

DEVELOPMENT OF IMMUNOLOGICAL-BASED ASSAY AND LOOP-MEDIATED
ISOTHERMAL AMPLIFICATION METHOD FOR SPECIFIC DETECTION OF
VIBRIO VULNIFICUS IN MARINE ANIMALS



Presented in Partial Fulfillment of the Requirements for the
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DEVELOPMENT OF IMMUNOLOGICAL-BASED ASSAY AND LOOP-MEDIATED
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VIBRIO VULNIFICUS IN MARINE ANIMALS



A DISSERTATION
BY
THANAI SURASILP

Presented in Partial Fulfillment of the Requirements for the
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การพัฒนาการตรวจสอบเชื้อ *Vibrio vulnificus* ในสัตว์ทะเลด้วยวิธีทางภูมิคุ้มกันวิทยาและวิธี
loop-mediated isothermal amplification



เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
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Vibrio vulnificus เป็นแบคทีเรียที่ก่อให้เกิดโรค ในการศึกษานี้ได้ใช้โมโนโคลนอล
แอนติบอดี และ LAMP-LFD เพื่อทำการตรวจสอบเชื้อ *V. vulnificus* โมโนโคลนอลแอนติบอดีผลิต
โดยการนำเชื้อ *V. vulnificus* ในรูปแบบตายด้วยความร้อนและแบบตรึงด้วยฟอร์มาลินมาปลูกภูมิคุ้มกัน
ให้แก่หนูขาว โมโนโคลนอลแอนติบอดีที่ได้แบ่งออกเป็น 9 กลุ่ม โดยที่โมโนโคลนอลแอนติบอดี 5
กลุ่มแรกสามารถตรวจสอบเชื้อ *V. vulnificus* ได้ 1 ถึง 7 ไอโซเลตตามความจำเพาะของแต่ละกลุ่ม
โมโนโคลนอลแอนติบอดีกลุ่มที่ 6 สามารถตรวจสอบเชื้อ *V. vulnificus* ได้ครบทั้ง 40 ไอโซเลต โม
โนโคลนอลแอนติบอดีกลุ่มที่ 7 สามารถตรวจสอบเชื้อ *V. vulnificus* และ *V. fluvialis* ได้ โมโน
โคลนอลแอนติบอดีกลุ่มที่ 8 สามารถตรวจสอบเชื้อ *V. vulnificus* และ *V. alginolyticus* ได้ โมโน
โคลนอลแอนติบอดีกลุ่มที่ 9 สามารถตรวจสอบเชื้อ *V. vulnificus* และไวรัสชนิดอื่นได้ โมโน
โคลนอลแอนติบอดีทุกกลุ่มมีความไวในการตรวจสอบเชื้ออยู่ที่ 10^6 to 10^7 CFU ml⁻¹ โมโนโคลนอล
แอนติบอดีกลุ่มที่ 2 4 5 และ 9 สามารถตรวจสอบเชื้อ *V. vulnificus* ในเนื้อกุ้งขาวแปซิฟิกด้วยวิธี
immunohistochemistry ได้ ความไวของโมโนโคลนอลแอนติบอดีทุกกลุ่มในการตรวจสอบเชื้อ *V.*
vulnificus หลังจากบ่มเชื้อ 6 ชั่วโมงในอาหารเลี้ยงเชื้อบริสุทธิ์อยู่ที่ 10 CFU ml⁻¹ ในกรณีที่ย้อมเชื้อ
V. vulnificus ในอาหารเลี้ยงเชื้อที่เจือปนด้วยหอยนางรมสดมีความไวในการตรวจสอบเชื้อ *V.*
vulnificus น้อยกว่าในอาหารเลี้ยงเชื้อบริสุทธิ์ ส่วนวิธีตรวจสอบเชื้อ *V. vulnificus* ด้วยวิธี LAMP-
LFD ต่อยีน RNA polymerase subunit sigma factor S (*rpoS*) นั้นทำได้โดยการเพิ่มปริมาณ
ผลิตภัณฑ์ของ LAMP ที่สลายด้วยไบโอตินด้วยไพรเมอร์ 6 สาย จากนั้นนำผลิตภัณฑ์ที่ได้ไปผสม
กับ probe ที่สลายด้วย FITC แล้วตรวจสอบผลิตภัณฑ์ที่ได้ด้วยแท่ง LFD เวลาและอุณหภูมิที่
เหมาะสมในการทำ LAMP อยู่ที่ 90 นาทีและ 65 องศาเซลเซียสผสมลำดับ วิธี LAMP-LFD
สามารถตรวจสอบเชื้อ *V. vulnificus* ได้ทั้ง 14 ไอโซเลตและไม่เกิดปฏิกิริยาข้ามกับเชื้อไวรัสและ
แบคทีเรียชนิดอื่น ความไวของวิธี LAMP-LFD ในการตรวจสอบเชื้อ *V. vulnificus* ในอาหารเลี้ยง
เชื้อบริสุทธิ์อยู่ที่ 1.5×10^3 CFU ml⁻¹ หรือเทียบเท่า 2.8 CFU ต่อปฏิกิริยา ส่วนความไวในการ
ตรวจสอบเชื้อ *V. vulnificus* ในอาหารเลี้ยงเชื้อที่เจือปนด้วยหอยนางรมอยู่ที่ 1.2×10^4 CFU g⁻¹
หรือเทียบเท่า 11 CFU ต่อปฏิกิริยา จากผลการศึกษานี้จะเห็นได้ว่าวิธีการตรวจสอบเชื้อ *V.*
vulnificus ที่ใช้ในการศึกษานี้มีความไว รวดเร็ว ง่าย และเป็นวิธีที่มีคุณภาพ ประโยชน์ ดังนั้นวิธีการ
ตรวจสอบเชื้อ *V. vulnificus* ทั้งวิธีใช้โมโนโคลนอลแอนติบอดีและวิธี LAMP-LFD จึงสามารถ
นำไปใช้อย่างง่ายทั้งในห้องปฏิบัติการขนาดเล็กและในภาคสนาม

DEVELOPMENT OF IMMUNOLOGICAL-BASED ASSAY AND LOOP-MEDIATED
ISOTHERMAL AMPLIFICATION METHOD FOR SPECIFIC DETECTION OF
VIBRIO VULNIFICUS IN MARINE ANIMALS



AN ABSTRACT
BY
THANAI SURASILP

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Thanai Surasilp. (2012). *Development of immunological-based assay and loop mediated isothermal amplification method for specific detection of Vibrio vulnificus in marine animals*. Dissertation, Ph.D. (Biotechnology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Assoc. Prof. Dr. Parin Chaivisuthangkura , Prof. Dr. Paisarn Sithigorngul, Asst. Prof. Dr. Siwaporn Longyant.

Vibrio vulnificus is a pathogenic bacterium. To identify of *V. vulnificus*, monoclonal antibodies (MAb) against *V. vulnificus* and LAMP-LFD technique were used. MAbs against *V. vulnificus* were produced and developed using heat-killed and formalin-fixed form of *V. vulnificus* for mouse immunization. Nine different groups of monoclonal antibodies specific to *V. vulnificus* were investigated. The first five isolate-specific antibodies were isolates specific that recognized one to seven of *V. vulnificus* isolates. The sixth antibody group recognized forty isolates of *V. vulnificus*. The seventh groups co-detected *V. vulnificus* and *V. alginolyticus*. The eighth groups co-detected *V. vulnificus* and *V. fluvialis*. The last groups recognized all of *V. vulnificus* and non-*vulnificus* *Vibrio*. All MAbs could be used to identify *V. vulnificus* or other *Vibrio* spp. by dot blotting with a sensitivity ranging from 10^6 to 10^7 CFU ml⁻¹. Monoclonal antibodies in groups 2, 4, 5 and 9 could detect *V. vulnificus* infection in Pacific white shrimp tissues by immunohistochemistry. The detection capability could be improved to 10 CFU ml⁻¹ of the original bacterial content after pre-incubating samples in alkaline peptone water (APW) for 6 h prior to dot blotting. In the case of detection of *V. vulnificus* after pre-incubation in oyster homogenate-APW, the detection limit was lower than that of pre-incubation in APW. In a case of LAMP-LFD, biotinylated LAMP amplicons were produced by a set of six designed primers that recognized the *V. vulnificus* RNA polymerase subunit sigma factor S (*rpoS*) gene followed by hybridization with an FITC-labeled probe and LFD detection. The optimized time and temperature conditions for the LAMP assay were 90 min at 65 °C. The LAMP-LFD method accurately identified 14 isolates of *V. vulnificus* but did not detect non-*vulnificus* *Vibrio* isolates and non-*Vibrio* isolates. The sensitivity of LAMP-LFD for *V. vulnificus* detection in pure culture was 1.5×10^3 CFU ml⁻¹ or equivalent to 2.8 CFU per reaction. In the case of spiked oyster samples without enrichment, the detection limit for *V. vulnificus* was 1.2×10^4 CFU g⁻¹ or equivalent to 11 CFU per reaction. In summary, the methods for detection of *V. vulnificus* in this study are sensitive, rapid, simple and valuable tool for detection of *V. vulnificus*. Therefore, both MAbs and LAMP-LFD method can be easily used for small laboratory and field diagnosis.

The dissertation titled
“ Development of immunological-based assay and loop-mediated isothermal amplification
method for specific detection of *Vibrio vulnificus* in marine animals ”

by
Thanai Surasilp

has been approved by the Graduate School as partial fulfillment of the requirements for the
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Thanai Surasilp

TABLE OF CONTENTS

Chapter	Page
1 INTRODUCTION	1
Objectives	3
2 LITERATURE REVIEW.....	4
<i>Vibrio vulnificus</i>	4
Characteristics of <i>Vibrio vulnificus</i>	4
Geographical range distribution and environmental conditions.....	6
Illnesses caused by <i>V. vulnificus</i>	7
Virulence factors.....	8
Response of <i>Vibrio vulnificus</i> to various stresses.....	10
Diagnosis of <i>V. vulnificus</i>	12
Monoclonal antibodies	17
Loop-mediated isothermal amplification (LAMP)	21
The lateral-flow dipstick (LFD) technique.....	25
3 MATERIALS AND METHODS.....	27
Detection of <i>Vibrio vulnificus</i> using monoclonal antibodies (MAbs)	27
Detection of <i>Vibrio vulnificus</i> by loop-mediated isothermal amplification combined with lateral flow dipstick (LAMP-LFD) targeted to <i>rpoS</i> gene....	40
4 RESULTS.....	46
Detection of <i>Vibrio vulnificus</i> using monoclonal antibodies (MAbs).....	46
Immunization.....	46
Production of Monoclonal antibodies.....	46
Characterization of Monoclonal antibodies.....	46
Detection of <i>V. vulnificus</i> after pre-incubation in alkaline peptone water.	52
Detection of <i>V. vulnificus</i> after pre-incubation in oyster homogenate-APW.....	54

TABLE OF CONTENTS (CONTINUED)

Chapter	Page
4 (continued)	
Detection of <i>Vibrio vulnificus</i> by loop-mediated isothermal amplification combined with lateral flow dipstick (LAMP-LFD) targeted to <i>rpoS</i> gene...	56
LAMP primer design.....	56
Optimization of the temperature and time for <i>V. vulnificus</i> detection by LAMP.....	56
The optimal amount of FITC-labeled probe for lateral flow dipstick assay.....	58
Specificity for <i>V. vulnificus</i> detection by LAMP-LFD.....	58
Sensitivity for <i>V. vulnificus</i> detection by LAMP-LFD and PCR methods..	61
5 DISCUSSION.....	64
BIBLIOGRAPHY.....	69
APPENDICES.....	81
APPENDIX A.....	82
APPENDIX B.....	85
APPENDIX C.....	88
APPENDIX D.....	93
VITAE.....	95

LIST OF TABLES

Table	Page
1 Differentiation of three <i>Vibrio vulnificus</i> biogroups.....	6
2 List of bacterial isolates used in this study for immunological assay	28
3 Bacterial isolates used for LAMP-LFD assay	41
4 Primers for loop-mediated isothermal amplification (LAMP) designed from <i>rpoS</i> gene of <i>V. vulnificus</i>	43
5 Specificity of monoclonal antibodies against <i>V. vulnificus</i>	47
6 Specificity for <i>V. vulnificus</i> detection by PCR and LAMP	59



LIST OF FIGURES

Figure	Page
1 Monoclonal antibodies production	20
2 Loop mediated isothermal amplification for amplification of target DNA	23
3 Loop primers for decrease the total LAMP reaction time.....	24
4 Detection of LAMP products using lateral flow dipstick (LFD)	26
5 A flow chart of immunohistochemistry preparation	36
6 A flow chart of determination of antibody isotypes and subisotypes preparation	38
7 Cross-reactivity of MAbs determined by dot blotting	48
8 Western blot analyses of <i>V. vulnificus</i> using MAbs from each group	50
9 Immunohistochemistry	51
10 Sensitivity testing of MAbs for <i>V. vulnificus</i> as detected by dot blotting	52
11 Sensitivity test after pre-incubating in APW.....	53
12 Sensitivity test after pre-incubating oyster homogenate in APW.....	55
13 Determination of the optimal temperature for LAMP assay targeted to <i>rpoS</i> gene of <i>V. vulnificus</i>	57
14 Determination of the optimal time for LAMP assay targeted to <i>rpoS</i> gene of <i>V. vulnificus</i>	57
15 The various amount of FITC-labeled probe used in this study for lateral flow dipstick assay.....	58
16 Sensitivity comparison of loop-mediated isothermal amplification and PCR assays for the <i>V. vulnificus</i> detection in pure cultures.....	62
17 Sensitivity of LAMP combined with LFD for the detection of <i>V. vulnificus</i>	63

LIST OF ABBREVIATIONS AND SYMBOLS

CENTEX	=	Centex Shrimp, Faculty of Science, Mahidol University.
CPF	=	Charoen Pokphand Foods Public Co. Ltd.
DABU	=	Department of Aquatic Science, Burapa University.
DMSC	=	Department of Microbiology, Faculty of Science, Chulalongkorn University.
DMSM	=	Department of Microbiology, Faculty of Science, Mahidol University.
DMST	=	Department of Medical Science, Ministry of Public Health Thailand.
DBSWU	=	Department of Biology, Faculty of Science, Srinakharinwirot University, Thailand
GB	=	Ghent University, Belgium
LAMP	=	loop-isothermal amplification
PCR	=	polymerase chain reaction
PSU	=	Prince of Songkla University
SWU	=	Department of Biology, Srinakharinwirot University.
?	=	unknown
+	=	positive reaction
-	=	negative reaction

CHAPTER 1

INTRODUCTION

Vibrio vulnificus is a gram-negative bacterium that was first isolated by the US Centers of Disease Control (CDC) in 1964 (Strom; & Paranjpye. 2000: 177-188) and designated as *V. vulnificus* in 1980 (Farmer. 1980: 656). This bacterium is widely distributed in marine and estuarine environments worldwide and has been isolated from water, sediments and variety of seafood, such as shrimp, fish, oysters and clams (Jones; & Oliver. 2009: 1723-1733). Consumption of seafood (especially raw oysters) containing *V. vulnificus* can result a severe, fulminant systemic infection. Characteristics of this disease include fever, chills, nausea, hypotensive septic shock. The mortality rate of primary septicemia caused by *V. vulnificus* is more than 50% within days (Jones; & Oliver. 2009: 1723-1733).

Traditional identification of *V. vulnificus* is achieved through biochemical tests that are laborious and require several days to complete. Furthermore, several *Vibrio* species display similar biochemical characteristics that limit the identification of species by biochemical tests (O'Hara; et al. 2003: 5654-5659). Therefore, several molecular methods have been used for identification of *V. vulnificus* (Takahashi; et al. 2005: 77-85); (Panicker; et al. 2004: 7436-7444; Kumar; et al. 2006: 216-220; Kim; et al. 2008: 1841-1847). The conventional PCR method and real-time PCR for detection of bacterial pathogens have been shown to be highly specific and relatively less time-consuming than classical methods. However, for both PCR and real-time PCR, a dedicated thermal cycler is needed.

