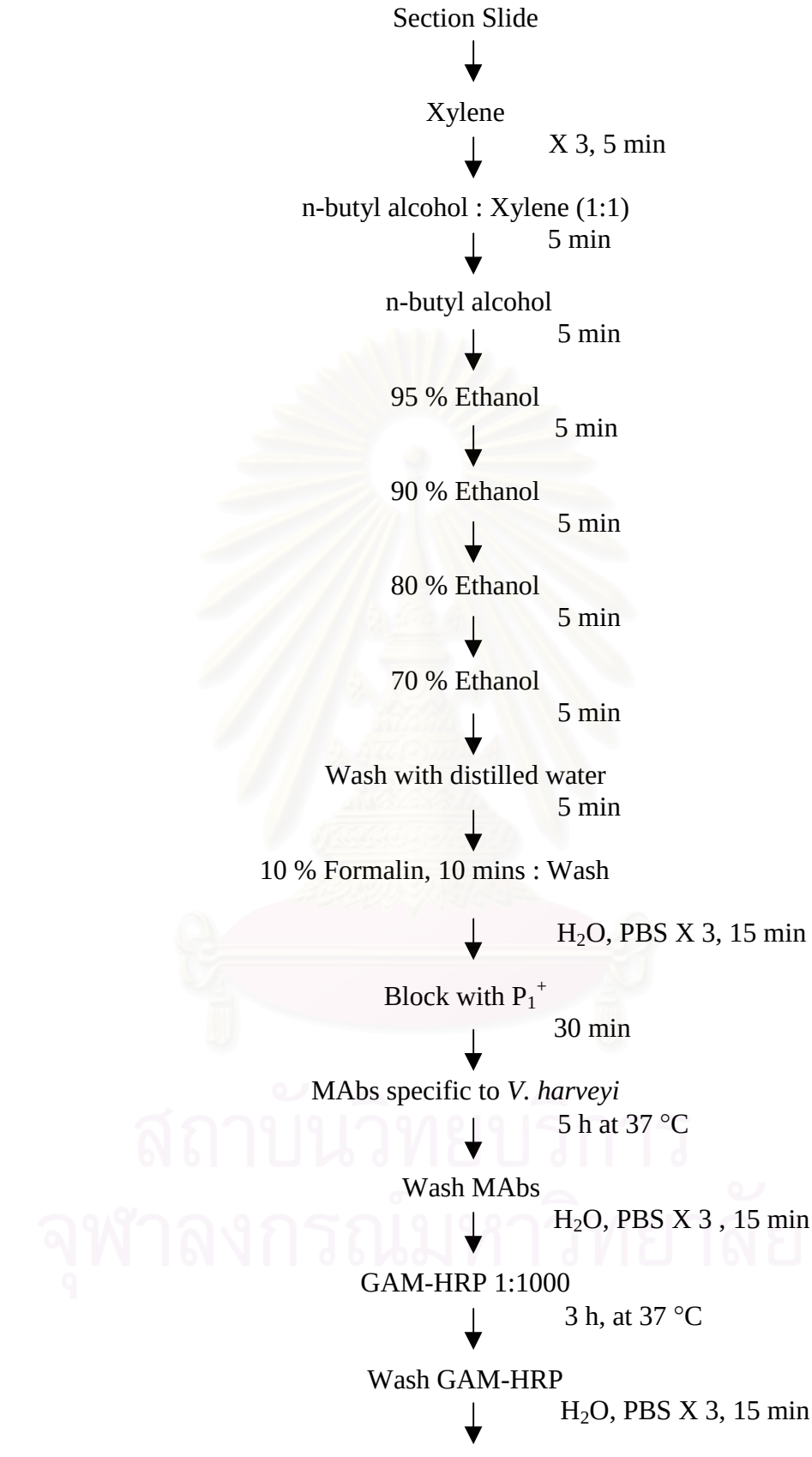

Figure 4.4 (Cont.)

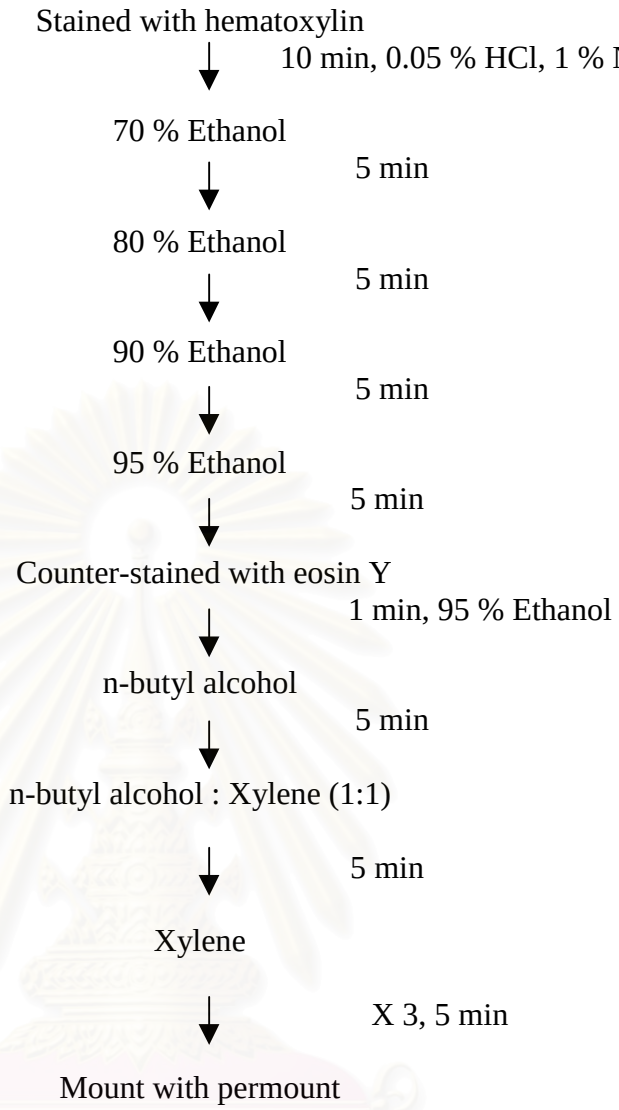

Figure 4.4 (Cont.)

RESULTS

Specificity of antisera

Mouse anti-*V. harveyi* 639 antisera were collected from 4 mice and preabsorped with *V. parahaemolyticus.* All antisera showed specific binding to all *V. harveyi* isolates and no cross-reactivity to *Vibrio* species and other bacteria was detected (Figure 4.5-1 and 4.6-1). Therefore, only one mouse was used as a spleen donor.

Hybridoma production

One fusion trial with 40 microculture plates yielded approximately 2500 culture wells, each giving 1 to 3 colonies of hybridoma cells. The first screening by dot blot revealed 75 hybridoma clones that gave strong reactions with *V. harveyi* 639 (Figure 4.5). After screening by Western blot and dot blot against various bacteria including several *Vibrio* species, 15 clones specific to *V. harveyi* were successfully established as permanent cell lines. Most antibodies specifically recognized *V. harveyi* and showed no cross-reactivity to other *Vibrio* spp., other Gram-negative and Gram-positive bacteria tested (Figure 4.5 and Table 4.1). Exception was MAb VH39-4E that showed slight cross-reactivity to *A. hydrophila* (Figure 4.5-5 and 4.6-5, Table 4.2). The cloned cell lines could be divided into 6 types according to their specificities to bacterial proteins as determined by Western blot (Table 4.2, Figure 4.6).

Monoclonal antibodies characterization

The first MAb type (3 clones) recognized a 49 kDa protein. The second MAb type (4 clones) recognized 37 and 32 kDa proteins. By immunohistochemistry, these antibodies could be used to localize *V. harveyi* 639 in various tissues such as the

lymphoid organ, hepatopancreas, heart, testis and muscle of infected *P. monodon* (Figure 4.7 and 4.8). The third MAb type (1 clone) also recognized 37 and 32 kDa proteins but its immunohistochemistry reactions differed from those of type 2 MAb in that positive signals occurred in different regions of the lymphoid organ and appeared as small condensed particles only in cells with condensed nuclei (Figure 4.7-2 and 4.7-4). The fourth MAb type (1 clone) recognized a 28 kDa protein and showed slight cross-reactivity to *A. hydrophila*. The fifth MAb type (1 clone) recognized a sharp protein band of 8 kDa and bound only lightly to another virulent isolate of *V. harveyi* 1526 (Figure 4.6-6). The sixth MAb type (5 clones) recognized a broad protein band around 8 kDa. These antibodies detected *V. harveyi* in dot blot assays with different sensitivities ranging from 10^4-10^9 CFU ml^{-1} (Table 4.2) and type 6 MAbs gave the highest sensitivity (Figure 4.6-7).

Isotype and subisotype determination

Isotype and subisotype of MAbs obtained were IgG_1, IgG_2a, IgG_2b, IgG_3 and IgM (Table 4.2).

Sensitivity of MAb for *Vibrio harveyi* detection by dot blot

These antibodies can be used to detect *V. harveyi* by means of dot blot assay with different sensitivity ranging from 10^4-10^9 CFU ml^{-1} (Table 4.2, Figure 4.10).

Table 4.1 The bacteria used in the characterization of the monoclonal antibodies (MAbs) and reactivity of MAbs (VH3-3H and VH 29-6D) specific to *V. harveyi* and other bacteria detected by dot blots.