In 2008, a real-time PCR detection of *V. vulnificus* has been developed by targeting an alternative gene called RNA polymerase subunit sigma factor S (*rpoS*) gene. This gene has been shown to be specific to *V. vulnificus* since in some cases mutation or rearrangements within the *vvhA* gene, a commonly used target for detection of *V. vulnificus*, can occur (Kim; et al. 2008: 1841-1847).

Although the sensitivity of PCR-based methods is very high but this method requires expensive equipment and may not be feasible for small laboratories or field diagnosis. Various immunological-based assays using monoclonal antibodies (MAbs) have been used for identification of several pathogens of aquatic organisms. MAbs are antibodies produced by one expanded clone of B-cells. The cell lines are produced by fusing B cells

from immunized animal with myeloma cell. All antibody molecules have the same amino acid sequence and belong to the same class of immunoglobulins with an identical antigen combining site. Recently, several researchers have developed MAbs to verify pathogenic *Vibrio* spp. such as *V. vulnificus* (Rengpipat; Pusiririt; & Rukpratanporn. 2008: 398-404) and *V. harveyi* (Sithigorngul; et al. 2007: 256-264).

Recently, a novel method termed loop-mediated isothermal amplification (LAMP) was applied by Notomi et al (2000: e63). This method is a technique for rapid and sensitive nucleic acid amplification for detection of aquatic animal pathogen. Since LAMP reaction requires isothermal temperature, the reaction can be carried out using a simple heat block or hot water bath. The method relies on auto-cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment and a set of two inner and two outer primers that recognize a total of six distinct sequences on the target DNA. The final products are stem-loop DNA with several inverted repeats of target DNA and cauliflower-like structures with multiple loops. There are several reports using this technique to detect *V. vulnificus* (Han; & Ge. 2008: 311-320; Ren; et al. 2009: 514-521; Li; et al. 2010: 93-97), non-*vulnificus* *Vibrio* and other bacteria such as *V. parahaemolyticus* (Nemoto; et al. 2009: 748-754; Yamazaki; et al. 2008a: 163; Yamazaki; et al. 2010: 820-828; Chen; & Ge. 2010: 41; Prompamorn; et al. 2011: 344-351), *V. harveyi* (Cao; et al. 2010: 24-29), *V. cholerae* (Srisuk; et al. 2010: 36-42), *Salmonella* (Hara-Kudo; et al. 2005: 155-161).

Analysis of LAMP products is carried out usually by agarose gel electrophoresis, followed by staining with carcinogenic ethidium bromide. Lateral-Flow Dipstick (LFD) is a new technique that can be used to detect LAMP products rapidly using the principles of DNA hybridization and chromatography in membrane strips. This technique can be completed within 10 minutes whereas, gel electrophoresis requires 45-60 minutes (Nimitphak; et al. 2010: 1-5).

In this study, the identification and detection of *V. vulnificus* will be performed using MAbs and LAMP-LFD techniques targeting *rpoS* gene in order to evaluate and compare the possibility to detect *V. vulnificus*.

Objectives

1. To produce monoclonal antibodies specific to *V. vulnificus*.
2. To develop a LAMP-LFD technique targeted to *rpoS* gene of *V. vulnificus* for rapid and specific detection of *V. vulnificus*.
3. To use both MAbs and LAMP-LFD technique to identify and detect *V. vulnificus* in order to evaluate and compare the ability of these two techniques in the detection of *V. vulnificus*.



CHAPTER 2

LITERATURE REVIEW

1. *Vibrio vulnificus*

The bacteria genus *Vibrio* is in the family Vibrionaceae, which also includes the genera *Plesiomonas*, and *Photobacterium*. All Vibrios are environmentally ubiquitous in the marine environment except *V. cholerae* and *V. mimicus*. Several *Vibrio* species are known to be pathogenic to human, fish and shellfish including *V. cholerae*, *V. mimicus*, *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. cincinnatiensis*, *V. hollisae*, *V. vulnificus*, *V. furnissii*, *V. damsela*, *V. metshnikovii*, and *V. carchariae*. All pathogenic vibrios have been reported to cause foodborne diseases; however, *V. cholerae* O1, *V. parahaemolyticus* and *V. vulnificus* are the most significant agents (Drake; DePaola; & Jaykus. 2007: 120-144; Gopal; et al. 2005: 151-159).

V. vulnificus is an opportunistic human pathogen that is highly lethal and is responsible for the overwhelming majority of reported seafood-related death in the United States. This bacterium is a part of the natural flora of coastal marine environment worldwide and has been isolated from water, sediment, and a variety of seafood, including shrimp, fish, clams and oyster (Jones; & Oliver. 2009: 1723-1733). Consumption of seafood, especially raw oyster, containing *V. vulnificus* can result in a severe, fulminant systemic infection. Characteristics of this disease include fever, chills, nausea, hypotensive septic shock, and the formation of secondary lesions on the extremities of patient (Oliver. 2006: 349-366; Strom; & Paranjpye. 2000: 177-188).

2. Characteristics of *Vibrio vulnificus*

Vibrio vulnificus is a Gram-negative, oxidase-positive, obligately halophilic, motile, curved bacillus with a single polar flagellum. This bacterium belongs to the family Vibrionaceae, and is distinguished from other members of the *Vibrio* genus by its ability to ferment lactose. This bacterium can grow in NaCl with a concentration as high as 5% and does not require Mg^{+2} or K^{+} for its growth. Consequently, *V. vulnificus* can easily be cultured in a variety of media. *Vibrio vulnificus* was first isolated by the US Centers of Disease Control (CDC) in 1964 (Strom; & Paranjpye. 2000: 177-188) and was initially

misidentified as *Vibrio parahaemolyticus*. The bacterium was designated as *V. vulnificus* in 1980 (Farmer. 1980: 656).

Vibrio vulnificus has been classified based on biotypes, lipopolysaccharide (LPS) antigens, capsule, and most recently DNA sequences. There are three biotypes of *V. vulnificus* according to their different biochemical and biological properties. Biotype 1 is ubiquitous in estuarine environments, is an opportunistic human pathogen, produces indole and ornithine decarboxylase, and exhibits several immunologically distinct lipopolysaccharide (LPS) types (Linkous; & Oliver. 1999: 207-214). Biotype 2 is primarily related with eels but can be pathogenic to humans, is characterized by a homogeneous lipopolysaccharide (LPS), and is negative for indole production and ornithine decarboxylase (Amaro; & Biosca. 1996: 1454-1457). The third group, biotype 3, was recently identified and causes wound infections and bacteremia in humans handling St. Peter's fish (*Tilapia* spp.) in Israel (Bisharat; et al. 1999: 1421-1424). Differential characteristics of the three biotypes of *V. vulnificus* are given in Table 1.

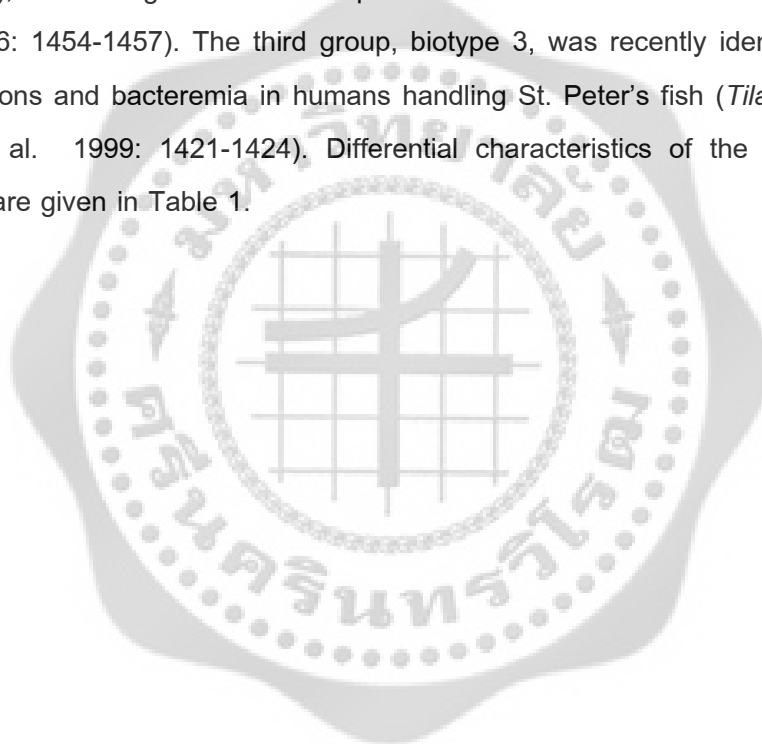


Table 1 Differentiation of three *Vibrio vulnificus* biotypes (Bisharat; et al. 1999: 1421-1424)

Test	Biotype		
	1	2	3
Citrate (Simmons)	+	+	-
Indole production	+	-	+
ONPG (o-Nitrophenyl--D-galactoside) test	+	+	-
Ornithine decarboxylase	+	-	+
Fermentation of :			
Cellobiose	+	+	-
Lactose	+	+	-
D-Manitol	+	-	-
Salicin	+	+	-
D-Sorbitol	-	+	-

3. Geographical range distribution and environmental conditions

The presence of *V. vulnificus* is not associated with pollution. This bacterium is normally found in estuarine environments, flourishes in warm temperatures. *V. vulnificus* is a part of the bacterial flora of the marine environment, and is distributed worldwide (Jones; & Oliver. 2009: 1723-1733). *Vibrio vulnificus* has been isolated from waters of widely varying temperatures and salinities (Blackwell; & Oliver. 2008: 146-153). Environmental conditions that correlate with increased prevalence of *V. vulnificus* include intermediate salinities (5-25 ppt), such as those found in estuaries, and higher water temperature (>20 °C), such as those observed during summer months (Motes; et al. 1998: 1459-1465). In fact, *V. vulnificus* optimal temperature growth is 35°C, and like all human pathogens can also grow at 37°C. However, this bacterium has never been responsible for human infections in that area where summer temperatures are optimal for its growth (Motes; & DePaola. 1996: 3875-3877). Under suboptimal temperatures the number of *V. vulnificus* increases during 6 days incubation at 13°C to 22°C, but *V. vulnificus* survives poorly at less than 8.5°C (Kelly.

1982: 820-824). Pfeffer et al. (2003: 3526-3531) reported that water temperature accounted for most of the variability in the concentration of *V. vulnificus*, and that low water temperatures (<10°C) have a negative effect for its isolation. There have been several theories to explain the seasonality observed in *V. vulnificus* isolation. One possible reason that might explain why *V. vulnificus* is difficult to culture during cold-water months is that this bacterium can enter a viable but non culturable (VBNC) state. It has been proven that *V. vulnificus* enters a VBNC state at temperatures below 10°C under low nutrient conditions (Oliver; et al. 1995: 2624-2630).

4. Illnesses caused by *V. vulnificus*

V. vulnificus can cause three different diseases including wound infection, gastroenteritis and primary septicemia. In addition to these three major diseases, *V. vulnificus* has been associated with other clinical syndromes, such as pneumonia, osteomyelitis, spontaneous bacterial peritonitis, eye infections, and meningitis (Daniels; et al. 2000: 1467-1470).

4.1 Wound infections

V. vulnificus has ability to enter to the body through a pre-existing wound. The symptoms begin within 24 hours of the incubation (CDC. 1996: 621-624). It usually starts as redness and swelling with the pain at the site of the wound. The illness will progressively affect the whole body and it is fatal about 20% of the time, to prevent death destructive surgical treatments are needed.

4.2 Gastroenteritis

This is the mildest human syndrome caused by *V. vulnificus*. Healthy individuals usually do not suffer from the other diseases but may suffer from light gastroenteritis. If the patients do get the illness, the symptoms occur within 16 hours of ingesting the organism. Many cases of *Vibrio* associated gastroenteritis are under recognized in clinical laboratories due to lack of the proper identification methods (Daniels; et al. 2000: 1467-1470). Therefore, rapid identification and enumeration of *V. vulnificus* from oysters are very desirable. A history of alcohol abuse and routine usage of antacid may also act as factors for gastroenteritis (Johnston; Becker; & McFarland. 1986: 336-338). The symptoms of *V. vulnificus* infection for the people with immunocompromising conditions may occur within two days.

4.3 Primary septicemia

Primary septicemia is the most momentous illness of *V. vulnificus* and the fatality rates of infections are 50- 60% mainly due to under cooked oysters. The symptoms occurred in immunocompromised patients within 36 hours of ingestion. Lipopolysaccharide (LPS) endotoxin in *V. vulnificus* is the major virulence factor for the disease. The immense numbers of cases occur in males over the age 53. It has been discovered that the major female hormone estrogen has an effect on LPS endotoxin and help to prevent action of the toxin (Merkel; et al. 2001: 6119-6122). The most commonly reported symptoms are fever, nausea and hypertension. The primary septicemia leads to secondary lesions filled with fluid (Chiang; & Chuang. 2003: 81-88).

5. Virulence factors

A variety of factors have been implicated as possible virulence determinants for *V. vulnificus*, including an exoenzyme, the ability to acquire iron from transferrin, the presence of a polysaccharide capsule and an endotoxic lipopolysaccharide. In addition, *V. vulnificus* strains demonstrate a variation between virulent and avirulent forms, with virulent forms being encapsulated, serum resistant, and possessing the ability to acquire iron from iron-saturated transferrin, while avirulent variants lack these characteristics.

5.1 Exoenzymes

Both clinical and environmental strains of *V. vulnificus* were tested for exoenzyme production and possible associated virulence. Several extracellular compounds are produced by this bacterium such as hemolysin, collagenase, DNase, lipase, elastase, phospholipase, chondroitinsulfatase, mucinase, fibrinolysin, hyaluronidase and a protease with activity against native serum albumin (Oliver; et al. 1986: 99-111).

The important virulence exoenzyme of *V. vulnificus* is hemolysin. Oliver et al. (1986: 99-111) found that hemolysin of *V. vulnificus* may be significant in fatal infection caused by *V. vulnificus*. Stelma et al. (1992: 2776-2782) reported that virulent *V. vulnificus* produced high titres of hemolysin activity and suggested that protein that occupies hemolysin activity might be potential virulent factor. A pore-forming hemolysin (*vvhA*) is known to be cytolytic to Chinese hamster ovary (CHO) cell and to disrupt the membrane of various mammalian erythrocytes (Kim; et al. 1993: 571-577). However, although correlation between hemolysin and the virulence of *V. vulnificus* strain have been reported as

described above, a mutant deficient in production of hemolysin (*vvhA*) was still virulent in mice, as assayed in the other animal (Wright; & Morris. 1991: 192-197).

5.2 Iron utilization

The amount of iron available in the host is an important factor influencing the lethality of *V. vulnificus*. While some strains of *V. vulnificus* are able to overcome the iron sequestering mechanisms, e.g. transferrin, lactoferrin, haptoglobin, of the human host, successful infection appears to require an increase in transferrin saturation. Wright et al. (1981: 503-507) have directly correlated virulence with host iron availability. The result showed that *V. vulnificus* does not grow in normal human serum and reported a lowered 50% lethal dose (LD50) caused by the injection of iron, in the form of ferric ammonium citrate, prior to the injection of bacterial cells in mice. This experiment regarding *V. vulnificus* infection has led to various studies of the organism's ability to obtain iron from mammalian hosts.