Bacteria tested	Number of strains tested	Number of strains react with MAb	Source
Vibrio harveyi 639, 1526, VG, VG1	4	4	CENTEX Shrimp, Thailand
Vibrio harveyi 1A, 1B, 1L, 2U, 2C, 2E, 2D, 2K, 3O, 3M, 3N, 3R, 3T, 4G, 4J, 4Q, 4S, 4I	18	18	Chareon Phokphan Co. Ltd., Thailand
V. alginolyticus	1	0	Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand
V. parahaemolyticus AQ 453, AQ 4613	2	0	
V. vulnificus	1	0	
V. mimicus	1	0	
V. penaeicida	1	0	Department of Marine Biology, Israel
Aeromonas hydrophila	1	0	Thailand Institute Science and Technology Research, Thailand
Photobacterium damselae	1	0	
Escherichia coli	1	0	
Salmonella Typhimurium	1	0	
Bacillus cereus	1	0	
Staphylococcus aureus	1	0	

Table 4.2 Specificities of monoclonal antibodies tested by dot blot, Western blot and immunohistochemistry. The underlined clones are the representative MAbs used in various tests.

Group	MAbs (type)	Dot blot (CFU ml^{-1})	Western blot (kDa)	Immunohisto-chemistry	Remarks
1	VH13-4D (IgG_1), VH14-10G, VH25-8B (IgM)	~$5x10^5$	49	-	
2	VH27-3G (IgG_1), VH3-3H, VH28-3B, VH38-2D(IgG_2a)	~$5x10^5$	37, 32	+	Bound to unprocessed antigens
3	VH16-2A (IgG_2a)	~$1x10^9$	37, 32	+	Bound to processed antigens
4	VH39-4E (IgG_2b)	~$1x10^6$	28	-	Slight cross reactivity to *Aeromonas hydrophila*
5	VH24-8H (IgG_2a)	~$5x10^5$	8	-	Bound lightly to *V. harveyi* 1526
6	VH2-3B, VH2-6A, VH21-10G (IgG_3), VH28-8D, VH29-6D (IgG_1)	~$5x10^4$	~8	-	Different band from that of VH24-8H antibody

+: strong immunoreactivity ; - : no immunoreactivity

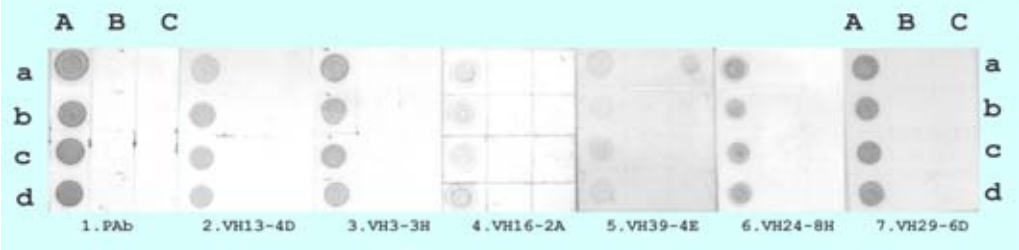

Figure 4.5 Dot blot analysis of *V. harveyi* 639. Various bacteria (~10^9 CFU ml^{-1}) were spotted on a nitrocellulose membrane (1 µl $spot^{-1}$) and treated with representative MAbs of types 2-7 and with mouse anti-*V. harveyi* 639 antiserum (1.PAb) absorbed with *V. parahaemolyticus*. Each bacterium was spotted onto each block of three columns as follows:

Column A: Row (a) *V. harveyi* 639 (b) *V. harveyi* 1526 (c) *V. harveyi* VG

(d) *V. harveyi* VG1.

Column B: Row (a) *V. alginolyticus* (b) *V. parahaemolyticus* (c) *V. vulnificus*

(d) *V. mimicus*.

Column C: Row (a) *Aeromonas hydrophila* (b) *E. coli* (c) *Photobacterium damselae*

(d) *Salmonella* Typhimurium.

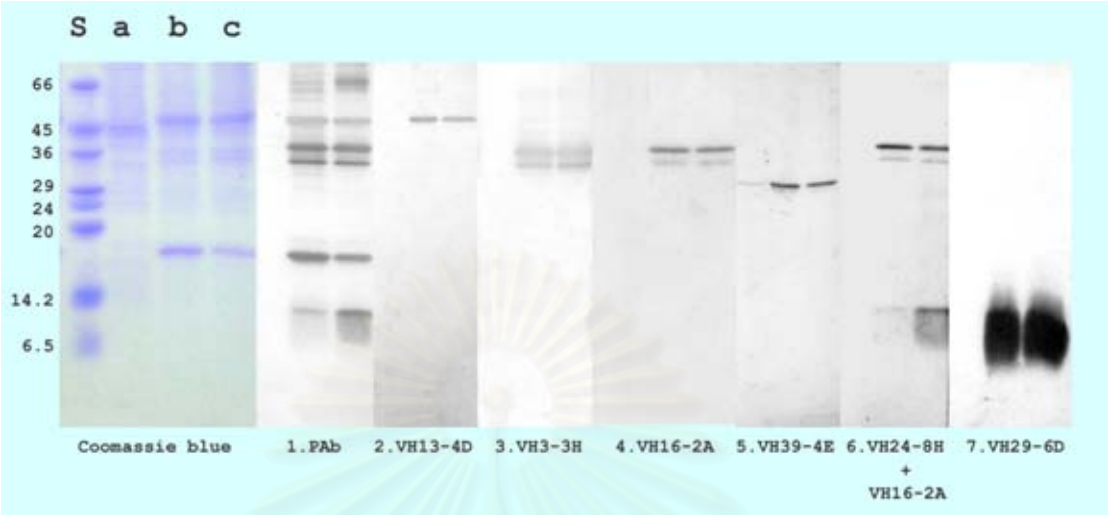

Figure 4.6 SDS-PAGE and Western blot of *V. harveyi* 639. Three bacteria (a) *Aeromonas hydrophila* (b) *V. harveyi* 1526 (c) *V. harveyi* 639 (~10^7 CFU $lane^{-1}$) were electrophoresed and stained with Coomassie blue or transferred to a nitrocellulose membrane and then treated with representative MAbs of types 2-7 or with mouse anti-*V. harveyi* antiserum absorbed with *V. parahaemolyticus* (1). S = low molecular weight standard proteins, the number denoted kDa. Nitrocellulose membrane No. 6 was first treated with MAb VH24-8H then reprobed with MAb VH16-2A to confirm the presence of equivalent amounts of antigens of *V. harveyi* 1526.

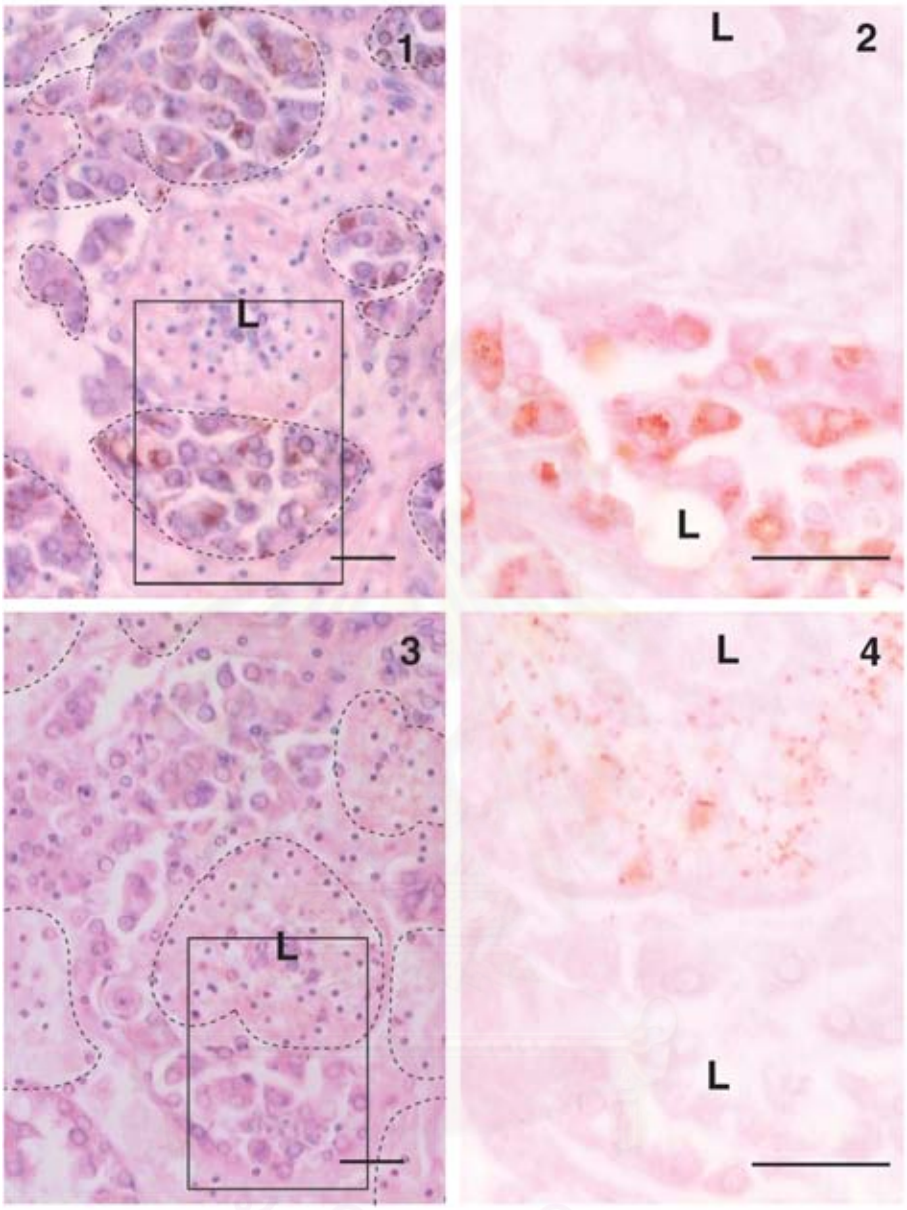

Figure 4.7 Immunohistochemistry of *P. monodon* lymphoid organ. Four consecutive sections from *P. monodon* injected with *V. harveyi* 639 at 3 h were treated with MAbs VH3-3H (1 and 2) or VH16-2A (3 and 4) and then counter stained with hematoxylin and eosin Y (1 and 3) or only eosin (2 and 4). Figure 2 and 4 show higher magnifications of the region in the rectangular frames in Figures 1 and 2. Immunoreactivity of the two MAbs occurred in different regions of the lymphoid tubules (dot surrounded areas). L = lumen of lymphoid tubule. Scale bar = 25 μm.

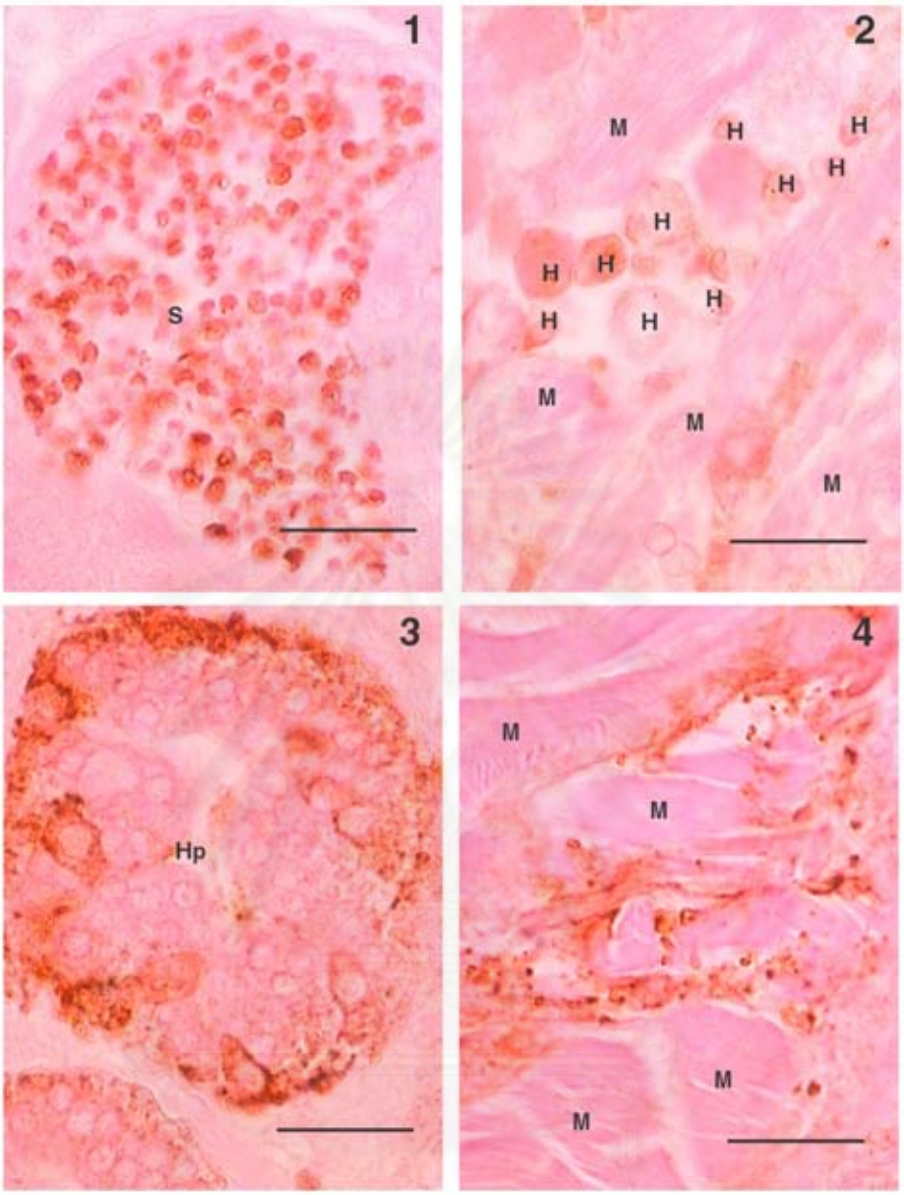

Figure 4.8 Immunohistochemistry of testis (1), heart (2), hepatopancreas (3) and muscle (4). Sections from *P. monodon* infected with *V. harveyi* 639 at 3 h were treated with MAb VH3-3H and counter stained with eosin Y. Sections were from the same block shown in Figure 4.7 Immunoreactivity was observed in both sperm (S), cardiac muscle (M), hemocytes (H) and hepatopancrease (Hp). Scale bar = 25 μm.

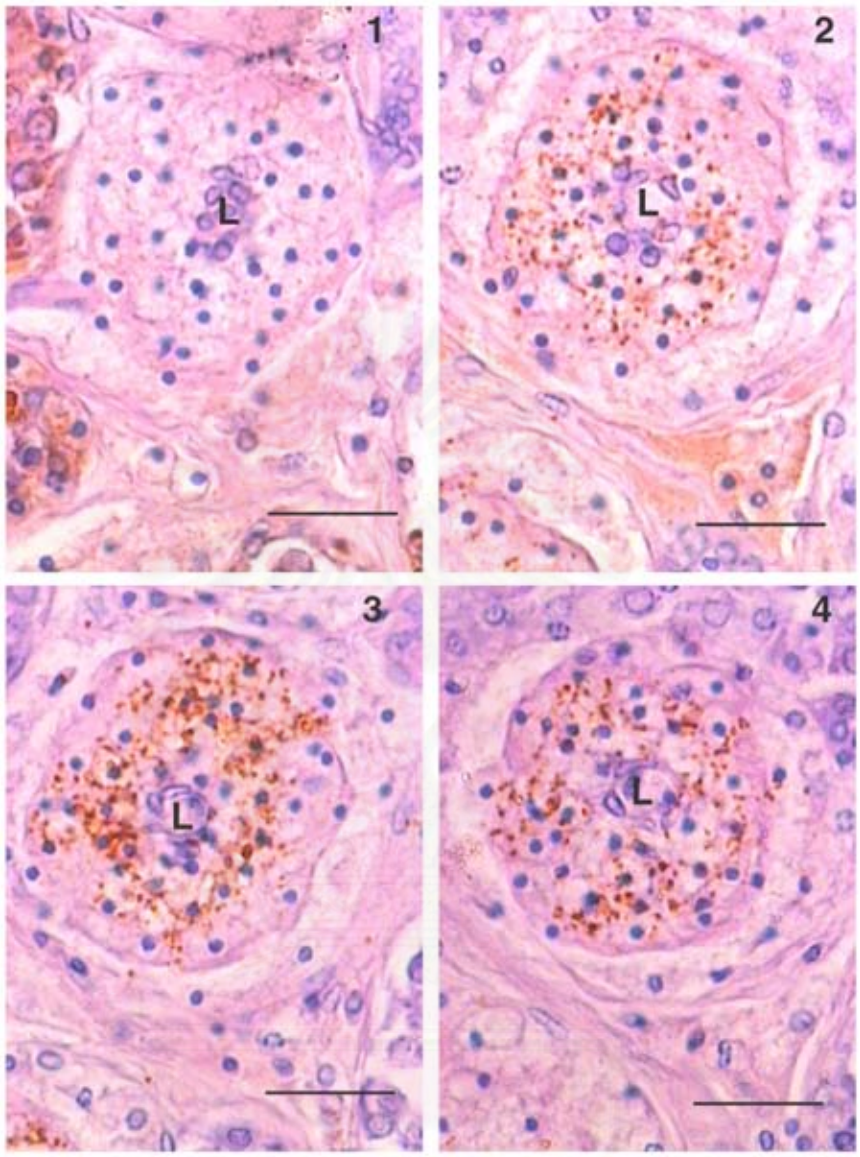

Figure 4.9 Immunohistochemistry of *P. monodon* lymphoid organ. Four consecutive sections from *P. monodon* injected with *V. harveyi* 639 at 3 h were treated with MAbs VH3-3H (1), VH16-2A (2) and Mabs against normal hemocyte HC 249d (3), HC 47d (4) and then counter stained with hematoxylin and eosin Y. Sections were from the same block shown in Figure 4.7. Figures 1 and 2. Immunoreactivity of the two MAbs occurred in different regions of the lymphoid tubules. L = lumen of lymphoid tubule. Scale bar = 25 μm. MAb against normal hemocyte confirmed that the immunoreactivity of VH16-2A to *V. harveyi* was digested in the normal cell but not in the abnormal tubule (spheroid).