V. vulnificus simultaneously produces both phenolate and hydroxamate siderophores, with the phenolate siderophore enabling virulent isolates to acquire iron from highly saturated transferrin (Morris; et al. 1987: 55-59). Similar results have been reported for other iron binding proteins such as lactoferrin and ferritin (Simpson; & Oliver. 1987: 155-157). It appears that cleavage of these proteins is caused by an exoprotease, thereby releasing iron to the siderophores. To confirm the iron requirement is needed for *V. vulnificus* virulence, Litwin et al. (1996: 2834-2838) reported that a mutagenized virulent strain which loss phenolate siderophore production was no longer able to use transferrin at 100% iron saturation, and displayed reduced virulence.

5.3 Capsule

The presence of a capsule, which is also related to colony opacity, is probably the best known virulence factor for *V. vulnificus*. *V. vulnificus* is an extra cellular pathogen that relies on its polysaccharide capsule to avoid phagocytes of the host cells (Linkous; & Oliver. 1999: 207-214; Strom; & Paranjpye. 2000: 177-188). The transformation of encapsulated isolates to the nonencapsulated form is dependent on growth phase and temperature, which in turn affect bacterial cell morphology. Wright et al (1990: 1769-1773) discovered an increase in the expression of capsular polysaccharide (CPS) during the logarithmic growth phase and a decrease during the stationary phase of growth for a clinical isolate of *V. vulnificus*. Also, there was significant expression of CPS observed for cell grown at 30 °C as compare to those grown at 37 °C. Encapsulated isolates have opaque

colony morphology but can undergo a reversible phase variation to the translucent colony phenotype, which is correlated with reduced CPS production (Wright; et al. 1990: 1769-1773; Strom; & Paranjpye. 2000: 177-188). Wright et al (1990: 1769-1773) reported that nonencapsulated strains (clinical) produced by transposon mutagenesis had a lethal dose over 4 times higher than that of the encapsulated strains. Research has showed that infection with *V. vulnificus* elicits an antibody response specific to the capsule (Foire; et al. 1992: 428-431) and *V. vulnificus*, like other bacteria, relies on the capsule to resist host defenses during systematic disease.

5.4 Endotoxin

The presence of capsule is essential for the ability of *V. vulnificus* to avoid host phagocytosis, and thus initiate infection, the factor that most likely causes the shock and death associated with *V. vulnificus* infections appears to be the cell's Lipopolysaccharide (LPS), or endotoxin. The major symptoms associated with *V. vulnificus* infections, including fever, tissue edema, hemorrhage and hypotension, are classical symptoms associated with endotoxin shock. McPherson et al. (1991: 272-273) found that injection of purified LPS extracted from *V. vulnificus* resulted in a rapid decline in heart rate and blood pressure in rats, with death resulting within 60 minutes. Elmore et al. (1992: 391-397) used an inhibitor of the LPS-induced enzyme (nitric oxide synthase) responsible for this symptoms and found complete inhibition of these toxic effect.

6. Response of *Vibrio vulnificus* to various stresses

Vibrio vulnificus can be found in seawater, oysters and other molluscan shellfish as described above. In its natural habitat, food processing environment or during human infection, *V. vulnificus* encounters different environmental stresses. The response of *V. vulnificus* to various stresses has been examined, including starvation (Bang; & Drake. 2002: 975-980), acid, heat, cold (Bang; & Drake. 2002: 975-980; Bryan; et al. 1999: 168-175), hyperosmolarity (Simpson; Engle; & Oliver. 1994: 209-214) and low salinity (Wong; & Liu. 2008: 416-419). Environmental stress often induces the expression of specific sigma factors which regulate the expression of factors involved in stress protection as well as virulence of pathogenic bacteria. Rpos is the sigma factor frequently produced in stationary phase and in cell responding to different stresses (Jishage; et al. 1996: 5447-5451).

6.1 RNA polymerase sigma factor S gene (*rpoS*)

The *rpoS* gene encodes a sigma factor, σ^S , RpoS, which is required for expression of a large number of genes in response to various stresses, including nutrient limitation, osmotic challenge, and during growth into stationary phase (Loewen; et al. 1998: 707-717). The *rpoS* gene has been found in a variety of Gram-negative bacteria, and the function and regulation of this gene have been studied extensively in the enteric species *E. coli* and *Salmonella enterica* serovar Typhimurium. RpoS can act as a virulence factor since it activates some of the genes necessary for pathogenesis in several bacteria, including *E. coli* and *Shigella flexneri* (Olsén; et al. 1993: 523-536; Small; et al. 1994: 1729-1737). In fact, *rpoS* expression is induced when these bacteria enter mammalian cells (Chen; et al. 1996: 4739-4743). The *rpoS* levels tend to be very low in rapidly growing cells not exposed to any stress. However, the *rpoS* levels increase in response to a variety of environmental stresses, which included starvation for limiting nutrients, stationary phase, high osmolarity, and high or low temperature. RpoS is a highly unstable protein under non-stress conditions, but exposure to various stresses differentially affects *rpoS* transcription and translation, as well as the rate of proteolysis. Research on the regulation of *rpoS* gene expression has mainly focused on the control of RpoS translation and RpoS proteolysis (Loewen; et al. 1998: 707-717; Lange; & Hengge-Aronis. 1994: 1600-1612)

6.2 DNA-binding transcriptional regulator gene (*oxyR*)

The gene named *oxyR* is a peroxide sensor and a transcriptional activator of the oxidase stress response. This gene has been proved to be a positive regulator of the expression of hydrogen-peroxide-inducible genes, including catalase (*katG*), glutathione reductase (*gor*), glutaredoxin (*grxA*), alkyl hydroperoxide reductase (*ahpCF*), *oxyS* (a regulatory RNA), *fur* (ferric uptake regulation), and *dps* (a nonspecific DNA binding gene) (Christman; et al. 1985: 753-762; Morgan; et al. 1986: 8059-8063). OxyR-regulated enzymes are involved in the defense against reduced-oxygen compounds. OxyR is required for adaptation to H₂O₂ (Jamet; et al. 2005: 376-381). The *oxyR* gene has been identified in several bacterial genomes that included *V. vulnificus* (del Camen Vargas; et al. 2003: 1165-1176; Kong; et al. 2004: 133-142). The position of this gene is variable in the several bacterial genomes, but it is often next to a gene involved in oxidative stress protection and regulated by OxyR. The expression of peroxide defense genes is activated by OxyR in the exponential phase in *E. coli*. In the stationary phase, expression of *oxyR* is controlled by the starvation-induced sigma factor RpoS. OxyR mediates a response to oxidative stress in *E.*

coli. The change in *oxyR* expression during aerobic growth is completely abolished in the absence of oxygen (Gonzalez-Flecha; & Demple. 1997: 6181-6186).

6.3 catalase/hydroperoxidase HPI(I) gene (*katG*)

Most bacteria appear to exhibit one or more catalases in response to peroxidase stress. *V. vulnificus* has been shown to have only one catalase gene encoding the catalase/ hydroperoxidase (*katG*) (Kong; et al. 2004: 133-142). This gene is induced during the bacterial exponential phase (Triggs-Raine; & Loewen. 1987:121-128). The expression of the *katG* is controlled by the positive transcriptional activator OxyR, and it is induced in response to exogenous H₂O₂ (Morgan; et al. 1986: 8059-8063), which directly sense oxidative stress (Kim; et al. 2002: 383-396; Zheng; Åslund; & Storz. 1998: 1718-1722). Bacteria produce H₂O₂ under aerobic conditions to be toxic, and *katG* is primarily responsible for its detoxification (Panek; & O'Brian. 2004: 7874-7880).

7. Diagnosis of *V. vulnificus*

The family Vibrionaceae includes the genera, *Vibrio*, *Plesiomonas* and *Photobacterium*. Members of the family are usually found in aquatic environments but can also be isolated from human and other animals in which they may be pathogenic (Baumann; Furniss; & Lee. 1984: 518-538). *Vibrio* spp. often occurs in association with plankton and their numbers increase in parallel with seasonal planktonic bloom (Twedt. 1989: 544-568; West. 1989: 1-34). Various methods have been developed for rapid diagnosis of *Vibrio* spp.

7.1 Bacterial culture

Several isolation and identification techniques have been developed to distinguish *V. vulnificus* from other *Vibrios*. Thiosulfate citrate bile salts sucrose (TCBS) agar was originally used for isolation of pathogenic vibrios and became widely used for the isolation of *Vibrio* spp. from environmental samples. This medium differentiates *V. cholerae* from *V. parahaemolyticus* and *V. vulnificus* on the basis of sucrose fermentation (Kelly. 1982: 820-824; Roberts; et al. 1982: 299-312). Sodium dodecyl sulfate-polymyxin B-sucrose agar (SPS) was used to separate *V. vulnificus* from *V. parahaemolyticus*, which are both negative for sucrose fermentation and had similar morphology on TCBS (Kitaura; et al. 1983: 205-209). Several selective and differential media have been developed for *V. vulnificus* isolation including *V. vulnificus* (VV) agar (Brayton; et al. 1983: 1039-1044), cellobiose-polymyxin B colistin (CPC) agar (Massad; & Oliver. 1987: 2262-2264), modified

CPC (mCPC) agar (Tamplin; et al. 1991: 1235-1240), *V. vulnificus* agar (VVA) (Wright; et al. 1993: 541-546), *V. vulnificus* enumeration (VVE) agar (Miceli; Watkins; & Rippey. 1993: 3519-3524), cellobiose-colistin (CC) agar (Hoi; Dalsgaard; & Dalsgaard. 1998: 1721-1724), *V. vulnificus* medium (VVM) (Cerdeña-Cuellar; Jofre; & Blanch. 2000: 855-859) and modified VVM (VVMc) agar (Cerdeña-Cuellar; et al. 2001: 322-327). Selective media used to isolate *V. vulnificus* from other non-*vulnificus* *Vibrio* in infected animals and environment samples are time-consuming for bacterial growth and not suitable for routine use by farmers.

7.2 Immunodiagnosis

Several immunological based assays have been developed for detection of *V. vulnificus* and non-*vulnificus* *Vibrio*. This method does not required special instruments and highly skilled personnel. Therefore, immunological based methods may be feasible for small laboratories or field diagnosis.

Simonson and Siebeling (1986: 1299-1304) used anti-H antibodies for identification of *V. vulnificus*. *Staphylococcus aureus* Cowan I cells were armed with anti-flagellar (anti-H) antibody produced in rabbits immunized with flagellar core protein prepared from *V. vulnificus*. After the addition of the anti-*V. vulnificus* H coagglutination, 99.3% of *V. vulnificus* isolates were agglutinated in the slide test within 2 min. Other *Vibrio* spp. recovered from seafood and the marine environment did not agglutinate after the addition of this reagent. The serological resolution of the coagglutination reaction was enhanced by suspending in 0.1 M Tris buffer-0.1 mM EDTA-1.0% Triton X-100 (TET) for 24 h before serological examination. The TET buffer also increased the sensitivity of the coagglutination reaction 100-fold over that for isolates suspended in 0.3% formalinized phosphate-buffered saline before testing.

Martin and Siebeling (1991: 1684-1684) produced monoclonal antibodies against lipopolysaccharide for identification of *V. vulnificus* O serovars. Rabbits were immunized with heat-killed whole-cell *V. vulnificus* for production of polyclonal antibodies. BALB mice were immunized with formalin-killed *V. vulnificus* cells for production of monoclonal antibodies. Spleen cells of BALB mice were fused with SP2/O-Ag14 myeloma cells. Hybridomas were screened by using LPS and whole cells ELISA to identify clones secreting antibodies specific to LPS of *V. vulnificus*. Monoclonal antibodies identified five serogroup of *V. vulnificus* LPS and a single group of each *V. damsela* and *V. hollisae*. Cross reactivity between *V. vulnificus*, *V. damsela* and *V. hollisae* were not performed.

Rengpipat, Pusiririt and Rukpratanporn (2008: 398-404) produced monoclonal antibodies against two isolates of *V. vulnificus* (VVC and VVB). MAbs were divided into five groups according to their specificities to different *V. vulnificus* isolates and apparent protein antigens with ranged from 3-50 kDa. The sensitivity testing for detection of *V. vulnificus* with dot blot assay ranged from 1.6×10^5 to 4×10^8 CFU ml⁻¹ depending on the group of MAbs.

In 2010, six monoclonal antibodies specific to *V. vulnificus* flagella were produced by immunizing mice with purified *V. vulnificus* ATCC 27562 flagellin (Jadeja; Janes; & Simonson. 2010: 1288-1293). Antibodies that exhibited high titres were coated onto Cowan I *Staphylococcus aureus* cells and sheep anti-mouse immunoglobulin G immunomagnetic beads. The two reagents were used to determine the species specificity of the selected antibodies, which positively identified and coagglutinated 70 *V. vulnificus* isolates and did not react with other *Vibrio* species. Immunomagnetic separation (IMS) protocol was optimized for PBS and oyster homogenate spiked with three different strains of *V. vulnificus*. IMS with *V. vulnificus*-spiked PBS yielded binding of 19-57%, and IMS with spiked oyster homogenate carried out at two *V. vulnificus* levels exhibited binding of 25-57%.

7.3 DNA hybridization

An assay based on DNA probe and DNA hybridization has been used to enumerate *V. vulnificus* in oysters in several studies (Wright; et al. 1993: 541-546). This method provides a more rapid and accurate evaluation of post-harvest treatment of oysters (Harwood; Gandhi; & Wright. 2004: 301-316). The sensitivity of DNA probe hybridization assays for detection of vibrios in oyster homogenates approaches 10 CFU/g oyster homogenate. The identification of bacteria by DNA probe hybridization is based on the presence and absence of particular genes. The physical basis for gene probe tests stem from the structure of DNA molecules themselves. DNA hybridization tests may be performed in many ways; one technique is the colony hybridization assay (Miceli; Watkins; & Rippey. 1993: 3519-3524). For example, an aliquot of a homogenized food is spread on the appropriate agar plate medium. After incubation, the colony pattern is transferred to a solid support (usually membrane or paper filter). Then, the cells are lysed by a combination of high pH and temperature, which also denature and fix the DNA to the support. The solid supports with the attached target DNA are incubated with an enzyme-labeled specific probe. The labeled probe failing to attach to the target is removed by stringent washing (USFDA. 2001: online). The radioactive probe DNA that is bound to the target DNA on the support is often detected by a colorimetric reaction. A chromogenic substrate then is added

to the membrane. Where the probe-associated enzyme is present, a color spot will develop. Each spot represents a bacterial colony that has arisen from a single cell. The number of cells harboring the target gene in the original sample can be calculated by multiplying the number of spots by the dilution factor (USFDA. 2001: online).

7.4 Polymerase chain reaction (PCR)

The molecular based methods especially PCR may enable easy and rapid identification of many pathogenic organisms in seafood and aquatic animal. An important factor in evaluating PCR is the specificity of the DNA sequence chosen for the gene of interested strains. Size of amplified fragment should be verified by hybridization to a specific DNA probe, digestion with a restriction enzyme or DNA sequencing of the DNA fragment (Olson. 1995: 394-396).

In 1991, Hill et al used PCR targeted to cytotoxin-hemolysin gene to identify *V. vulnificus* in artificially contaminated oyster. After 24 h of incubation in alkaline peptone water, positive PCR results can be obtained with DNA extracted from oyster homogenates seeded with 10^2 CFU of *V. vulnificus* per g of oyster.

Brauns, Hudson and Oliver (1991: 2651-2655) used PCR for detection of *V. vulnificus* targeted to cytotoxin-hemolysin gene. As little as 72 pg of DNA from culturable cells and 31 ng of DNA from nonculturable cells could be detected.

Brasher et al (1998: 101-107) used multiplex PCR for detection of microbial pathogens in shellfish. In the case of *V. vulnificus* detection, a set of primers targeted to cytolsin-hemolysin gene was designed. The sensitivity of detection was $\leq 10^1 - 10^2$ CFU. This gene was well known as hemolysin gene (*vvh*) and this primer set was usually used for detection of *V. vulnificus* by several researchers.