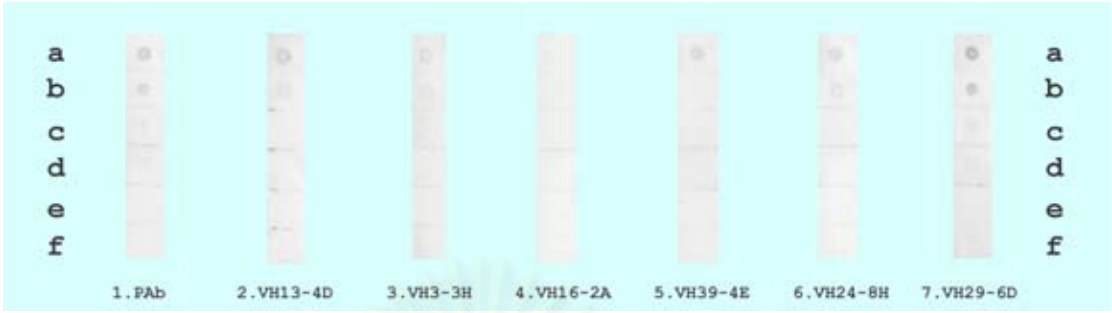

Figure 4.10 Sensitivity of MAb for *V. harveyi* 639 as detected by dot blot. Dilution of bacteria (from 10^6 to 10^3 CFU ml^{-1}) were spotted on a nitrocellulose membrane (1 µl $spot^{-1}$) and treated with representative MAbs of types 2-7 and with mouse anti-*V. harveyi* 639 antiserum (1.PAb) absorbed with *V. parahaemolyticus*. Each dilution was spotted onto each block of six rows as follows:

Row a : $1x10^6$ CFU ml^{-1}

Row b : $5x10^5$ CFU ml^{-1}

Row c : $1x10^5$ CFU ml^{-1}

Row d : $5x10^4$ CFU ml^{-1}

Row e : $1x10^4$ CFU ml^{-1}

Row f : $5x10^3$ CFU ml^{-1}

DISCUSSION

Fifteen MAbs were obtained from a mouse immunized with *V. harveyi* 639 and can be divided into six groups according to their specificities to various proteins ranging from 8-49 kDa. Most of the selected MAbs bound specifically to all *V. harveyi* isolates without any cross reactivity to other *Vibrio* spp., Gram-negative and Gram-positive bacteria tested (Table 4.1), except for one MAb, VH39-4E antibody that showed slight cross-reactivity to *A. hydrophila* (Table 4.2, Figure 4.5-5 and 4.6-5). Some MAbs can be used to detect the infection of *V. harveyi* in various tissues of *P. monodon* by means of immunohistochemistry. It was surprising that MAb types 2 and 3 recognized the same proteins (37 and 32 kDa) but gave different immunohistochemical reactions. The differences could be due to different epitope targets. Since the type 2 MAb (VH3-3H) gave strong immunoreactivity in dot blots of heat killed (Figure 4.5-3) and intact *V. harveyi* (not shown), it recognized an exposed epitope. By contrast, the type MAb (VH16-2A) bound relatively intact bacteria in dot blots but strongly to protein in Western blots. It also bound strongly to particulate material only in lymphoid organ cells with condensed nuclei or in a few hemocytes. This evidence suggested that it recognized a hidden epitope that became exposed after denaturation during Western blot preparation or after partial digestion in phagocytic cells. These two groups of antibodies may show their potential for diagnosis of *V. harveyi* infection and for the study of immune mechanisms against *V. harveyi* infection in various organisms. Use of MAbs to follow antigen in various tissues such as the gut, kidney and spleen after oral vaccination has been demonstrated successfully with *A. hydrophila* in several species of carp (Azad et al., 2000). MAb immunohistochemistry has also been used to follow *Photobacterium damselae* ssp. *piscicida* infection in kidney, spleen and liver of sea bass (Jung et al., 2001).

We confirmed that MAb types 2 and 3 recognized the same proteins (37 and 32 kDa) but gave different immunohistochemical reactions by using MAbs specific to hemocyte, HC249d and HC47d (Winotaphan et al., in press), that gave strong reaction in the normal tubule of lymphoid organ (Figure 4.9-3 and 4.9-4). From this result, we suggested that MAb types 2 recognized an exposed epitope because it gave strong reaction with spheroid cell that did not kill bacteria (Figure 4.9-1). Another MAb type 3 recognized a hidden epitope that became exposed after partial digestion in phagocytic cells and gave strongly reaction in the normal tubule of lymphoid organ in the same area with MAb HC249d and HC47d (Figure 4.9-2).

Because of their high sensitivity (down to $5x10^4$ CFU ml^{-1} or ~50 cells $spot^{-1}$), type 6 MAbs revealed to be the most suitable for further development for diagnostic techniques in aquaculture and in aquatic habitats. Combination with enrichment methods in specific selective media could augment the detection of *V. harveyi* in the environment. For example, MAbs specific to *Cytophaga johnsonae, Comamonas acidovorans* and *A. hydrophila* have been used for identification, quantification and study of depth distribution in a lake (Faude and Höfle, 1997).

Since there were no cross-reactivity with 4 closely related *Vibrio* species and with several other Gram-negative bacteria, it is unlikely that most of the obtained MAbs will cross-react with other bacteria. Since MAbs are most likely to cross-react with antigens from closely related bacteria, the exceptional cross-reactivity of VH39-4E with *A. hydrophilla* but not with 4 other *Vibrio* species tested was unexpected. On the other hand, both *Vibrio* and *Aeromonas* species are included in the family Vibrionaceae based on small-subunit rRNA sequences (Krieg and Holt, 1984) and it is possible that they may share some similar proteins. MAbs against Non-01 heat stable enterotoxin of *V. cholerae* cross-reacted with antigens from *V. mimicus*

and *Yersinia enterocolitica* (Takeda et al., 1990). MAbs against non-membrane damaging cytotoxin from *V. cholerae* O26 cross-reacted with antigens from *V. parahaematolyticus*, *Aeromonas* species and *Shigella* species (Saha and Nair, 1997). MAbs against lipopolysacacharide of *E. coli* O26 cross-reacted with antigens of *E. coli* 0111 and *Salmonella* O35 (Rivera-Betancourt and Keen, 2000) and MAbs against *Bacillus cereus* cross-reacted with antigens from *B. thuringiensis* (Charni et al., 2000).

Although we did not identify the target antigens for our MAbs, many potential antigens have been reported for *Vibrio harveyi*. It produced specific polysaccharide antigen (O-Ag), flagella protein antigen (H-Ag) and extracellular products (ECPs) (Liu et al., 1996a; Liu, Lee, Yii et al., 1996b) including proteases, hemolysins and cytotoxins that allow the bacteria to survive and multiply within host tissues (Ellis, 1991). We revealed intracellular *V. harveyi* in many cell types by immunohistochemical localization using type 2 MAb (VH3-3H). Proteases, phospholipases, hemolysins or exotoxins (ECPs) may play roles in the pathogenicity of *V. harveyi* in black tiger shrimp *P. monodon* (Liu et al., 1996a; Liu, Lee, Yii et al., 1996b; Lee, Chen et al., 1997a; Liu, Lee, Tu et al., 1997).

Our type 1 MAb recognized a 49 kDa protein whereas Fukasawa, Nakamura, Kamii et al. (1988a) and Fukasawa, Nakamura, Miyahira et al. (1988b) have reported the presence of three metal chelator-sensitive proteases in ECPs produced from environmental (water) isolates of *V. harveyi* with molecular weights of 84, 49 and 46 kDa. They suggested that these molecules comprise a major product of *V. harveyi*. Our type 2 and type 3 MAbs recognized 37 and 32 kDa proteins. Zhang et al. (2001) found 2 closely related hemolysin genes designated *vhhA* and *vhhB* encoding 47.3 kDa proteins in *V. harveyi* VIB 645 that caused severe hemorrhagic septicemia in

salmonids. The majority of other strains examined carried only a single hemolysin gene. Since our both proteins are smaller, they are unlikely to be double bands of hemolysin. Our type 5 and type 6 MAbs recognized proteins in the range of 8 kDa. We cannot speculate on the nature of the sharp band obtained with MAb type 5 and the reason for the difference in intensity of reaction between *V. harveyi* 1526 and *V. harveyi* 639. This could be explained by differences in epitope recognition or expression levels of the same protein. However, the broad reaction band for type 6 MAbs was suggestive of reaction with lipopolysaccharides. In *Photobacterium damselae* ssp. *piscicida*, 2 MAbs have been reported to recognize a low molecular weight material running at the dye front and are believed to be LPS (Jung et al., 2001). Lee, Liu et al. (1997b) demonstrated successful protection of *P. monodon* against vibriosis by passive immunization using antisera against *V. harveyi* antigens. However, the specificity of the antisera was not characterized. Broad-range detection was possible because of cross-reactivity between species and the use of a polyclonal antibody. MAbs against *V. harveyi* exotoxin subunits (58, 48, 47, 46, 45, 39 kDa) have been reported (Munro et al., 2003), but their cross-reactivity and ability for protection was not tested. Nor can we speculate whether our MAb would be protective.

MAbs can be used as a very specific immunological tools to accurately differentiate *V. harveyi* from other *Vibrio* species and other bacteria and may be suitable for replacement of laborious and time consuming biochemical identifications. Further development of a very sensitive assay such as sandwich ELISA would facilitate the identification and quantitative analysis of vibriosis.

CHAPTER V

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST *Vibrio harveyi* 1526

INTRODUCTION

Vibrio harveyi was identified as predominant microorganism that cause significant mortalities associated with luminescence in larval culture of *Peneaus monodon* in the Philippines (Baticados et al. 1990). Outbreaks of disease due to *V. harveyi* had also been observed in India (Karunasagar et al., 1994), Indonesia (Sunaryanto and Mariam, 1986), Australia (Pizzutto and Hirst, 1995), Taiwan (Liu et al., 1996a) and Thailand (Jiravanichpaisal and Miyazaki, 1994).

Conventional methods for diagnosis of *V. harveyi* infection were based on clinical signs and luminescence in hatcheries and farm (Jiravanichpaisal and Miyazaki, 1994). These methods are not highly specific and are not useful for detecting early stage of infections. A selective medium, such as thiosulfate citrate bile salts sucrose (TCBS) agar, eliminates most non-target bacteria in clinical samples but was not satisfactory for environmental samples because many bacteria present in natural water sources can produce colonies on TCBS agar whose appearance is similar to the appearance of *V. harveyi* (Choopun et al., 2002). By contrast, in other studies conventional method and biochemical detection by API strip test was confirmed and directly showed evidence of infection in *P. monodon* (Liu, Lee, Yii et al., 1996b; Alvarez et al., 1998; Alcaide et al., 2001). These methods were very slow, mis-identified and must use shrimp to confirm the pathogenicity. Even though these techniques were directly confirm for bacteria detection, there were practical limitation

for their widespread commercial application. These limitations include the need for special media, highly trained personnel, time consuming, and high cost. Immunological techniques are widely used for diagnosis of many bacterial diseases in both food industry and agriculture. Due to high sensitivity and specificity, immunological methods can be simplified to obtain results quickly at relatively low cost (Sithigorngul et al., 2002).

The use of monoclonal antibodies to detect other bacteria has been recently reported (Okrend et al., 1990; Adam et al., 1995; He et al., 1996; Jaradat and Zawistowski, 1996; Saha and Nair, 1997; Charni et al., 2000; Rivera-Betancourt and Keen, 2000; Jung et al. 2001). Munro et al. (2003) produced monoclonal antibodies specific to the exotoxin sub-units from *V. harveyi* strain 642. However, the assay (Western blot analysis) was not highly sensitive and could not be easily applied during field examinations.

The same results of biochemical and conventional tests of *V. harveyi* 639 and 1526 were shown in Table 3.1 and 3.2 of Chapter III. Obviously gelatinase and hemolysis activity (Table 3.3) in *V. harveyi* 639 were more pronounced than those of *V. harveyi* 1526. Moreover, LD_{50} at 10^7 CFU ml^{-1} of *V. harveyi* 639 on black tiger shrimp could cause shrimp death in shorter time than that of *V. harveyi* 1526 (Figure 3.2). However, there was a history of the big outbreak of *V. harveyi* 1526 in 1998 among shrimp hatcheries and growout prawn (personnel communication). Besides the successful preparation of MAbs specific to *V. harveyi* 639 as reported in Chapter IV, we also attempt to prepare MAbs specific to *V. harveyi* 1526 on the purpose of obtaining varieties of MAbs for use as antibody probes against the species specific antigens and potential protective antigens and to differentiate *V. harveyi* 1526 from *V. harveyi* 639.

MATERIALS AND METHODS

Followed the procedures on page 52-63 in Chapter IV, except using *Vibrio harveyi* 1526 for antigen preparation and immunization.

Vibrio harveyi 1526, isolated from *Penaeus monodon* infected with luminous vibriosis from a shrimp farm in Thailand (provided by CENTEX shrimp, Thailand), was cultured as described previously (page 52).

RESULTS

Specificity of antisera

Mouse anti-*V. harveyi* 1526 antisera were collected from 4 mice and preabsorbed with *V. parahaemolyticus.* All antisera showed specific binding to all *V. harveyi* isolates without cross-reactivity to *Vibrio* species or other tested bacteria (Figure 5.1-1 and 5.2-1). Therefore, only one mouse was used as a spleen donor.

Hybridoma production

One fusion trial with 30 microculture plates yielded approximately 1500 culture wells, each giving 1 to 6 colonies of hybridoma cells. The first screening by dot-blot revealed 67 hybridoma clones that gave stronger reaction with *V. harveyi*, strain 1526 (Figure 5.1). After screening by Western blot, supernatants from approximately 30 clones produced specific and intensity band. Only 16 clones were successfully established as permanent cell lines. Most antibodies specifically recognized *V. harveyi* and showed no cross-reactivity to other *Vibrio* spp., Gram-negative and Gram-positive bacteria (Figure 5.1, Table 5.1). Exceptions were MAb VH15-6H antibody that shows cross-reactivity to *E. coli, S.* Typhimurium (Figure 5.1-2, 5.2-2) and MAb VH7-6F antibody that shows cross-reactivity to *E. coli, S.* Typhimurium and *A. hydrophila* (Figure 5.1-3, 5.2-3). These cloned cell lines could be divided into 6 groups according to their specificities to bacterial structures as determined by Western blot (Table 5.2, Figure 5.2).

Monoclonal antibodies characterization

The first group (1 clone) recognized the 95 kDa protein which VH15-6H antibody shows cross-reactivity to *E. coli, S.* Typhimurium (Figure 5.1-2, 5.2-2). The second group (1 clone), MAb VH7-6F recognized the 95 kDa protein and antibody

shows cross-reactivity to *E. coli*, *S.* Typhimurium and *A. hydrophila* (Figure 5.1-3 and 5.2-3). The third group (5 clones) recognized the 49 kDa proteins (Figure 5.2-4). The fourth group (2 clones) recognized the 37, 32 kDa proteins (Figure 5.2-5). The fifth group constituted the major antibodies obtained in this fusion (5 clones) and recognized the 18 kDa protein and showed slightly cross-reactivity to another virulent strain of *V. harveyi* VG and 639 (Figure 5.2-6 and 5.4). The sixth group (2 clones) recognized the 8 kDa protein (Figure 5.2-7). All of MAbs did not show reaction by immunohistochemistry (Table 5.2). The MAbs of group 3, 4 and 6 have the same specificities as MAb obtained from mouse immunized with *V. harveyi* 639.

These antibodies detected *V. harveyi* in dot blot assays with different sensitivities ranging from 10^4-10^6 CFU ml^{-1} (Table 5.2) and the sixth group of MAbs gave the highest sensitivity (Figure 5.2-7).

The MAb VH24-8H specific to *V. harveyi* 639 binds lightly to *V. harveyi* 1526. The MAb VH23-11B bound to the antigen that specific to *V. harveyi* 1526 but not to the antigens of *V. harveyi* 639 and VG. This MAb can be used to differentiate *V. harveyi* 1526 from *V. harveyi* 639 and *V. harveyi* VG (Figure 5.4II).

Isotype and subisotype determination

Isotype and subisotype of monoclonal antibodies obtained belonged IgG_1, IgG_2a, IgG_2b and IgM (Table 5.2).

Sensitivity of MAb for *Vibrio harveyi* detection by dot blot

These antibodies can be used to detect of *V. harveyi* by means of dot blot assay with different sensitivity ranging from 10^4-10^6 CFU ml^{-1} (Table 5.2, Figure 5.3).

Table 5.1 The bacteria used in the characterization of the monoclonal antibodies (MAbs) and reactivity of MAbs (VH30-10D and VH26-11E) specific to *V. harveyi* and other bacteria detected by dot blots.

Bacteria tested	Number of strains tested	Number of strains react with MAb	Source
Vibrio harveyi 639, 1526, VG, VG1	4	4	CENTEX Shrimp, Thailand
Vibrio harveyi 1A, 1B, 1L, 2U, 2C, 2E, 2D, 2K, 3O, 3M, 3N, 3R, 3T, 4G, 4J, 4Q, 4S, 4I	18	18	Chareon Phokphan Co. Ltd., Thailand
V. alginolyticus	1	0	Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand
V. parahaemolyticus AQ 453, AQ 4613	2	0	
V. vulnificus	1	0	
V. mimicus	1	0	
V. penaeicida	1	0	Department of Marine Biology, Israel
Aeromonas hydrophila	1	0	Thailand Institute Science and Technology Research, Thailand
Photobacterium damselae	1	0	
Escherichia coli	1	0	
Salmonella Typhimurium	1	0	
Bacillus cereus	1	0	
Staphylococcus aureus	1	0	

Table 5.2 Specificities of monoclonal antibodies tested by dot blot, Western blot and immunohistochemistry. The underlined clones are the representative MAbs used in various tests. EC= *Escherichia coli*, ST= *Salmonella* Typhimurium, AH= *Aeromonas hydrophila*

Group	MAbs (type)	Dot blot (CFU ml^{-1})	Western blot (kDa)	Immunohisto-chemistry	Remarks
1	VH15-6H (IgG_2b)	~$5x10^5$	95	-	Cross-react to EC, ST
2	VH7-6F (IgG_2a)	~$5x10^5$	95	-	Cross-react to EC, ST and AH
3	VH10-7H (IgG_2a), VH17-8G (IgG_2a), VH18-4B (IgG_2a), VH23-3D (IgG_1), VH30-1D (IgG_1)	~$5x10^5$	49	-	Similar to MAb of *V. harveyi* 639
4	VH19-10B (IgG_2b) VH30-10D (IgG_2a)	~$5x10^5$	37,32	-	Similar to MAb of *V. harveyi* 639
5	VH2-1B,VH11-1A, VH11-3A (IgM), VH23-11B (IgG_2b), VH30-8G(IgG_2b)	~$1x10^6$	18	-	Bound lightly to *V. harveyi* VG and *V. harveyi* 639
6	VH8-12A(IgG_2b), VH26-11E (IgG_2b)	~$5x10^4$	8	-	Similar to MAb of *V. harveyi* 639

- : no immunoreactivity

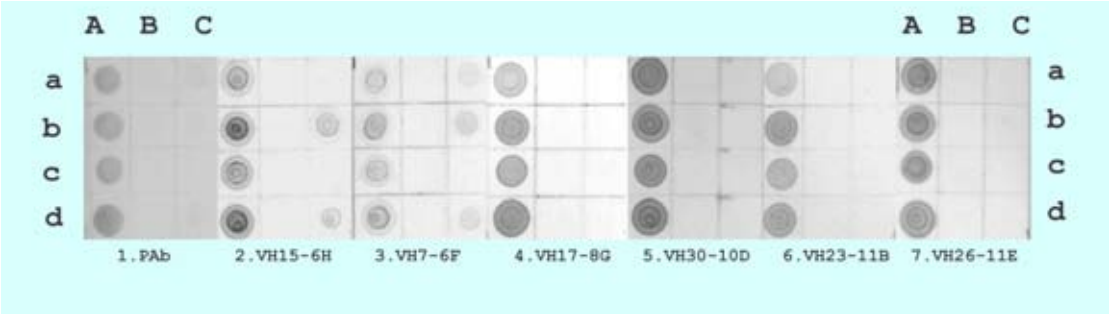

Figure 5.1 Dot blot analysis of *V.harveyi* 1526. Various bacteria (~10^9 CFU ml^{-1}) were spotted on a nitrocellulose membrane (1 µl $spot^{-1}$) and treated with representative MAbs of types 2-7 and with mouse anti-*V. harveyi* 1526 antiserum (1.PAb) absorbed with *V. parahaemolyticus*. Each bacterium was spotted onto each block of three columns as follows:

Column A: Row (a) *V. harveyi* 639 (b) *V. harveyi* 1526 (c) *V. harveyi*, VG

(d) *V. harveyi*, VG1

Column B: Row (a) *V. alginolyticus* (b) *V. parahaemolyticus* (c) *V. vulnificus*

(d) *V. mimicus*

Column C: Row (a) *Aeromonas hydrophila* (b) *Escherichia coli*

(c) *Photobacterium damselae* (d) *Salmonella* Typhimurium

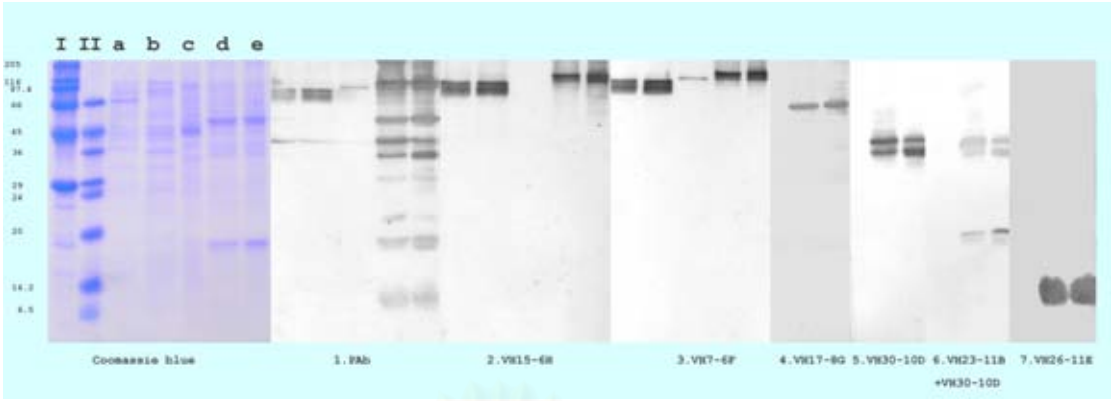

Figure 5.2 SDS-PAGE and Western blot of *V. harveyi* 1526. Six bacteria (a*) Salmonella* Typhimurium (b) *E. coli* (c) *Aeromonas hydrophila* (d) *V. harveyi* VG (e) *V. harveyi* 1526 (~10^7 CFU $lane^{-1}$) were electrophoresed and stained with Coomassie blue or transferred to a nitrocellulose membrane and then treated with representative MAbs of types 2-7 or with mouse anti-*V. harveyi* 1526 antiserum absorbed with *V. parahaemolyticus* (2). I and II = high and low molecular weight standard proteins, the number denoted kDa. Nitrocellulose membrane No. 6 was first treated with MAb VH23-11B then reprobed with MAb VH30-10D to confirm the presence of equivalent amounts of antigens of *V. harveyi* VG.

Figure 5.3 Sensitivity of MAb for *V. harveyi* 1526 as detected by dot blot. Dilution of bacteria (from 10^6 to 10^3 CFU ml^{-1}) were spotted on a nitrocellulose membrane (1 µl $spot^{-1}$) and treated with representative MAbs of types 2-7 and with mouse anti-*V. harveyi* 1526 antiserum (1.PAb) absorbed with *V. parahaemolyticus*. Each dilution was spotted onto each block of six rows as follows:

Row a : $1x10^6$ CFU ml^{-1}

Row b : $5x10^5$ CFU ml^{-1}

Row c : $1x10^5$ CFU ml^{-1}

Row d : $5x10^4$ CFU ml^{-1}

Row e : $1x10^4$ CFU ml^{-1}

Row f : $5x10^3$ CFU ml^{-1}

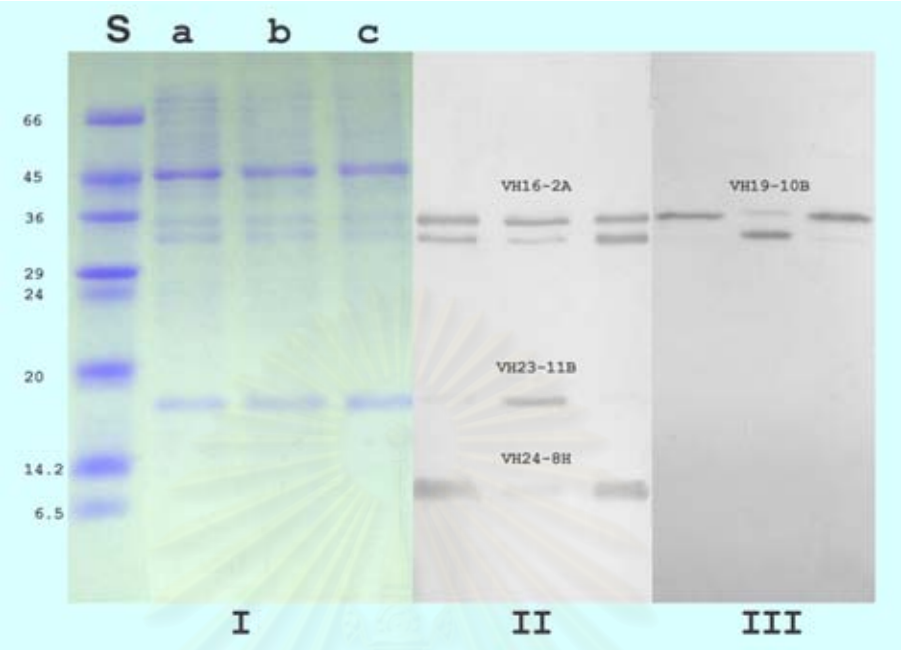

Figure 5.4 SDS-PAGE and Western blot of MAbs specific to *V. harveyi* 639 and 1526. Three bacteria (a) *V. harveyi* 639 (b) *V. harveyi* 1526 (c) *V. harveyi* VG (~10^7 CFU $lane^{-1}$) were electrophoresed and stained with Coomassie blue (I) or transferred to a nitrocellulose membrane and then treated with representative MAbs, VH24-8H + VH23-11B. Nitrocellulose membrane was first treated with MAb VH24-8H + VH23-11B then reprobed with MAb VH16-2A to confirm the presence of equivalent amounts of antigens of *V. harveyi* (II). Another nitrocellulose membrane was treated with VH19-10B antibody (III). S = low molecular weight standard proteins, the number denoted kDa.

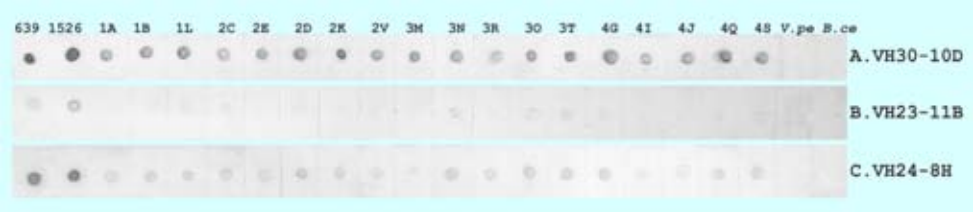

Figure 5.5 Dot blot for detection of other *V. harveyi* (column 1-20), *V. penaeicida* (column 21) and *Bacillus cereus* (column 22). Bacteria (~10^9 CFU ml^{-1}) were spotted on a nitrocellulose membrane (1 µl $spot^{-1}$) and treated with representative MAbs, (A) VH30-10D, (B) VH23-11B and (C) VH24-8H. Each bacteria was spotted onto each block of column.

DISCUSSION

Sixteen MAbs were obtained from mice immunized with *V. harveyi* 1526 and can be divided into six groups according to their specificities to various proteins ranging from 8-95 kDa. Most of the selected MAbs bound specifically to all *V. harveyi* isolates without any cross-reactivity to other *Vibrio* spp., Gram-negative and Gram-positive bacteria tested (Table 5.1 and Figure 5.5). However, the first two groups of MAbs recognized the 95 kDa protein in which VH15-6H antibody shows cross-reactivity to *E. coli, S.* Typhimurium (Table 5.2, Figure 5.1-2 and 5.2-2) and VH7-6F antibody shows cross-reactivity to *E. coli, S.* Typhimurium and *A. hydrophila* (Table 5.2, Figure 5.1-3 and 5.2-3). According to high specificity and high sensitivity, MAbs in the sixth group can be used to detect *V. harveyi* specifically at low number down to $5x10^4$ CFU ml^{-1} (~50 cells $spot^{-1}$) by dot blot assay. Therefore, these MAbs can be used for diagnosis of bacterial diseases in aquaculture and in aquatic habitat with high accuracy. Combination of this technique with an enrichment of the bacteria in specific selective media will augment the accuracy and quantitative determination of the *V. harveyi* in the environment. MAbs specific to *Cytophaga johnsonae, Comamonas acidovorans* and *A. hydrophila* were used for identification, quantification and depth distribution of the bacteria in the lake (Faude and Höfle 1997).

In this experiment, a series of MAbs specific only to *V. harveyi* were produced. The selection was performed against other 4 closely related *Vibrio* spp. and several groups of diverse bacteria in order to eliminate the potentiality of MAbs against common epitopes. Therefore, it is unlikely that these MAbs will show cross-reactivity to other bacteria. However, a MAb that we intended to select, VH15-6H antibody shows cross-reactivity to *E. coli, S.* Typhimurium and VH7-6F antibody

shows cross-reactivity to *E. coli, S.* Typhimurium and *A. hydrophila* but still did not showed cross-reactivity to the antigens of the other four *Vibrio* spp. tested. The two MAbs VH15-6H and VH7-6F recognized the 95 kDa protein. The protein bands recognized by these two MAbs displayed the same molecular weight in all *V. harveyi* tested, *E. coli, S.* Typhimurium and *A. hydrophila*, suggesting an antigenic relationship among Gram negative bacteria. It is possible that they may share some epitopes of subunit of similar proteins. Similarly in whole cell lysates polypeptides with molecular weight of 60-97 kDa were highly cross-reactive suggesting the common distribution of antigens in the bacterial species (Swain et al., 2003). On the other hand, both *Vibrio* spp. and *Aeromonas* spp. are included in family Vibrionaceae based on small-subunit rRNA sequences (Krieg and Holt, 1984). The close-relateness between these two species may explain the non-specific nature of the reaction observed in this study by both dot blot and Western blot.