Campbell and Wright (2003: 7137-7144) developed a *TaqMan* real-time PCR assay targeted to *vvhA* gene for detection of *V. vulnificus*. Probe specificity was confirmed by amplification of 28 *V. vulnificus* templates and by the lack of a PCR product with 22 non-*V. vulnificus* strains. Detection of *V. vulnificus* in pure cultures was observed over a 6-log-unit linear range of concentration (10^2 to 10^8 CFU ml^{-1}), with a lower limit of 72 fg of genomic DNA/ μl of PCR mixture or the equivalent of six cells. Viable but nonculturable cells were also enumerated by real-time PCR and confirmed by the *BacLight* viability assay.

In 2004, Panicker et al developed a multiplex PCR for the detection of pathogenic *Vibrio* spp. from coastal water and shellfish (Panicker; et al. 2004: 7436-7444). Primer sets have been designed to detection of targeted genes such as *vvh* and

viuB genes to verify *V. vulnificus*; *ompU*, *toxR*, *tcpl*, and *hlyA* genes to verify *V. cholera*; and *tlh*, *tdh* and *trh* to verify *V. parahaemolyticus*. The result revealed that the detection sensitivity for pure cultures without enrichment was $10^2 - 10^3$ CFU/ml, and the specificity was 100%. The sensitivity for 5h spiked oyster samples was 10^1 CFU per g of oyster tissue homogenate.

Panicker and Bej (2005: 5702-5709) compared three sets of oligonucleotide primers and two probes designed for *V. vulnificus* hemolysin A gene (*vvhA*) for TaqMan-based real-time PCR method for detection of *V. vulnificus* in oysters. Two sets of primers with a specific probe (F-vvh758/R-vvh990 and F-vvh731/R-vvh1113 primer with P-vvh874 probe) were specific to detection of all 81 *V. vulnificus* isolates. Cross reactivity were not performed. However, the third set of primers with a probe (F-vvh1059/R-vvh1159 primer with P-vvh1109 probe) exhibited positive reactivity to other *Vibrio* spp. The detection limit of both F-vvh758/R-vvh990 and F-vvh731/R-vvh1113 primer with P-vvh874 probe were 1 pg of genomic DNA and 10^3 CFU ml⁻¹ in pure cultures. In the case of detection of *V. vulnificus* in seeded oyster tissue homogenate, the detection limit was 3×10^3 CFU *V. vulnificus* resulting from an initial cell count of 1 CFU in 1 g of seeded oyster tissue samples after 5 h of enrichment.

Dalmasso et al (2009: 21-25) used PCR method for the detection of *rpoA* genes for the screening of *Vibrio* genus. The *rpoA* primers correctly amplified all of *Vibrio* species considered. No cross reaction was observed when tested against closely related species. The sensitivity was determined using serial dilutions of difference pure cultures of certified strains, resulted to 10^3 CFU/ml⁻¹.

Takahashi et al (2005: 77-85) designed specific primers and probe for real-time PCR targeted to *toxR* gene for detection of *V. vulnificus* in seawater and oyster samples. The result showed that the assay was very sensitive, detecting as few as 10 microbes per milliliter of seawater and oyster homogenate. The amounts of *V. vulnificus* counted by TaqMan assay were similar to those by a culture method in most of the samples.

Kumar et al (2006: 216-220) developed PCR for detection of *V. vulnificus* in oyster samples. A set of primers has been designed for amplified specific region of *gyrB* gene of *V. vulnificus*. The sensitivity of *gyrB* PCR to detection of *V. vulnificus* in pure culture was 3 CFU/reaction. The sensitivity of *gyrB* PCR in spiked oyster samples without enrichment was 300 CFU/g, whereas with 18 h enrichment in alkaline peptone water was 30 CFU/g.

The detection of *V. vulnificus* by real-time PCR targeted to *rpoS* gene has been developed by Kim et al. (2008: 1841-1847). The result showed that *rpoS* PCR was highly specific to detection of *V. vulnificus*. Cross reactivity was not performed. The sensitivity of detection by real-time PCR was 2 pg of purified genomic DNA, or 10^3 *V. vulnificus* cells from pure culture and 10^3 cells in 1 g of oyster tissue homogenate.

8. Monoclonal antibodies

Monoclonal antibodies are antibodies produced by one expanded clone of B-lymphoid cells (blast cells and/or plasma cells). All antibody molecules have the same amino acid sequence and same class of immunoglobulins with an identical antigen combining site (Edwards; et al. 1982: 272-277; Kennedy; & Dreesman. 1983: 385-389). They are directed to a single epitope on the antigen and they are available in essentially unlimited quantities as the major component of ascites of fluid-derived immunoglobulin.

In 1975 Kohler and Milstein made a report on the continuous culture of fused cells secreting antibody of predefined specificity (Kohler; & Milstein. 1975: 495-497). They reported on the fusion of two cell populations which yielded a cell line with the desirable characteristics of both parent cells i.e., B lymphocyte hybridomas are made by fusing B lymphocytes with myeloma cells. The lymphocytes contribute specific antibody producing genes to the hybridoma while the myelomas provide genes that allow the hybridoma to divide indefinitely and continue to express the immunoglobulin gene.

8.1 Principle of monoclonal antibody production

The fusion of B lymphocyte cells and myeloma cells is a relative rare event, even when PEG or Sendai virus is used. Thus, if myeloma cells are fused with B lymphocyte from spleen, the culture will be rapidly overgrown by unfused myeloma cells. Selective medium is needed in this step is a way to suppress the myeloma growth but ensure that only the hybrids will grow. By far the popular selective systems are the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and the adenine phosphoribosyl transferase (APRT) selective system (Littlefield. 1964: 709-710). The mechanism of hybridoma production was described below.

The main biosynthetic pathways of purine and pyrimidine can be blocked by the folic acid antagonist, aminopterin, and that of purine can be blocked by glutamine antagonist and azaserine. As a safe guard for survival, the normal cells possess alternative salvage pathways to overcome this biosynthesis. In these pathways, the recycle

synthesis is carried out from single building stones which include diphosphoribose phosphate, glutamine, glycine and other free bases from nucleic acid degradation via the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) which catalyzes the formation of inosinate (IMP) and guanylate (GMP) from hypoxanthine and guanine, respectively. The enzyme adenine phosphoribosyl transferase (APRT) catalyzes the formation of adenylates (AMP) from free adenine bases. Salvage pathway also depends on the enzyme thymidine kinase (TK); but selection by TK is more difficult, because the occurrence of TK negative variant is rare events (Goding. 1980: 285-308). In selective system, the myeloma cells which are defective in the enzyme HGPRT (HGPRT⁻), die in tissue culture medium containing hypoxanthine (H), aminopterin (A) and thymidine (T) or HAT (Littlefield. 1964: 709-710) (Figure 1). They cannot multiply in the presence of aminopterin, even when supplied with hypoxanthine and thymidine. The cell which produces enzyme TK and HGPRT can survive. Thus only the hybrids between the myelomas and spleen cells survive in the selective medium, since the myelomas provided the ability to grow in tissue culture and spleen cells contributed the functional HGPRT enzyme necessary to overcome the aminopterin block. In fact, this system is a half-selective medium, since the spleen cells which are normal cells die off naturally.

Another selective system was based on the property of the enzyme APRT. The medium contains azaserine to block de novo purine synthesis. Myeloma cell line lacking this enzyme (APRT) cannot complete their nucleic acid synthesis and die off. Mutant myeloma cells lacking HGPRT are selected by the use of toxic drug to incorporate into DNA of the myeloma cells via HGPRT. The HGPRT producing cells die and only the mutant that lost of HGPRT will survive for selection. Production of the cells that lack of HGPRT is relatively easy, since the enzyme is coded for by a gene on X-chromosome, and thus only a single mutation is needed to result in total loss of the enzyme (Littlefield. 1964: 709-710).

8.2 Fusion of myeloma cells and immune cells

Spontaneous fusion of cells is usually rare except that of sperms, eggs, osteoblasts, myotubes, foreign body giant cells or certain virus infected cells (syncytium formation) (Ringertz; & Savage. 1976: 366). However, normal cell fusion can possibly be made by addition of cell fusogens such as Sendai virus (Knutton; & Pasternak. 1979: 220-223), polyethylene glycol (PEG) (Pontecorvo. 1975: 397-400) or lysolecithin. PEG is now the reagent of choice. The mechanism of fusion is poorly understood (Poste; &

Nicolson. 1978:); (Knutton; & Pasternak. 1979: 220-223; White; & Helenius. 1980: 3273-3277). However, when cells are treated with fusogen, their embranes fuse and multinucleated cells, called heterokaryons, are formed (Ringertz; & Savage. 1976: 366). Later, the nuclei of heterokaryons fused. At the next cell division, the hybrid cells possess a more or less equal share of the genetic material (Goding. 1980: 285-308).



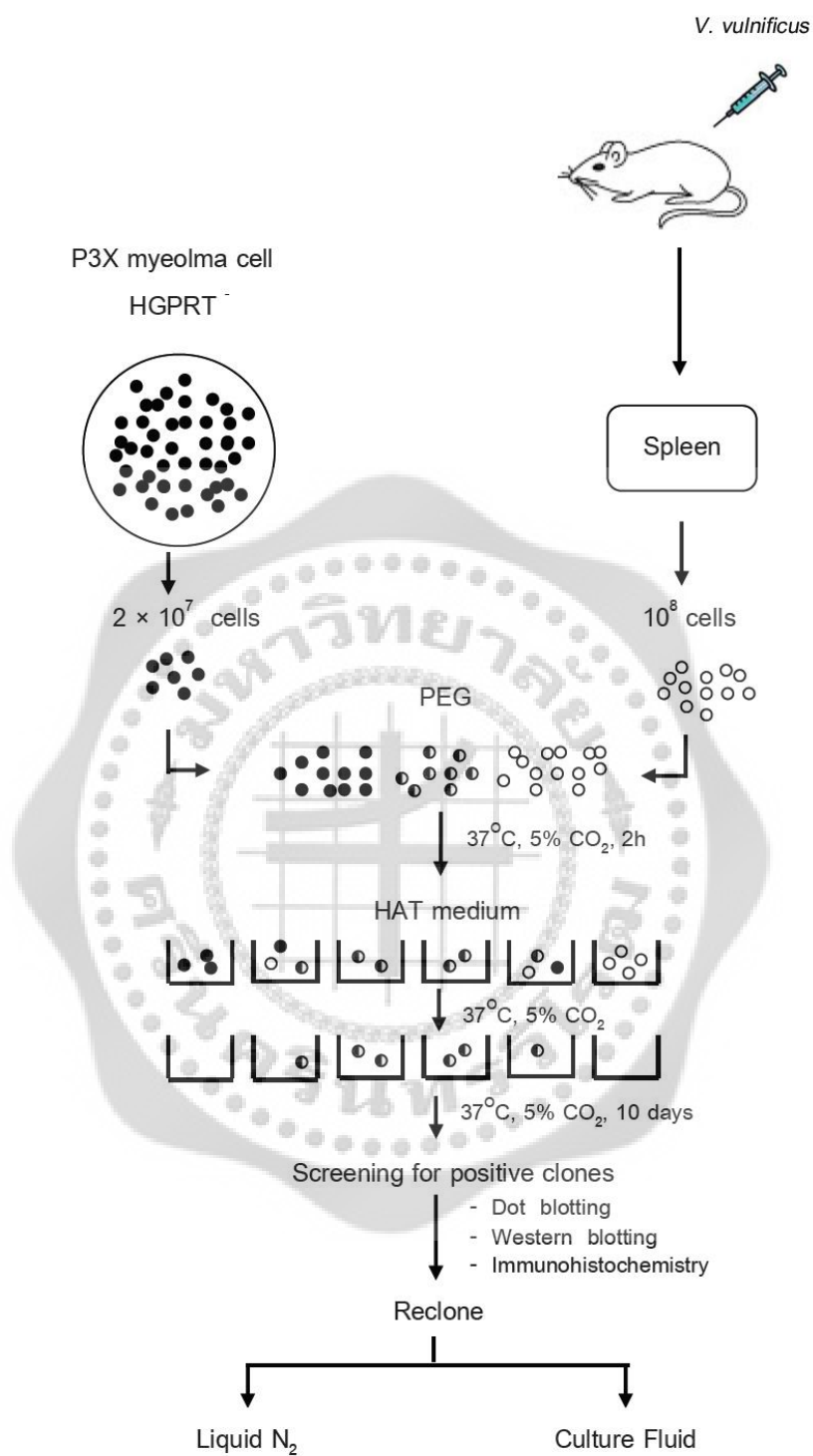


Figure 1 Monoclonal antibodies production (Sithigorngul; et al. 2000b: 27-34)

9. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) assays allow amplification of nucleic acid with high specificity, sensitivity and rapidity under isothermal conditions. LAMP originally described by Notomi et al. (2000: e63). This method can amplify target nucleic acid to 10^9 copies at 60–65 °C within 60 minutes. The method relies on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment, a DNA polymerase with high strand displacement activity, and a set of two specially designed inner primers and two outer primers. LAMP is highly specific because of the recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences in the later stages of the LAMP reaction. As the reaction is conducted under isothermal conditions, LAMP can be performed with a simple and inexpensive water bath and a thermal cycler is not required. As there is no time-loss for thermal changes, amplification efficiency is extremely high (Parida; et al. 2004: 257-263; Savan; et al. 2005: 573-581).

Analysis of LAMP products (amplicons) is usually carried out by agarose gel electrophoresis, followed by ethidium bromide staining. The principle of the loop-mediated isothermal amplification (LAMP) method is described below. For ease of explanation in primer design of the LAMP reaction, six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c and B3 from the 5' end. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) consists of two distinct sequences corresponding to F2 and F1c sequences in the target, with a TTTT spacer in between. Similarly, BIP consists of the complementary sequence to B1c, a TTTT spacer and the sense B2 sequence. FIP and BIP initiate LAMP cycling by structuring the loop while the outer primers initiate the strand displacement of DNA (Notomi; et al. 2000: e63).

The LAMP reaction begins when the inner primers (FIP) hybridizes to F2c in the target DNA and initiates complementary strand synthesis (Figure 2). The outer forward primer (F3), which is a few bases shorter and lower in concentration than FIP, slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end (structure 4). This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of dumb-bell shape (structure 6). Then it is quickly converted into a stem-loop DNA by self-primed DNA synthesis (structure 7). This stem-loop DNA then serves as the

starting material for LAMP cycling, the second stage of LAMP reaction. To initiate LAMP cycling, FIP hybridizes to the loop in the stem-loop DNA (structure 7) and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem-loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via the BIP sequence (structure 8). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA (structure 10) and one gap repaired stem-loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end (structure 9). Both these products then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles, a part of which is designated the elongation and recycling step, illustrated in the right half of Figure. Therefore, in LAMP the target sequence is amplified 3-fold every half cycle. (Notomi; et al. 2000: e63). When the LAMP amplicons are visualized by agarose gel electrophoresis, a ladder-like pattern of different size fragments is seen in each single well.

Nagamine et al. (2002: 223-229) have been developed loop primers to decrease the total LAMP reaction time. The loop primers (either LF or LB) contain sequences complementary to the single stranded loop region (either between the F1 and F2 regions, or between the B1 and B2 region) on the 5' end of the dumbbell-like structure and provide an increased number of starting points for DNA synthesis in the LAMP reaction. In the original LAMP method, only two of these loops would be used, but by using loop primers, all of the single-stranded loops can be used as starting points for DNA synthesis (Figure 3). The use of loop primers (rapid method) can shorten the total amplification time to one-third to one-half of that without loop primers.