The fifth group of MAbs constituted the major antibodies obtained in this fusion (5 clones) and recognized the 18 kDa protein and bound lightly to another virulent isolate of *V. harveyi* VG. Therefore this MAb can be used to differentiate *V. harveyi* 1526 from *V. harveyi* VG. However, the difference was not distinguished whether it was due to the difference in recognized epitope or different expression levels of the protein. The LPS antigens approximately 14 kDa of *V. salmonicida* and *A. salmonicida* have earlier been recorded as strong immuno-reactive antigens (Hoel, Reitan and Lillehaugh, 1998).

MAbs VH19-10B and VH30-10D did not work in IHC indicating that the different epitopes of the antigen prepared by IHC techniques were not recognized by these MAbs. Therefore, the combination of MAbs may help to increase the sensitivity.

In Western blot analysis, the MAbs of group 3, 4 and 6 recognized the same proteins that have the same molecular weight in both *V. harveyi* 639 and *V. harveyi* 1526, suggesting an antigenic relationship between the two *V. harveyi*. However, some MAbs demonstrated different immunoreactivities against *V. harveyi* 1526, 639 and VG (Figure 5.4II), for example VH23-11B (this study) recognized the 18 kDa protein, VH24-8H (previous study) recognized the 8 kDa protein. This evidence indicated the different epitope natures of the similar proteins between these two isolate groups.

Surprisingly, another MAb in the fourth group (VH19-10B) which recognized the 32 and 37 kDa proteins also demonstrated different patterns of staining between *V. harveyi* 1526 and 639 and VG. In *V. harveyi* 1526 the antibody demonstrated stronger immunoreactivity for the 32 kDa protein than the 37 kDa protein, whereas in *V. harveyi* 639 and VG the antibody demonstrated stronger immununoreactivity for the 37 kDa protein than the 32 kDa protein (Figure 5.4III). From the immunoreactivity data demonstrated by these three antibodies indicated that serotype of *V. harveyi* 1526 is different from the serotypes of *V. harveyi* 639 and VG.

Moreover, most of the antibodies obtained from both set seem to recognized these four isolates stronger than other 18 *V. harveyi* isolates (Table 5.1) especially the VH24-8H and VH23-11B antibodies demonstrated distinct stronger immunoreactivities (Figure 5.5). It is possible that those 18 isolates may belong to different serotypes from the four isolates used in this study.

In this study we are able to isolate various monoclonal antibodies which can be use not only for identification of *V. harveyi* but some of them can be used to identify the different isolates (serotypes) among *V. harveyi* as well.

CHAPTER VI

SUMMARY

1. Level of pathogenicity depends on the type of *V. harveyi* isolates and their virulent substance productions; for example, protease and hemolysin. From our studies, *V. harveyi* 639 produced higher protease (gelatinase) and hemolysin (tested on shrimp hemolymph) activities than those of the other *V. harveyi* tested.
2. LD_{50} at ~ 10^7 CFU ml^{-1} of *V. harveyi* 639, 1526, VG and VG1 in shrimp in 39, 43, 49 and 50 h were observed, respectively.
3. Thirty-one clones of monoclonal antibodies (MAbs) were classified into 9 groups based on the recognition of proteins ranging from 8-95 kDa.
4. MAbs specific to 8 kDa can be used to detect *V. harveyi* with high sensitivity up to $5x10^4$ CFU ml^{-1} or 50 cells $spot^{-1}$ by dot blotting.
5. VH3-3H and VH16-2A antibodies were specific to *V. harveyi* 639 and could be used to localize *V. harveyi* infection in the tissues of black tiger shrimp *Penaeus monodon* by means of immunohistochemistry.
6. VH24-8H antibody was specific to *V. harveyi* 639, VG but slightly bound to other 18 isolates.
7. VH23-11B antibody was specific to *V. harveyi* 1526, but slightly bound to *V. harveyi* 639 and VG.
8. VH19-10B antibody demonstrated dual specificity in *V. harveyi* 1526 in which the antibody demonstrated stronger immunoreactivity to the 32 kDa protein than the 37 kDa protein and vice versa for *V. harveyi* 639 and VG.

9. VH39-4E antibody was specific to *V. harveyi* 639 but cross-reacted to *A. hydrophila*. The other two MAbs against *V. harveyi* 1526, VH15-6H and VH7-6F cross-reacted to *E. coli, S.* Typhimurium and *E. coli, S.* Typhimurium and *A. hydrophila*, respectively.
10. VH3-3H, VH29-6D antibodies specific to *V. harveyi* 639 and VH26-11E, VH30-10D antibodies specific to *V. harveyi* 1526 were selected to be further developed a test kit based on sandwich ELISA technique.

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APPENDICES

APPENDIX A

ORIGINAL DATA FROM EXPERRIMENT IN CHAPTER III

Table 1A Hemolysis and pathogenic characteristics exhibited by various *Vibrio harveyi* isolates.

V. harveyi isolates	Protease against		Phospho-lipase against egg yolk	Lipase against tween 80	Chitinase against chitin	Hemolysis against hemolymph from		
	Casein	Gelatin				sheep	shrimp	crab
639	1.5	1.6	0.8	1.6	1.6	2.5	1.5	1
1526	1.1	1.1	0.7	1.6	0.8	2.5	1	0.8
VG	0.6	1.4	0.7	1.4	0.7	2	0.8	0.6
VG1	0.6	1.4	0.7	1.4	0.7	2.2	0.8	0.6

Table 2A Survival (%) of *P. monodon* at various time (h) after being infected by immersion with *V. harveyi* at concentration of ~ 10^7 CFU ml^{-1}; *V. harveyi* 639 (VH 639), *V. harveyi* 1526 (VH 1526), *V. harveyi* VG (VH VG) and *V. harveyi* VG1 (VH VG1).

Hours	VH 639	VH 1526	VH VG	VH VG1
0	100	100	100	100
24	75	79	84	82
48	16	32	38	44
72	0	2	18	22
96	0	2	6	4
108	0	0	6	4

APPENDIX B

MEDIA PREPARATION

1. Tryptic soy broth

Tryptone	17.0	g
Soytone	3.0	g
Dextrose	2.5	g
NaCl	5.0	g
K_2HPO_4	2.5	g

Adjust pH = 7.3 $\pm$ 0.2

2. Thiosulfate citrate bile salt agar

Yeast extract	5.0	g
Proteose peptone No.3	10.0	g
$HOC(COONa)(CH_2COONa)_2$	10.0	g
$Na_2S_2O_3$	10.0	g
Oxgall	8.0	g
Saccharose	20.0	g
NaCl	10.0	g
$C_6H_5O_7Fe.5H_2O$	1.0	g
Bromthymol blue	0.04	g
Agar	15.0	g

Adjust pH = 8.6 $\pm$ 0.2

3. Motility medium

Tryptone	10.0	g
NaCl	5.0	g
Agar	5.0	g

Adjust pH = 7.2 $\pm$ 0.2

4. Hugh and Leifson's O-F medium

Tryptone	2.0	g
NaCl	5.0	g
K_2HPO_4	0.3	g
Bromthymol blue	0.08	g
Agar	2.0	g

Adjust pH = 6.8 $\pm$ 0.2

5. MR-VP medium

Buffer peptone	7.0	g
K_2HPO_4	5.0	g
Glucose	5.0	g

6. Christen's urea

Peptone	1.0	g
Glucose	1.0	g
NaCl	5.0	g
KH_2PO_4	2.0	g
Phenol red	0.012	g
Agar	20.0	g

7. Nitrate broth

Beef extract	5.0	g
Peptone	5.0	g
KNO_3	1.0	g
Adjust pH = 7.0 $\pm$ 0.2		

8. Simmon's citrate agar

$MgSO_4.7H_2O$	0.2	g
$(NH_4)_2\ SO_4$	1.0	g
K_2HPO_4	1.0	g
$HOC(COONa)(CH_2COONa)_2$	2.0	g
NaCl	5.0	g
Bromthymol blue	0.08	g
Agar	15.0	g

9. Gelatin medium

Beef extract	3.0	g
Peptone	5.0	g
Gelatin	5.0	g
Adjust pH = 7.0 $\pm$ 0.2		

10. Sodium caseinate agar

Sodium caseinate	10.0	g
Glucose	1.0	g
K_2HPO_4	1.0	g
$MgSO_4.6H_2O$	0.2	g
$FeSO_4.7\ H_2O$	0.01	g
Agar	15.0	g

11. Starch agar

Soluble starch	2.0	g
Yeast extract	3.0	g
Peptone	5.0	g
Agar	15.0	g

Adjust pH = 7.0 $\pm$ 0.2

12. Tween 80 agar

Peptone	10.0	g
NaCl	5.0	g
$CaCl_2.2H_2O$	0.1	g
Tween 80	10.0	g
Agar	15.0	g

Adjust pH = 7.0 $\pm$ 0.2

13. Egg yolks agar

Peptone	1.0	g
Yeast extract	0.5	g
Phenol red	0.025	g
Agar	15.0	g

After autoclaved, added 0.2 % egg yolk and polymyxin B sulfate 10 μg/ml

14. Blood agar

Beef heart, Infusion from	500.0	g
Tryptone	10.0	g
NaCl	5.0	g
Agar	15.0	g

After autoclaved, added 2% sterile defribrinated sheep blood, 45 ^{o}C

15. Hemolymph shrimp and crab agar

Tryptone	17.0	g
Soytone	3.0	g
Dextrose	2.5	g
NaCl	5.0	g
K_2HPO_4	2.5	g
Agar	15.0	g