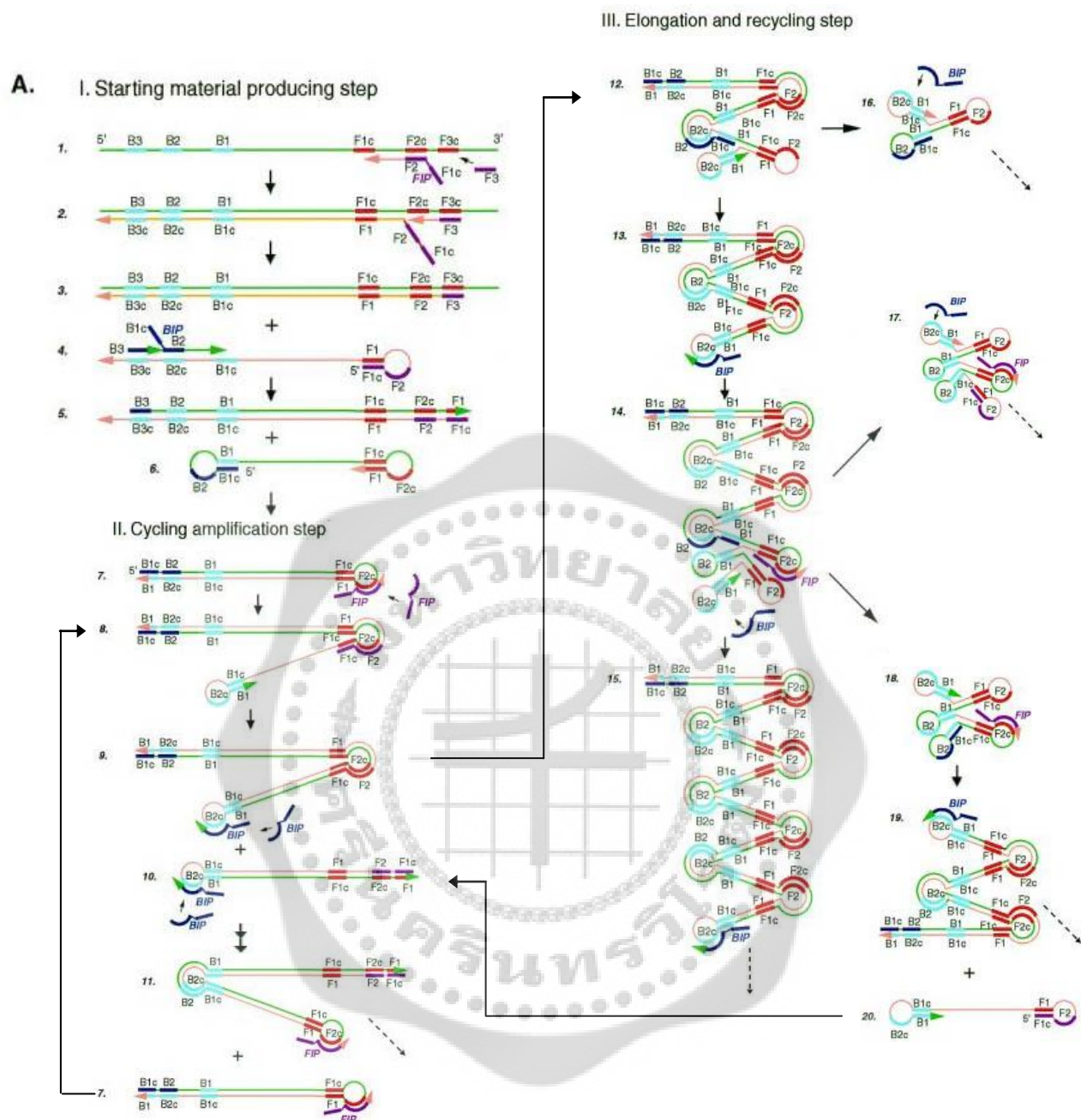


Figure 2 Loop mediated isothermal amplification for amplification of target DNA.

(Notomi; et al. 2000: e63)

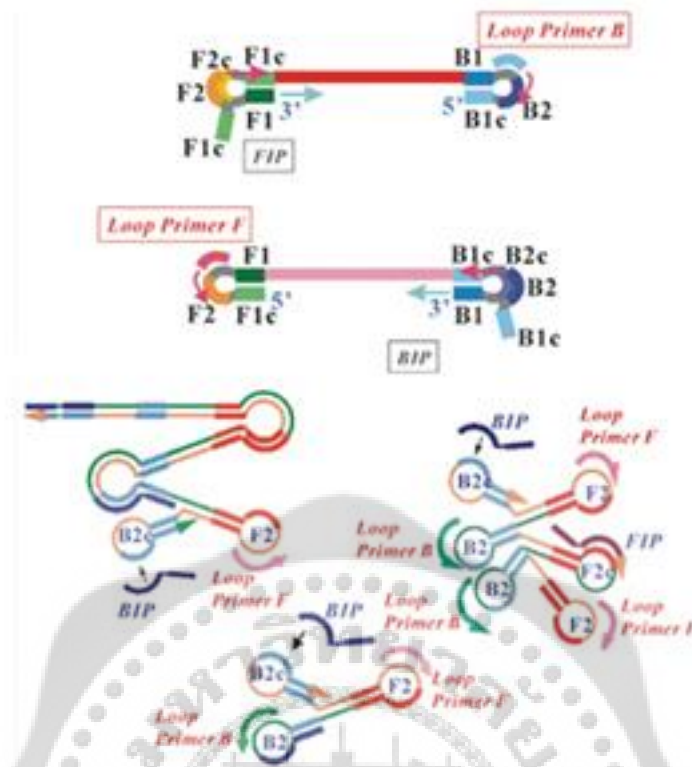


Figure 3 Loop primers for decrease the total LAMP reaction time
(Nagamine et al. 2002: 223-229)

9.1 Detection of *V. vulnificus* using LAMP

Several techniques had been used for identification of *V. vulnificus* as described above. The new technique termed LAMP has been developed for detection of *V. vulnificus* in several reports.

Han and Ge (2008: 311-320) designed a set of LAMP primers targeted to cytolyisin/hemolysin gene (*vvhA*) for detection of *V. vulnificus*. No false-positive or false-negative results were observed. The detection limit of the LAMP assay was approximately 20 CFU in pure cultures, 10-fold more sensitive than PCR. In the case of spiked oyster homogenate sample, the LAMP assay had a detection limit of approximately 10^7 CFU/g. After 5-hour enrichment, LAMP was capable of detecting 7 CFU of *V. vulnificus* per gram of oyster tissue.

Ren et al (2009: 514-521) developed a LAMP technique for detection of *V. vulnificus* targeted to cytolyisin gene. The LAMP reaction was processed in a heat block at 65°C for 60 minutes. The results showed that the LAMP reaction was highly specific to *V. vulnificus*. This method was 10-fold more sensitive than conventional PCR.

Li et al (2010: 93-97) used the LAMP and PCR targeted to virulent-correlated gene (*vcgC*) of *V. vulnificus*. No cross-reaction was observed in both LAMP and PCR assay. The detection limits were 10^2 CFU ml⁻¹ (16 copies per reaction) and 10^3 CFU ml⁻¹ (180 copies per reaction) for LAMP and PCR, respectively.

Han and Ge (2010: 60-66) developed the LAMP assay coupling with either fluorescence- or turbidity-based real-time platforms for the quantitative detection of *V. vulnificus* in raw oysters. Both real-time platform assays possessed good specificity, with no false positive or false negative results among 38 *V. vulnificus* and 42 other bacteria isolates. Comparable sensitivity was obtained using the two real-time platforms, with the assay limits of detection to be approximately 1–10 CFU/reaction of *V. vulnificus* in pure culture, up to 100-fold more sensitive than PCR. When applied to spiked oyster samples, the real-time LAMP assays were able to detect 6.4×10^4 CFU/g of oyster without enrichment, 1000 fold more sensitive than PCR.

Han et al (2011: 2589-2595) designed a set of primers targeted to *vcgC* gene for detection of potentially virulent *V. vulnificus* strains in raw oysters. Cross reactivity was not observed. The detection limit was 5.4 CFU per reaction for a virulent *V. vulnificus* strain in pure culture, 100-fold more sensitive than that of PCR. In the case of spiked raw oysters, the assay was capable of detecting 2.5×10^3 CFU/g of *V. vulnificus*, while showing negative results for a nonvirulent *V. vulnificus* strain spiked at 10^7 CFU/g. After 6 h of enrichment, the LAMP assay could detect 1 CFU/g of the virulent *V. vulnificus* strain.

10. The lateral-flow dipstick (LFD) technique

Non-specific amplification products from LAMP reactions may cause false positive results using gel electrophoresis. To solve this problem, the identity of LAMP products can be confirmed by restriction endonuclease digestion (Notomi; et al. 2000:e63) and hybridization with specific probes (Mori; Hirano; & Notomi. 2006: 3). In order to further simplify and speed up the total time for the LAMP-based assay, amplicon detection by an affinity chromatography technique combined with the lateral-flow technology was developed. Lateral-flow dipstick (LFD) is a novel technique that can be used to detect target genes rapidly using the principle of DNA hybridization and chromatography in membrane strips.

In principle, generic LFD strips (Milenia® GenLine HybriDetect) were used to detect biotin labeled amplicons hybridized with an FITC labeled DNA probe linked to a nanogold-conjugated primary antibody (1° Ab) or anti-FITC (fluorescein isothiocyanate)

antibody (e.g. goat anti-mouse IgG or goat antirabbit IgG antibodies), that binds specifically with a FITC labeled on 5' DNA probe. The test line of the membrane is coated with streptavidin whereas the control line is coated with a respective secondary antibody (2° Ab) against the gold- labeled 1° Ab.

To visualize LAMP products using LFD, the LAMP reaction must be carried out using biotin-labeled FIP or BIP. After amplification, an FITC-DNA probe is added to the LAMP reaction vial and allowed to hybridize with any biotin-labeled LAMP amplicons. After that, the LFD is dipped into a tube containing the mixture of applied buffer and LAMP products. As a result of capillary force, the triple complex (biotin-labeled LAMP product that has been hybridized with an FITC-DNA probe complex and bound to a gold-labeled anti-FITC antibody) is trapped at the test line by a molecule specific to biotin and a reddish-purple nanogold color develops at the test line. Non-hybridized FITC-probe is bound by the gold-labeled anti-FITC to form a double complex without biotin and moves through the test line to be trapped at the control line by the 2° antibody and a reddish-purple color develops at the control line (Figure. 4). Any residual biotin-labeled primer is also bound by streptavidin at the test line, but it has no color and the reaction remains undetected.

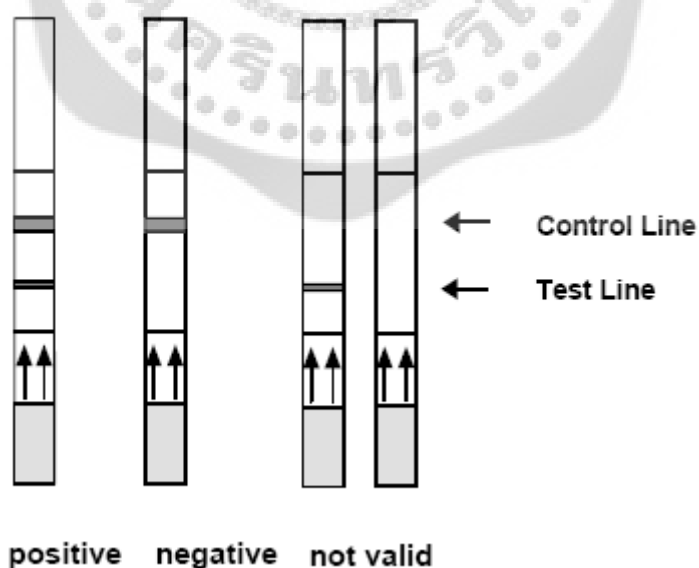


Figure 4 Detection of LAMP products using lateral flow dipstick (LFD). (Milenia® GenLine HybriDetect)

CHAPTER 3

MATERIALS AND METHODS

1. Detection of *Vibrio vulnificus* using monoclonal antibodies (MAbs)

1.1 Production of monoclonal antibodies against *Vibrio vulnificus*

1.1.1 Preparation of antigen

V. vulnificus from stock cultures were grown overnight on thiosulphate citrate bile salt sucrose agar (TCBS) and subcultured to obtain the circular and low convex with entire margin colonies before transferring the colony to tryptic soy broth (TSB). TSB were supplemented with 2% NaCl for the growth of bacteria. Bacteria were harvested by centrifugation at 3000 x g for 10 minutes and resuspended in phosphate buffer saline (PBS), pH 7.2 to a density of 1×10^9 CFU ml⁻¹. For antigen preparation, the suspension were heat-killed at 60°C for 60 minutes, then stored at 4°C until used. Half of the heat-killed antigen were denatured by addition of formalin at a ratio of 1:10 (v/v) and dialyzed against dH₂O for a week. Both heat-killed and formalin-treated bacteria were used as antigens for immunization. All isolates of *V. vulnificus* and other bacteria used in this study for immunological assay were shown in Table 2.

1.1.2 Immunization

Four ICR mice were intra-peritoneally injected with 100 µl of heat-killed and formalin-treated *V. vulnificus* mixed with complete Freund's adjuvant in a 1:1 ratio. Mice were subsequently injected three more times with the treated-bacteria mixed with incomplete Freund's adjuvant at 2 week intervals. Seven days after the forth injection, mice antisera were collected and tested against *V. vulnificus* using Westen blotting. The best performing mice was later boosted 3 days before hybridoma production.

Table 2 List of bacterial isolates used in this study for immunological assay

No.	Bacteria	Code	Institute	Source
1.	<i>Vibrio vulnificus</i> (VVB)	VV1	DABU	Sea bass
2.	<i>V. vulnificus</i> MT1506	VV2	DABU	?
3.	<i>V. vulnificus</i> 4907001	VV3	SWU	<i>P. vannamei</i>
4.	<i>V. vulnificus</i> 4907006	VV4	SWU	<i>P. vannamei</i>
5.	<i>V. vulnificus</i> 4907011	VV5	SWU	<i>P. vannamei</i>
6.	<i>V. vulnificus</i> 5258	VV6	DMST	Hemoculture
7.	<i>V. vulnificus</i> 19346	VV7	DMST	Hemoculture
8.	<i>V. vulnificus</i> 22441	VV8	DMST	Hemoculture
9.	<i>V. vulnificus</i> 24726	VV9	DMST	Hemoculture
10.	<i>V. vulnificus</i> 24727	VV10	DMST	Blood
11.	<i>V. vulnificus</i> 24728	VV11	DMST	Hemoculture
12.	<i>V. vulnificus</i> 24729	VV12	DMST	Hemoculture
13.	<i>V. vulnificus</i> 26351	VV13	DMST	Hemoculture
14.	<i>V. vulnificus</i> 27562	VV14	ATCC	?
15.	<i>V. vulnificus</i> M06-24/O	VV15	DABU	Patient
16.	<i>V. vulnificus</i> PSU 001	VV16	PSU	Oyster
17.	<i>V. vulnificus</i> PSU 005	VV17	PSU	Soil
18.	<i>V. vulnificus</i> PSU 0010	VV18	PSU	Shrimp
19.	<i>V. vulnificus</i> PSU 0011	VV19	PSU	Fish
20.	<i>V. vulnificus</i> PSU 0013	VV20	PSU	Crab
21.	<i>V. vulnificus</i> PSU 0015	VV21	PSU	Shrimp
22.	<i>V. vulnificus</i> PSU 0016	VV22	PSU	Shrimp
23.	<i>V. vulnificus</i> PSU 0025	VV23	PSU	Oyster
24.	<i>V. vulnificus</i> PSU 0026	VV24	PSU	Soil
25.	<i>V. vulnificus</i> PSU 0027	VV25	PSU	Fish
26.	<i>V. vulnificus</i> PSU 0029	VV26	PSU	Shrimp
27.	<i>V. vulnificus</i> PSU 0032	VV27	PSU	Oyster
28.	<i>V. vulnificus</i> PSU 0046	VV28	PSU	Oyster
29.	<i>V. vulnificus</i> PSU 0048	VV29	PSU	Shrimp
30.	<i>V. vulnificus</i> PSU 0054	VV30	PSU	Crab
31.	<i>V. vulnificus</i> PSU 0063	VV31	PSU	Shrimp
32.	<i>V. vulnificus</i> PSU 0070	VV32	PSU	Oyster
33.	<i>V. vulnificus</i> PSU 0078	VV33	PSU	Oyster
34.	<i>V. vulnificus</i> PSU 0086	VV34	PSU	Shrimp
35.	<i>V. vulnificus</i> PSU 0092	VV35	PSU	Shrimp
36.	<i>V. vulnificus</i> PSU 00159	VV36	PSU	Oyster
37.	<i>V. vulnificus</i> PSU 00227	VV37	PSU	Shrimp
38.	<i>V. vulnificus</i> PSU 00247	VV38	PSU	Blood
39.	<i>V. vulnificus</i> PSU 00252	VV39	PSU	Blood

Table 2 (continued)

No.	Bacteria	Code	Institute	Source
40.	<i>V. vulnificus</i> PSU 00258	VV40	PSU	Blood
41.	<i>V. fluvialis</i> 22085	VF1	DMST	Rectal swab
42.	<i>V. fluvialis</i> 22086	VF2	DMST	Stool
43.	<i>V. fluvialis</i> 22087	VF3	DMST	Stool
44.	<i>V. fluvialis</i> 24049	VF4	DMST	Stool
45.	<i>V. fluvialis</i> 24050	VF5	DMST	Stool
46.	<i>V. fluvialis</i> 24051	VF6	DMST	Stool
47.	<i>V. fluvialis</i> 24052	VF7	DMST	Stool
48.	<i>V. fluvialis</i> 5406001	VF8	SWU	Mussel
49.	<i>V. fluvialis</i> 5406002	VF9	SWU	Clam
50.	<i>V. fluvialis</i> 5406003	VF10	SWU	Mussel
51.	<i>V. parahaemolyticus</i> 22091, O8:K21	VP1	DMST	Stool
52.	<i>V. parahaemolyticus</i> 22092, O2:K3	VP2	DMST	Food
53.	<i>V. parahaemolyticus</i> 22093, O2:K3	VP3	DMST	Food
54.	<i>V. parahaemolyticus</i> 1 (VPV) O5:K33	VP4	VMARC	aquatic animals
55.	<i>V. parahaemolyticus</i> 2 (VPB) O5:K33	VP5	DABU	<i>P. monodon</i>
56.	<i>V. parahaemolyticus</i> 5665 O10:KUT	VP6	DMSC	Stool
57.	<i>V. parahaemolyticus</i> 45034 O10:KUT	VP7	DABU	?
58.	<i>V. parahaemolyticus</i> 23794 O3:K54	VP8	DMST	Stool
59.	<i>V. parahaemolyticus</i> 23795 O10:KUT	VP9	DMST	Stool
60.	<i>V. parahaemolyticus</i> 23796 O2:K28	VP10	DMST	Stool
61.	<i>V. parahaemolyticus</i> 23797 O10:KUT	VP11	DMST	Stool
62.	<i>V. alginolyticus</i> 22082	VA1	DMST	Stool
63.	<i>V. alginolyticus</i> 22083	VA2	DMST	Food
64.	<i>V. alginolyticus</i> 22084	VA3	DMST	Food
65.	<i>V. alginolyticus</i> 14800	VA4	DMSC	Seafood
66.	<i>V. alginolyticus</i> Y16	VA5	SWU	Shrimp pond
67.	<i>V. alginolyticus</i> BY2	VA6	SWU	Shrimp pond
68.	<i>V. alginolyticus</i> Y2	VA7	SWU	Seawater
69.	<i>V. alginolyticus</i> Y3	VA8	SWU	Seawater
70.	<i>V. alginolyticus</i> Y4	VA9	SWU	Seawater
71.	<i>V. alginolyticus</i> 24045	VA10	DMST	Food
72.	<i>V. alginolyticus</i> 24046	VA11	DMST	Food
73.	<i>V. alginolyticus</i> 24047	VA12	DMST	Stool
74.	<i>V. alginolyticus</i> 24048	VA13	DMST	Water
75.	<i>V. cholerae</i> 22115 O1,El Tor, Inaba	VC1	DMST	Stool
76.	<i>V. cholerae</i> 22116 O1,El Tor, Inaba	VC2	DMST	Stool
77.	<i>V. cholerae</i> 22117 O1,El Tor, Inaba	VC3	DMST	Stool
78.	<i>V. cholerae</i> 22118 O1,El Tor, Inaba	VC4	DMST	Stool

Table 2 (continued)

No.	Bacteria	Code	Institute	Source
79.	<i>V. cholerae</i> 22125 O1,El Tor, Ogawa	VC5	DMST	Rectal swab
80.	<i>V. cholerae</i> 22126 O1,El Tor, Ogawa	VC6	DMST	Rectal swab
81.	<i>V. cholerae</i> 22127 O1,El Tor, Ogawa	VC7	DMST	Rectal swab
82.	<i>V. cholerae</i> 22128 O1,El Tor, Ogawa	VC8	DMST	Rectal swab
83.	<i>V. cholerae</i> 22135 O139	VC9	DMST	Stool
84.	<i>V. cholerae</i> 22136 O139	VC10	DMST	Stool
85.	<i>V. cholerae</i> 22137 O139	VC11	DMST	Stool
86.	<i>V. cholerae</i> 22138 O139	VC12	DMST	Stool
87.	<i>V. cholerae</i> 22413 O141	VC13	DMST	?
88.	<i>V. cholerae</i> 22140 NVC	VC14	DMST	Food
89.	<i>V. cholerae</i> 22141 NVC	VC15	DMST	Food
90.	<i>V. cholerae</i> 22142 NVC	VC16	DMST	Food
91.	<i>V. cholerae</i> 22143 NVC	VC17	DMST	Stool
92.	<i>V. cholerae</i> 22144 NVC	VC18	DMST	?
93.	<i>V. cholerae</i> 27424 NVC	VC19	DMST	Stool
94.	<i>V. cholerae</i> 27425 NVC	VC20	DMST	Stool
95.	<i>V. cholerae</i> 25087 NVC	VC21	DMST	?
96.	<i>V. cholerae</i> B20 NVC	VC22	SWU	<i>P. vannamei</i>
97.	<i>V. cholerae</i> 4907014 NVC	VC23	SWU	<i>P. vannamei</i>
98.	<i>V. cholerae</i> 510601 NVC	VC24	SWU	<i>P. vannamei</i>
99.	<i>V. cholerae</i> 510602 NVC	VC25	SWU	<i>P. vannamei</i>
100.	<i>V. mimicus</i> 15142	VM1	DMSC	Patient
101.	<i>V. mimicus</i> 22088	VM2	DMST	Food
102.	<i>V. mimicus</i> 22089	VM3	DMST	Food
103.	<i>V. mimicus</i> 22090	VM4	DMST	Food
104.	<i>V. mimicus</i> 24053	VM5	DMST	Food
105.	<i>V. mimicus</i> 24054	VM6	DMST	Water
106.	<i>V. mimicus</i> 24055	VM7	DMST	Water
107.	<i>V. mimicus</i> 24056	VM8	DMST	Food
108.	<i>V. mimicus</i> B1.4	VM9	SWU	<i>P. vannamei</i>
109.	<i>V. mimicus</i> 27430	VM10	DMST	Rectal swab
110.	<i>V. harveyi</i> 639	VH1	CENTEX	<i>P. monodon</i>
111.	<i>V. harveyi</i> 1526	VH2	CENTEX	<i>P. monodon</i>
112.	<i>V. harveyi</i> 114 (VHS1)	VH3	CENTEX	<i>P. monodon</i>
113.	<i>V. harveyi</i> 47666-1	VH4	DABU	?
114.	<i>V. harveyi</i> MM30	VH5	GB	?
115.	<i>V. harveyi</i> MM77	VH6	GB	?
116.	<i>V. harveyi</i> 22891	VH7	GB	?
117.	<i>V. harveyi</i> 22893	VH8	GB	?

Table 2 (continued)

No.	Bacteria	Code	Institute	Source
118.	<i>V. harveyi</i> 22894	VH9	GB	?
119.	<i>V. harveyi</i> 22895	VH10	GB	?
120.	<i>V. harveyi</i> 120	VH11	GB	?
121.	<i>V. harveyi</i> 152	VH12	GB	?
122.	<i>V. harveyi</i> 170	VH13	GB	?
123.	<i>V. harveyi</i> 612	VH14	GB	?
124.	<i>V. harveyi</i> 886	VH15	GB	?
125.	<i>V. harveyi</i> H1	VH16	CPF	Shrimp pond
126.	<i>V. harveyi</i> H3	VH17	CPF	Shrimp pond
127.	<i>V. harveyi</i> H4	VH18	CPF	Shrimp pond
128.	<i>V. harveyi</i> H5	VH19	CPF	Shrimp pond
129.	<i>V. campbellii</i> 21361	Vcamp1	GB	?
130.	<i>V. campbellii</i> 21362	Vcamp2	GB	?
131.	<i>V. campbellii</i> 21363	Vcamp3	GB	?
132.	<i>V. shilonii</i>	Vshi	SWU	<i>P. vannamei</i>
133.	<i>V. ordalii</i> (VIB 02)	Vord	DABU	?
134.	<i>V. penaeicida</i>	Vpen	DMSC	?
135.	<i>V. anguillarum</i> AVL01	Vang	DABU	?
136.	<i>Aeromonas hydrophila</i> 22097	AH	DMST	?
137.	<i>A. caviae</i> 22106	AC	DMST	?
138.	<i>A. sobria</i> 22100	AS	DMST	?
139.	<i>A. veronii</i> 21255 ATCC 35624	AV	DMST	?
140.	<i>A. jandaei</i> 21256 ATCC 49568	AJ	DMST	?
141.	<i>Plesiomonas shigelloides</i> 22107	-	DMST	?
142.	<i>Enterobacter cloacae</i>	-	DMSM	?
143.	<i>Escherichia coli</i> ATCC 25922	-	CPF	?
144.	<i>Klebsiella pneumonia</i>	-	DMSM	?
145.	<i>Morganella morganii</i>	-	DMSM	?
146.	<i>Proteus vulgaris</i>	-	DMSM	?
147.	<i>Pseudomonas aeruginosa</i>	-	DMSM	?
148.	<i>Salmonella Enteritidis</i> 7108	-	DMST	?
149.	<i>Salmonella Typhi</i>	-	DMSM	?
150.	<i>Salmonella Typhimurium</i> ATCC 1408	-	CPF	?
151.	<i>Shigella flexneri</i>	-	DMSM	?
152.	<i>Photobacterium damsela</i>	-	DABU	Sea bass
153.	<i>Yesinia ruckeri</i>	-	DABU	?

1.1.3 Myeloma cell lines

The myeloma partner used for fusion was P3X. The cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) in a humidified incubator in the presence of 5% CO₂ at 37°C. The myelomas were maintained in exponential phase growth by means of daily subculture for one week prior to fusion. The medium volume in the petri dishes was maintained at approximately 15 ml.

1.1.4 Production of hybridoma

Hybridomas were produced according to Köhler and Milstein (1975: 495-497) with some modification (Mosmann; Bauma; & Williamson. 1979: 511-516).

V. vulnificus immunized mice was euthanized by cervical dislocation. It was sprayed liberally with 70% ethanol. The spleen was collected aseptically and gently washed two times in sterile petri dish containing PBS to minimize the chance of contamination. After that, the spleen was transfer in a sterile petri dish containing RPMI 1640 medium. During extensive soak the connective tissues were carefully removed as much as possible.

To accomplish a single cell suspension, spleen was clipped off and minced using a 5 ml syringe plunger through a sterile grid. The suspension was filtered through the grid several times by means of a pipette. Ten ml of spleen suspension was added into a 50 ml of a polypropylene tube containing approximately 10⁷ cells of P3X myeloma. It was centrifuged for 5 minutes at 1,500 rpm and the supernatant was discarded as completely as possible. To facilitate fusion, one ml of pre-warmed (37°C) 50% (w/v) polyethylene glycol (PEG 1500, Sigma) was added to the cell suspension, and the tube was slightly agitated simultaneously. This step was performed for 1 minute. Thirty-nine ml of HT medium was added into the suspension, and the mixture was incubated at the humidified incubator for 2 hours. The cell suspension was centrifuged at 1,500 rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in HAT medium supplemented with 20% FBS. One hundred µl of the mixed cell suspension was dispensed into each well of the twenty 96-well culture plates, and they were kept in the humidified incubator.

Six days after fusion, growth of hybridomas were examined under inverted microscope; if visible, cell supernatant were screened for anti *V. vulnificus* MAbs activity as described below. The selected positive hybridomas were cloned in new 96-well culture plates by limiting dilution method to obtain a single clone per well. Wells which contained a single colony were further propagated in 20% FBS RPMI 1640 media and cell supernatants were tested by dot blotting and Western blotting.

1.1.5 Cloning of hybridoma cell by the limiting dilution method

After identification of desirable cloned, all the cells from each positive well were transferred into separated well of 96 well plates with 100 μ l of RPMI media was added per well followed by serial dilution (i.e. by the limiting dilution method). Approximately 24 hr prior to seeding the wells with the isolated hybridoma cells, media was prepared and placed into the humidified incubator. From each positive well, 10 μ l of the culture suspension containing approximately 100 viable cells were drawn off and thoroughly blended with 7.5 ml of media. One hundred μ l of the mixture was dispensed into 48 wells of the 96-well plate and more 2.5 ml of the media was added into the remaining mixture to lower the concentration of hybridoma cells. The plates were incubated in a humidified incubator for 7 days. Cell growth in the bottom of the wells was monitored using an inverted microscope. Wells that showed a single confluent clone were screened for MABs.

1.1.6 Cryopreservation

The mid-log phase proliferating hybridomas were harvested and centrifuged at 1,500 rpm for 5 minutes at room temperature. The supernatants were kept at -20°C for any further assay and the hybridomas pellet were thoroughly mixed with 1 ml of freezing media, which was kept cold at 4°C at 5×10^6 cell ml^{-1} . About 0.5 ml of the suspension was transferred into 2 cryopreservation tubes. After the caps were closed rapidly and tightly, the tubes were left in a deep freeze (-80 °C) overnight and then transferred into a container of liquid N₂ (-196°C), where they could be kept for a long period. For thawing hybridomas, frozen hybridoma cells kept in liquid nitrogen tank was thawed in a 37°C water bath and transferred into 10 ml RPMI 1640 medium in a sterile tube. The cell suspension was centrifuged at 1,500 rpm for 5 minutes. Supernatant was discarded and the cells were re-suspended in a new sterile petri dish with 15 ml of 20% FBS RPMI media and placed in a 37°C, 5% CO₂ incubator.

1.1.7 Screening methods for Positive Hybridoma Clones

1.1.7.1 Dot blotting

To determine positive or negative cell lines after the fusion, hybridoma supernatants were screened using a dot blotting. The nitrocellulose membrane of 0.45 μ m average pore size was used as a solid phase in the dot blotting. *V. vulnificus* heat-killed form were spotted onto the nitrocellulose membrane (1 μ l/spot), then incubated with hybridoma-conditioned media from each culture wells (1:20 dilution in 5% blotto) for 8 hr. After extensive washing in PBS, the membrane were incubated in goat anti-mouse IgG

heavy and light chain specific antibody conjugated horseradish peroxidase (GAM-HRP; BioRad) at the dilution of 1:1,500 for 3 hours. The membranes were then washed extensively and incubated in a substrate mixture containing 0.006% hydrogenperoxide, 0.03% diaminobenzidine (DAB), 0.05% cobalt chloride in PBS (Sithigorngul; et al. 2006: 436-442). Hybridoma clones that displayed immunoreactivity against *V. vulnificus*, but not against other bacteria, will be confirmed for bacterial specificity by Western blot and immunohistochemistry before cloning and cryopreservation for further investigation.