After autoclaved, added 2% hemolymph shrimp and crab, 45 ^{o}C

16. Chitin agar

Chitin	3.0	g
Yeast extract	3.0	g
Peptone	5.0	g
Agar	15.0	g

Adjust pH = 7.0 $\pm$ 0.2

17. Phenol red broth base

Beef extract	1.0	g
Preteose peptone No.3	10.0	g
NaCl	5.0	g
Phenol red	0.018	g

Add 1% glucose, dextrose, arabinose, mannose, maltose, mannitol, lactose, sucrose, xylose and ribose and adjust pH = 7.4 $\pm$ 0.2

All media added 2% NaCl, prepareed per litre and autoclaved at 121 ^{o}C, 15 min except Thiosulfate citrate bile salt agar

APPENDIX C

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

APPENDIX D

REAGENT PREPARATION FOR HYBRIDOMA PRODUCTION

1. RPMI medium

The medium consisted of the following ingredients:

RMPI 1640 (Roswell Park Memorial Institute – Gibco BRL, USA)	10.4	g
D-glucose (Sigma)	3.6	g
L-glutamine (Sigma)	0.2923	g
Sodium pyruvate ($C_3H_3O_3Na$) (Sigma)	1.1005	g
$NaHCO_3$	2.0160	g
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma)	5.9525	g
H_2O (Meri Q water)	1000.0	ml

The solution of penicillin G, streptomycin and kanamycin were added to the final concentration of 20,000 units, 200 mg and 200 mg per liter, respectively. The medium was sterilized by millipore membrane (pore size 0.22 μm) filtration and stored at 4 ^{o}C.

2. RPMI medium with serum

The medium consisted of the following ingredients:

RPMI medium (1)	80.0	ml
Fetal calf serum (FCS, Starrate, Australia)	20.0	ml
Or Bovine calf serum (BCS, Starrate, Australia)		
100 x HT supplement (Gibco BRL, USA)	1.0	ml
-10 mM Sodium hypoxanthine		
-1.6 mM Thymidine		

3. Hybridoma selective medium (HAT medium)

The medium consisted of the following ingredients:

RPMI medium (1)	80.0	ml
FCS	20.0	ml
HT supplement	1.0	ml
50 X Aminopterin (Sigma)	2.0	ml
1 % Mouse red blood cell		

4. Fusion solution (40% polyethylene glycol)

Polyethylene glycol (PEG)	4.0	g

The solution was prepared by dissolving 4.0 g of polyethylene glycol in 6 ml of RPMI medium (1). The solution was incubated at 37 ^{o}C in CO_2 incubator before use.

5. Freezing medium (12% DMSO)

Dimethylsulfoxide (DMSO, Sigma)	12.0	ml
RPMI medium (1)	88.0	ml

The medium was stored in cold (4 ^{o}C) before use.

APPENDIX E

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	µl
TEMED	20.0	µl

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	µl
TEMED	10.0	µl

Table 1E Preparation of separating gel 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 µl	100 µl
TEMED	20 µl	10 µl
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-250	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 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 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, the was dried in a gel air dryer (BIO-RAD)

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

-Myosin, rabbit muscle	205	kDa
-β-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
-α-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 wing 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.

APPENDIX F

REAGENT FOR DETERMINATION OF ISOTYPE AND SUBISOTYPE OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURED ON ANTI-Ig ANTIBODIES

Hybridoma sub-isotyping kit, mouse (Zymed) contents:

A) Rabbit anti-Mouse IgG1 (γ1 chain specific)

B) Rabbit anti-Mouse IgG2a (γ2a chain specific)

C) Rabbit anti-Mouse IgG2b (γ2b chain specific)

D) Rabbit anti-Mouse IgG3 (γ3 chain specific)

E) Rabbit anti-Mouse IgA (α chain specific)

F) Rabbit anti-Mouse IgM (μ chain specific)

G) Rabbit anti-Mouse kappa light chain

H) Rabbit anti-Mouse lambda light chain

I) Normal Rabbit Serum, (Negative Control)

J) Positive Control, Monoclonal Mouse IgG1
(Mouse IgG1 is in RPMI-1640 with 10 % FBS)

K) Substrate Buffer, Concentration (10X)
(1 M citrate, pH 4.2, containing 0.03 % H_2O_2)

L) ABTS Substrate, Concentrated (50X)
(2,2 – azino-di [3-ethylbenzthiazoline sulfonic acid])

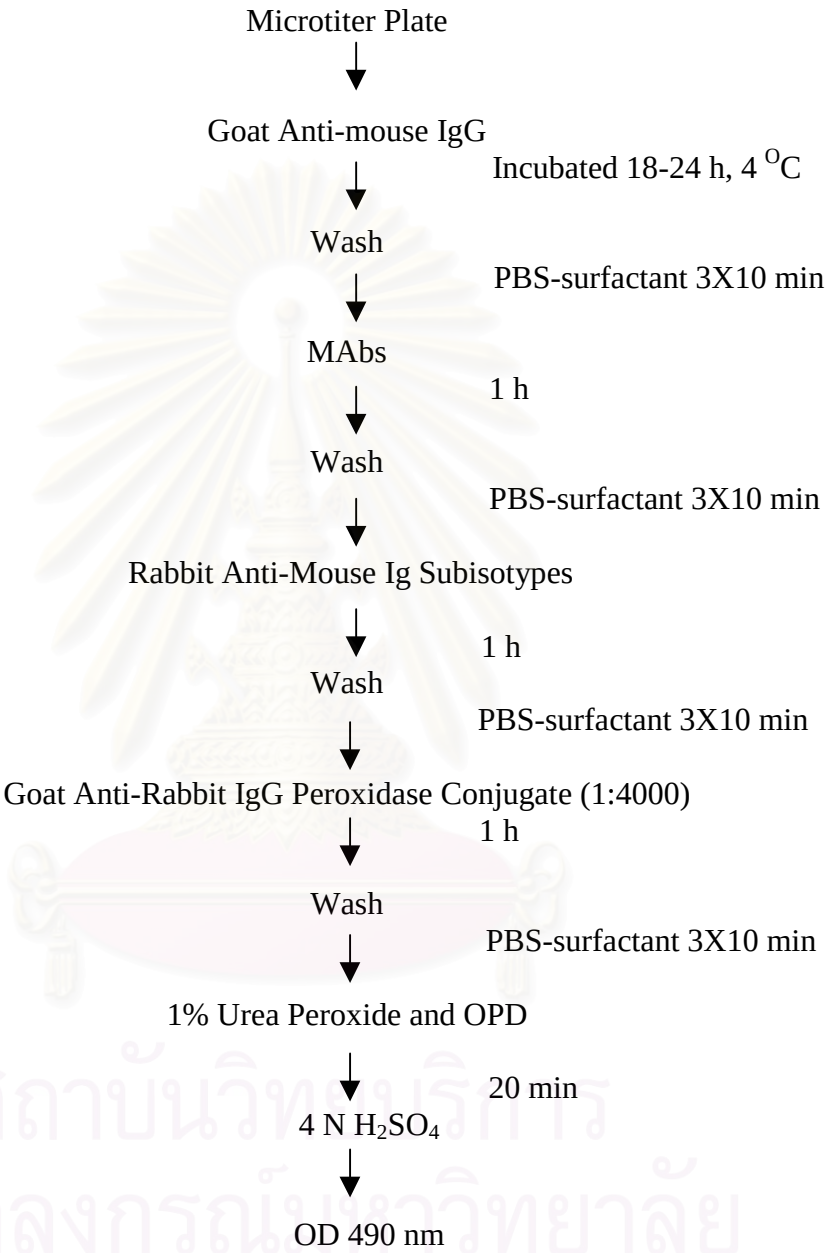
M) Blocking Solution, Concentration (50X)
(25% BSA in PBS and 0.05% NaN_3)

N) HPR-Goat anti-Rabbit IgG (H+L), Concentrated (50X)

O) Goat anti-Mouse IgGAM, Concentrated (50X)
(0.5 mg/ml in PBS containing 10% glycerol and 0.05% NaN_3)

P) 50% Tween 20

Method of sandwich ELISA for determination of antibody isotype and subisotype (Longyant, 1999).



APPENDIX G

BUFFRS AND SOLUTION FOR ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

1. 5% Blotto solution (Longyant et al., 1999)

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

2. Washing solution (0.5% Blotto)

5% Blotto solution (4.1)	50.0	ml
PBS 0.15 M pH 7.2	950.0	ml

3. 0.1 M Citrate Buffer pH 4.5

Sodium citrate	29.41	g
1% Merthiolate	10.0	ml
H_2O (distilled water) adjust volume to	1000.0	ml

The pH of the buffer was adjusted to 4.5 with 0.1 M HCl

4. 1 N H_2SO_4

Concentrated H_2SO_4	27.0	ml
H_2O (distilled water) adjusted volume to	1000.0	ml

5. O-Phenylenediamine dihydrochloride (OPD)

APPENDIX H

REAGENT AND SOLUTION FOR IMMUNOHISTOCHEMISTRY (IHC)

1. Coated slide solution

Gelatin	1.0	g
Clone 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% (P_1^+)

Calf serum	10.0	ml
PBS	100.0	ml

5. Enrilich'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

Methods of immunohistochemistry and indirect immunoperoxidase (Sithigorngul et al., 2000; 2002).

1. Immunnohistochemistry (IHC)

1.1 Cephalothoraces of uninfected and *V. harveyi* 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°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°C and incubated slide at 50°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 Removed 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 MAb specific to *V. harveyi*).

2.2.3 Incubated at 37°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°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% 3',3 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

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ACADEMIC PUBLICATION :

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