1.1.7.2 Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) and Western Blot

The identification and characterization of the antigens was determined by SDS-PAGE. A 15% SDS-PAGE separating gel, together with 4% stacking gel was used (Sithigorngul; et al. 2006: 436-442). The standard molecular weight markers were included. Samples were electrophoresed at 100V/slab gel until the tracking dye reached the separating gel. The electric current was decreased to 70 V until the tracking dye was about 10 mm from the bottom edge of the separating gel. The gel was removed from the chamber and protein was visualized using 0.1% Coomassie brilliant blue, then protein molecular mass was determined by comparison with mobility of protein markers. For Western blotting, the *V. vulnificus* antigen resolved in the polyacrylamide gel was electroblotted onto a nitrocellulose membrane using a Transblot apparatus (BioRad). After blotting, the nitrocellulose membrane was incubated with each monoclonal antibody and processed as dot blotting method that described above.

1.1.7.3 Molecular weight determination

A procedure used for molecular weight (MW) determination was modified according to the methods Weber and Osborn (1969: 4406-4412) and Davies and the Stark (1970: 651-656). The molecular weight of the sample could be determined by comparing their electrophoretic mobilities with those of protein markers of known molecular masses. A linear relationship was obtained when the relative mobility of the standard markers was plotted against the logarithmic value of their molecular weight. The molecular weight markers used in this study are serum albumin (66 kDa), ovalbumin, chicken egg (45 kDa), glyderaldehyde-3-phosphate dehydrogenase, rabbit muscle (36 kDa), carbonic anhydrase, bovine erythrocytes (29 kDa), trypsin, bovine pancreas (24 kDa), trypsin inhibitor, soybean (20 kDa) and α lactalbumin, bovine milk (14.2 kDa). The relative mobility of the sample was calculated according to the following formula:

Relative mobility (R_m) = Distance of protein migration/Distance of dye migration

1.1.7.4 Immunohistochemistry

Cephalothoraces from *L. vannamei* injected with *V. vulnificus* were cutted and fixed in Davidson's fixative solution for 24 hours before processing. Then, continuously rinsed with running tap water for 2 hours, and dehydrated with graded series of ethyl alcohol: 70%, 90% and 95% concentration for 3 hr each; N-butyl alcohol for 1 hour; and N-butyl alcohol in xylene for 1 hour; 3 changes of xylene. The tissue was completely infiltrated with paraffin and placed in a mold containing molten paraffin. The paraffin block was then allowed to cool at 20 °C before sectioning. Serial sections were prepared and processed for indirect immunoperoxidase staining using various MAbs and GAM-HRP diluted with 1:1,000 with 10%FBS in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparation will be dehydrated in grade ethanol series, counter-stained with Haematoxylin and eosin Y (H&E), cleared in xylene and mounted in Permount (Sithigorngul et al. 2000b: 27-34; 2002: 71-76) (Figure 5). Positive reactions were visualized as brown coloration against pink and purple colors of H&E.

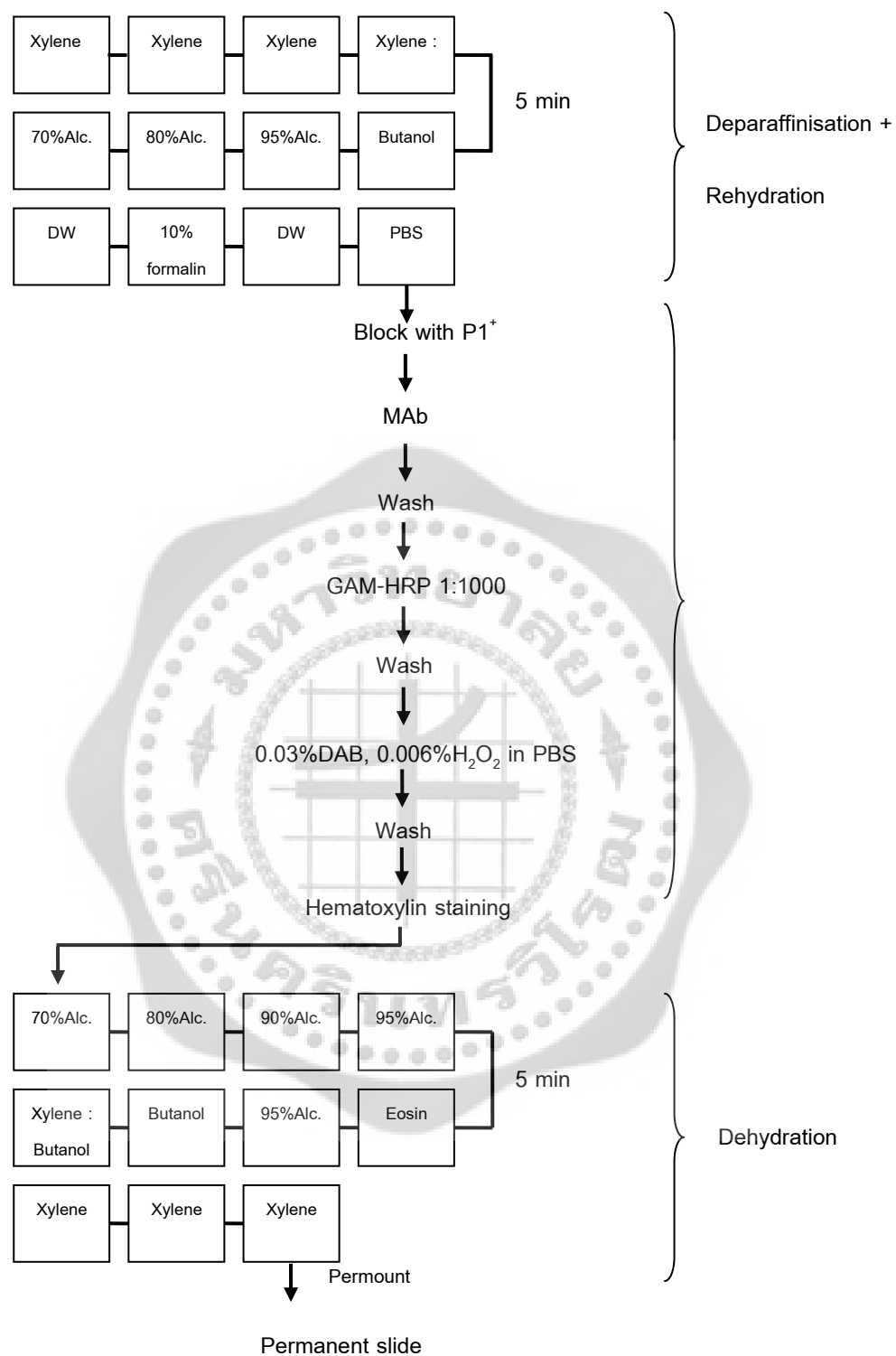


Figure 5 A flow chart of immunohistochemistry preparation (Sithigorngul; et al. 2000b: 27-34)

1.1.8 Determination of isotype and subisotype

Isotype and subisotype of mouse immunoglobulin produce by hybridomas were determined by sandwich ELISA using Zymed's mouse monoAb ID Kit (HRP). The assay is based on principle of an indirect sandwich ELISA (Figure 6). Rabbit anti-mouse class and subclass specific antibodies were employed in isotyping the mouse immunoglobulins secreted into the culture media. Peroxidase-labelled goat anti-mouse immunoglobulin serves as the signal generating reagent. The procedure was conducted following the manufacturer's recommendations with slight modification. Briefly, 50 μ l of goat anti-mouse Ig (GAM-Ig) dilution were added to the microtiter wells. After the plates were incubated in a humidified box at 4°C overnight, the solution were decanted and the plate will be slapped onto paper towel until dry, and immediately all wells were washed with 150 μ l of 0.5 % blotto and left at room temperature for 5 minutes. This step was repeated 4 times. Then, 100 μ l of diluted MABs were added into wells of each column and left at room temperature for 5 hours. Washing steps were repeated for 4 times as described above. Fifty μ l of subclass specific rabbit anti-mouse immunoglobulins (IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, K and L) were added into each well of the row, respectively. The plates were left at 4°C overnight in the humidified box. Washing steps were repeated for 4 times. After that, 100 μ l of diluted goat anti-rabbit conjugated horseradish peroxidase (GAR-HRP) was added into each well and the plates were left at room temperature for 3 hour. The reaction was obtained when each well were added 80 μ l of O-phenylenediamine dihydrochloride (OPD) working substrate solution and left for 5 minute. To stop the reaction, 80 μ l of 1N H₂SO₄ was added to each well. The optical density (OD) was measured at 490 nm using ELISA reader (Sithigorngul; et al. 2000b: 27-34).

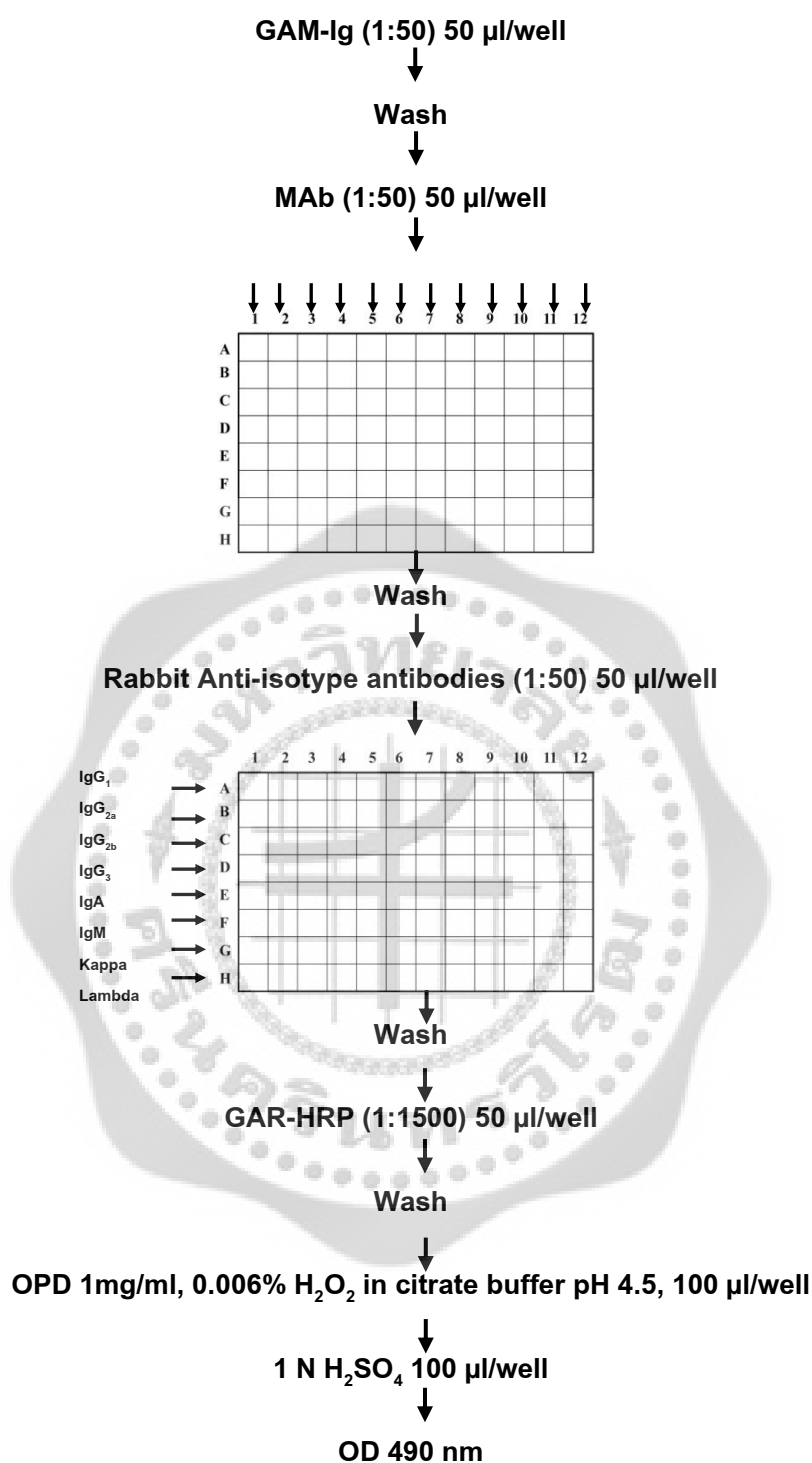


Figure 6 A flow chart of determination of antibody isotypes and subisotypes preparation

1.1.9 Sensitivity of MABs for *Vibrio vulnificus* detection by dot blot

Serial dilution of *V. vulnificus* in PBS was prepared and 1 µl of each dilution was spotted onto nitrocellulose membrane and fixed in 10% formalin for 30 minute 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.

1.1.10 Detection of *V. vulnificus* after pre-incubation in alkaline peptone water (APW)

An isolate of *V. vulnificus* VVS4907001 was spiked into 5 ml of the APW at dilutions from 0 - 10⁷ CFU ml⁻¹ and incubated at 37 °C. Aliquots of 0.5 ml from each tube at 0, 3, 6, 12 and 24 h were collected and immediately heat killed at 60 °C for 60 minutes before storage at -20 °C prior to testing by dot blotting.

For dot blotting, 1 µl sample from each incubation period in each treatment was spotted onto a nitrocellulose membrane, baked at 60 °C for 10 minutes and then processed using MAB specific to *V. vulnificus* to determine *V. vulnificus* in the homogenates. Two replications were performed to confirm the results.

1.1.11 Detection of *V. vulnificus* after pre-incubation in oyster homogenate-APW

Oyster samples were purchased at a local market in Bangkok, Thailand. The oyster samples were rinsed 3 times with sterile PBS and homogenized with glass homogenizer in 2% NaCl alkaline peptone water (APW) at 1:10 (weight : volume). The oyster homogenate-APW was used as enriched medium for *V. vulnificus* and performed the experiment as described above in 1.1.10.

2. Detection of *Vibrio vulnificus* by loop-mediated isothermal amplification combined with lateral flow dipstick (LAMP-LFD) targeted to *rpoS* gene

2.1 Bacteria isolates

A total of 76 bacterial isolates including 14 *V. vulnificus* isolates, 25 non-*vulnificus* *Vibrio* isolates and 37 non-*Vibrio* bacteria were used in this study (Table 3). The 16s rRNA gene amplifications were also performed to verify the bacterial identification as described in previous report (Weisburg; et al. 1991: 697-703). The origins and sources of all 39 *Vibrio* isolates tested were shown in Table 3. The other 37 non-*Vibrio* bacteria were obtained from clinical samples, food or environmental sources, as follows: four isolates of *Aeromonas hydrophila*, three isolates of *Aeromonas caviae*, three isolates of *Aeromonas sobria*, four isolates of *Pseudomonas aeruginosa*, two isolates of *Plesiomonas shigelloides*, one isolate each of *Aeromonas veronii*, *Aeromonas jandaei*, *Bacillus cereus*, *E. coli*, *Edwardsiella tarda*, *Photobacterium damsela* ssp. *damsela*, *P. damsela* ssp. *piscicida*, *Proteus vulgaris*, *Pseudomonas stutzeri*, *Pseudomonas chlororaphis*, *Pseudomonas acidovorans*, *Pseudomonas putida*, *Pseudomonas boreopolis*, *Pseudomonas oleovorans*, *Pseudomonas syringae*, *Pseudomonas japonica*, *Pseudomonas fluorescens*, *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *Staphylococcus aureus* and *Yersinia ruckeri*.

2.2 Culture conditions and DNA preparation

An isolate of *V. vulnificus* designated as VVS 4907001 (Table 3) was used for the assay of optimization and sensitivity testing with pure culture and spiked oyster samples. All *Vibrio* isolates were cultured using thiosulfate citrate bile salt sucrose agar (TCBS agar; Difco) at 37 °C for overnight. Non-*Vibrio* isolates were grown in tryptic soy agar (TSA; Difco) at 37 °C overnight. To extract bacterial DNA, a single loopful of culture on TCBS agar or TSA was used with QIAamp DNA mini kit (Qiagen) according to the manufacturer's manual. The extracted DNA was stored at -20 °C until use.

Table 3 Bacterial isolates used for LAMP-LFD assay

Bacterial isolates	Origin	Source
<i>Vibrio vulnificus</i> (n=14)		
DABU	Sea bass	DABU
MT 1506	Unknown	DABU
VVS 4907001	<i>Penaeus vannamei</i>	DBSWU
VVS 4907002	<i>P. vannamei</i>	DBSWU
VVS 4907009	<i>P. vannamei</i>	DBSWU
VVS 4907011	<i>P. vannamei</i>	DBSWU
DMST 5852	Hemoculture	DMST
DMST 19346	Hemoculture	DMST
DMST 22441	Hemoculture	DMST
DMST 26351	Hemoculture	DMST
DMST 27426	Hemoculture	DMST
DMST 27427	Blood	DMST
DMST 27428	Hemoculture	DMST
DMST 27429	Hemoculture	DMST
<i>V. fluvialis</i> (n=4)		
DMST 22086	Stool	DMST
DMST 22087	Stool	DMST
DMST 24049	Stool	DMST
DMST 24050	Stool	DMST
<i>V. cholerae</i> (n=3)		
DMST 22140	Food	DMST
DMST 22142	Food	DMST
DMST 22143	Stool	DMST
<i>V. mimicus</i> (n=3)		
DMST 22088	Food	DMST
DMST 22089	Food	DMST
DMST 22090	Food	DMST
<i>V. alginolyticus</i> (n= 3)		
DMST 22082	Stool	DMST
DMST 22084	Food	DMST
DMSC 14800	Seafood	DMSC

Table 3 (continued)

Bacterial isolates	Origin	Source
<i>V. parahaemolyticus</i> (n=3)		
DMST 15285	Food	DMST
DMST 21330 (O3:KUT)	Shrimp	DMST
DABU (O10:KUT)	<i>P. monodon</i>	DABU
<i>V. campbellii</i> (n=3)		
LMG 21361	Unknown	GB
LMG 21362	Unknown	GB
LMG 21363	Unknown	GB
<i>V. harveyi</i> (n=3)		
Centex 639	Unknown	Centex
Centex 1526	Unknown	Centex
LMG 22888	Unknown	GB
<i>V. shilonii</i> VS 4907012 (n=1)	<i>P. vannamei</i>	DBSWU
<i>V. anguillarum</i> AV L01 (n=1)	Unknown	DABU
<i>V. ordalii</i> Vib02 (n=1)	Unknown	DABU
Other non- <i>Vibrio</i> bacteria (n=37)		

2.3 Polymerase chain reaction

The DNA extracted from bacterial samples was used as a template for PCR amplification. The PCR reaction conditions for amplification of *rpoS* gene were described in a previous report (Kim; et al. 2008: 1841-1847). Briefly, the PCR reaction was carried out with primers vulrpoS-up (terminal sense; 5'-CAT GCG TGT TTC CTT GAT TC-3') and vulrpoS-rp (terminal antisense; 5'-TCC ATA GCC TTT TTT CTA TTG G-3') for 35 cycles, each of which consisted of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The PCR amplicon size was 273 bp.

The PCR reaction conditions for amplification of *vvhA* gene were described in a previous report (Panicker; et al. 2004: 7436-7444). Briefly, the PCR reaction was carried out with primers L-vvh (terminal sense; 5'- TTC CAA CTT CAA ACC GAA CTA TGA C-3') and R-vvh (terminal antisense; 5'-ATT CCA GTC GAT GCG AAT ACG TTG-3') for 35 cycles, each of which consisted of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. The PCR amplicon size was 205 bp.

2.4 LAMP primer design

A set of six primers for LAMP was designed according to the published sequence of *rpoS* coding region (GenBank ID: AY187681) using Primer Explorer ver. 4 (<http://primerexplorer.jp/elamp4.0.0/index.html>). To detect LAMP products by LFD, FIP inner primer was 5'-labeled with biotin for the use in subsequent LFD step. The details of primers are given in Table 4.

Table 4 Primers for loop-mediated isothermal amplification (LAMP) designed from *rpoS* gene of *V.vulnificus*.

Primer	Gene position	Sequence (5'-3')
F3	778-795	TTGGAAGAGCTCAATCCG
B3	948-969	ATTTTCCATATTCAGACCTTGT
Forward inner primer	797-816/TTTT/837-857	TCCAGCGTTGAAGGCTCATAATTTTAACAAAAAGAAGTGT TGGCA
Backward inner primer	928-946/TTTT/866-888	GGCAAGAGATTGGTTAACTCGCTTTTTGATCAGAACTTC ACGGAG
Loop forward	817-836	CCCAACAAACCAAGCGACG
Loop backward	906-926	TCAGGTTGAAGGGTTACGTCG
Forward inner primer-biotin	797-816/TTTT/837-857	TCCAGCGTTGAAGGCTCATAATTTTAACAAAAAGAAGTGT TGGCA
FICT probe	926-945	GCCTCCGTGAAGTTCTGATC

2.5 Optimization of LAMP reaction conditions

The LAMP assay was carried out in a total of 25 µl volume containing 40 pmol each of the inner primers (FIP and BIP), 5 pmol each of the outer primers (F3 and B3), 20 pmol each of loop primers (LF and LB), 1.4 mM dNTP mix (Fermentas), 6 mM MgSO₄, 0.8 M Betaine (Sigma-Aldrich), 8 U of *Bst* DNA polymerase (New England Biolabs) and 1x of supplied buffer and DNA template. The reaction temperature was optimized at 60, 63 and 65 °C, and LAMP was carried out on predetermined time (90 minutes) and terminated at 80 °C for 10 minutes. The LAMP products (2.5 µl) were analyzed by 2% agarose gel electrophoresis.

2.6 Design and optimization of FITC-labeled probe for lateral flow dipstick

(LFD) assay

In order to verify the result of LAMP obtained from agarose gel electrophoresis, the biotin-labeled LAMP reactions were carried out along with normal LAMP. In this condition, the biotin-labeled was added at the 5' end of the FIP inner primer (Table 4) but other primers and components used were the same as those described above. A DNA probe was designed from the *rpoS* sequence and it was labeled with fluorescein isothiocyanate (FITC) (Bio Basic Inc., Canada) at the 5' end. To determine the optimum concentration of FITC-probe for LAMP assay, three different concentrations of FITC-probe including 200, 20 and 2 pmol were used in the LAMP reaction. This labeled DNA probes were added to hybridize with the biotin-labeled LAMP products at 63 °C for 5 minutes. After hybridization, 8 µl of the hybridization solution was added to 150 µl of the assay buffer in a new tube. Finally, the LFD strip was dipped into the mixture for 5 minutes, after which the test line was observed for the presence of reddish-purple color indicative of a positive test reaction. The concentration of DNA probe that gave the strongest signal on the test line was determined to be the optimal concentration for LFD assay.

2.7 LAMP and PCR specificity test

The 76 bacterial isolates as shown in Table 3 were used to investigate the LAMP specificity. DNA templates isolated from bacterial cultures described earlier were subjected to both LAMP and PCR amplification.

2.8 Determination of sensitivity of LAMP and PCR with pure culture

The sensitivity of the LAMP assay for *V. vulnificus* in pure cultures was determined as previously described (Yamazaki; et al. 2008a: 163) with some modifications using known amounts of *V. vulnificus* VVS 4907001. Briefly, a single colony on TSA supplemented with 2% NaCl was inoculated in 4ml of tryptic soy broth (TSB; Difco) supplemented with 2% NaCl and incubated overnight at 37 °C with agitation. Then, 40 µl of TSB culture was transferred into a new 4 ml of TSB and incubated at 37 °C with shaking at 225 rpm to obtain mid-log phase cells (OD 600 nm equal to 0.5). Serial ten-fold dilutions of the cultures were prepared with PBS (Phosphate buffered saline). For preparation of DNA from pure cultures, 100 µl of each dilution was centrifuged at 18,000 x g for 5 minutes, then the pellet was resuspended in 50 µl of 25 mM NaOH and subsequently heated at 95 °C for 5 minutes. After neutralized with 4 µl of 1 M Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 18,000 x g for 5 minutes. For LAMP and PCR assay, 1 µl of each

supernatant was used as a template. The sensitivity tests were conducted in triplicate, the last dilution with all three samples tested positive was considered as the detection limit. The product from each reaction were analyzed by 2% agarose gel electrophoresis and by LFD using the appropriate amount of FITC-labeled DNA probe as described above. In parallel, to enumerate the bacteria, 100 μ l of each bacterial dilution was spread on TSA supplemented with 2% NaCl in duplicate and incubated at 37 °C for overnight. The colonies were counted at the dilution yielding 30-300 colony forming units (CFUs), and the CFU ml^{-1} of bacterial suspension was calculated.

2.9 Determination of sensitivity of LAMP and PCR with spiked oyster samples

Oyster samples were purchased at a local market in Bangkok, Thailand. The oyster samples were tested to be negative for *V. vulnificus* according to the microbiological examination by enrichment in APW (alkaline peptone water) overnight, and the oyster homogenate was plated onto the TCBS agar. The DNA samples of the green colonies were extracted by a QIAamp DNA mini kit (Qiagen) and tested for the presence of *V. vulnificus* by PCR targeted to *rpoS* gene (Kim; et al. 2008: 1841-1847). Only oyster homogenates that were negative for *V. vulnificus* were used in the following spiked oyster experiments.

The sensitivity of the LAMP assay for *V. vulnificus* in spiked oyster samples was determined as previously described (Yamazaki; et al. 2008a: 163) with some modifications using known amounts of *V. vulnificus* VVS 4907001. Nine milliliters of APW were added to 1 g of the oyster sample and homogenized thoroughly. Serial ten-fold dilutions of mid-log phase *V. vulnificus* were prepared as described earlier. One hundred microliters of each dilution of *V. vulnificus* with known amounts was spiked into 900 μ l of each of the oyster homogenates and mixed well. The oyster homogenate was centrifuged at 200 x g for 5 minutes to remove oyster tissues. The supernatant was transferred to a new tube and centrifuged at 18,000 x g for 5 minutes. After removal of the supernatant, the pellet was resuspended in 100 μ l of 25 mM NaOH, and the mixture was heated at 95 °C for 5 minutes. After neutralized with 8 μ l of 1 M Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 18,000 x g for 5 minutes. For LAMP and PCR, 1 μ l of each supernatant was used as a template. The sensitivity tests were conducted in triplicate, the last dilution with all three samples tested positive was considered as the detection limit. The product from each reaction was analyzed by 2% agarose gel electrophoresis and by LFD using the appropriate amount of FITC labeled DNA probe as described above.

CHAPTER 4

RESULTS

1. Detection of *Vibrio vulnificus* using monoclonal antibodies (MAbs)

1.1 Immunization

Seven days after the fourth injection of heat-killed and formalin-treated *V. vulnificus*, sera from four ICR mice were collected and adsorbed with excess heat-killed *A. hydrophila*. All antisera at 1:10,000 dilutions showed different degrees of immunoreactivity to multiple proteins in Western blot assay against whole-bacterial cell lysates of *V. vulnificus*, *V. fluvialis* and *V. parahaemolyticus*. The Western blot obtained from the sera of the mouse number 3 showed the most promising reactive bands with *V. vulnificus* without any cross-reactivity to the rest of the bacteria (data not shown). This mouse was used as the spleen donor in hybridoma production.

1.2 Production of Monoclonal antibodies

After fusion, there were approximately 1300 hybridoma-containing wells. Twenty eight hybridomas strongly reacted to *V. vulnificus* during dot blot screening. However, after screening against *Vibrio* spp. and various Gram negative bacteria, only 15 hybridoma clones produced monoclonal antibodies (MAb) with the highest avidity and demonstrated different specificity to *V. vulnificus*. These clones were selected for subsequent cloning by limiting dilution method and established as cell lines.

1.3 Characterization of Monoclonal antibodies

The MAbs were divided into nine different groups according to their specificity. As shown in Table 5 and Figure 7, monoclonal antibody (MAb) in group 1 consisted of one MAb (VV-5F2) that bound to only one isolate of *V. vulnificus* (VV2). MAbs in group 2 consisted of two MAbs (VV-4G2 and VV-19G12) that recognized two isolate of *V. vulnificus* (VV4 and VV5). MAb in group 3 (VV-6B9) recognized two isolates of *V. vulnificus* (VV6 and VV8). MAb in group 4 (VV-18H6) bound to two isolates of *V. vulnificus* (VV2 and VV11). MAb in group 5 (VV-10A6) recognized 7 isolates of *V. vulnificus* (VV1, VV3, VV6, VV8, VV10, VV15 and VV32). MAbs in group 6 (VV-14B2 and VV-20D1) bound to all of *V. vulnificus* isolates and did not showed cross reactivity to other non-*vulnificus* *Vibrio* isolates. MAbs in group 7 (VV-6D2, VV-7B6, VV-16A11 and VV-18B11) recognized all of *V. vulnificus* and *V. alginolyticus* isolates and did not show cross reactivity to other *Vibrio*

isolates. MAbs in group 8 (VV-3F11, VV-5B8 and VV-13B4) bound to all of *V. vulnificus* and *V. fluvialis* isolates and did not show cross reactivity to other *Vibrio* isolates. MAb in group 9 (VV-15E7) recognized all of *Vibrio* species.

Table 5 Specificity of monoclonal antibodies against *V. vulnificus*.

Group	MAb (isotype)	Antigen Western blotting (kDa)	IHC	Sensitivity Dot blotting (CFU/ml)	Bacterial immunoreactivity (Dot blotting)
1	VV-5F2 (IgG2b)	-	-	5×10^6	VV2
2	VV-4G2 (IgG2b)	65	+	5×10^6	VV4, 5
	VV-19G12 (IgM)			10^6	
3	VV-6B9 (IgM)	-	-	1.25×10^6	VV6, 8
4	VV-18H6 (IgM)	8-15	+	5×10^6	VV2, 11
5	VV-10A6 (IgG3)	8-11	+	1.25×10^6	VV1, 3, 6, 8, 10, 15, 32
6	VV-20D1 (IgG2b)	10, 13	-	5×10^6	VV all
	VV-14B2 (IgG2b)			10^7	
7	VV-6D2 (IgG2b)	10, 13	-	2.5×10^6	VV all, VA all
	VV-7B6 (IgG2a)			1.25×10^6	
	VV-16A11 (IgG2b)			1.25×10^6	
	VV-18B11 (IgG2b)			1.25×10^6	
8	VV-5B8 (IgG1)	20-25	-	5×10^6	VV all, VF all
	VV-13B4 (IgG2b)			5×10^6	
9	VV-15E7 (IgG2a)	20-25	+	2.5×10^6	<i>Vibrio</i> spp.

In Table 5, the binding of antibodies to various bacteria was determined by dot blotting using bacteria at approximately 10^8 CFU/ml. + = staining, - = not staining. IHC = immunohistochemistry, VV = *V. vulnificus*, VA = *V. alginolyticus*, VF = *V. fluvialis*. The number of each isolate was assigned as appeared in Table 1.

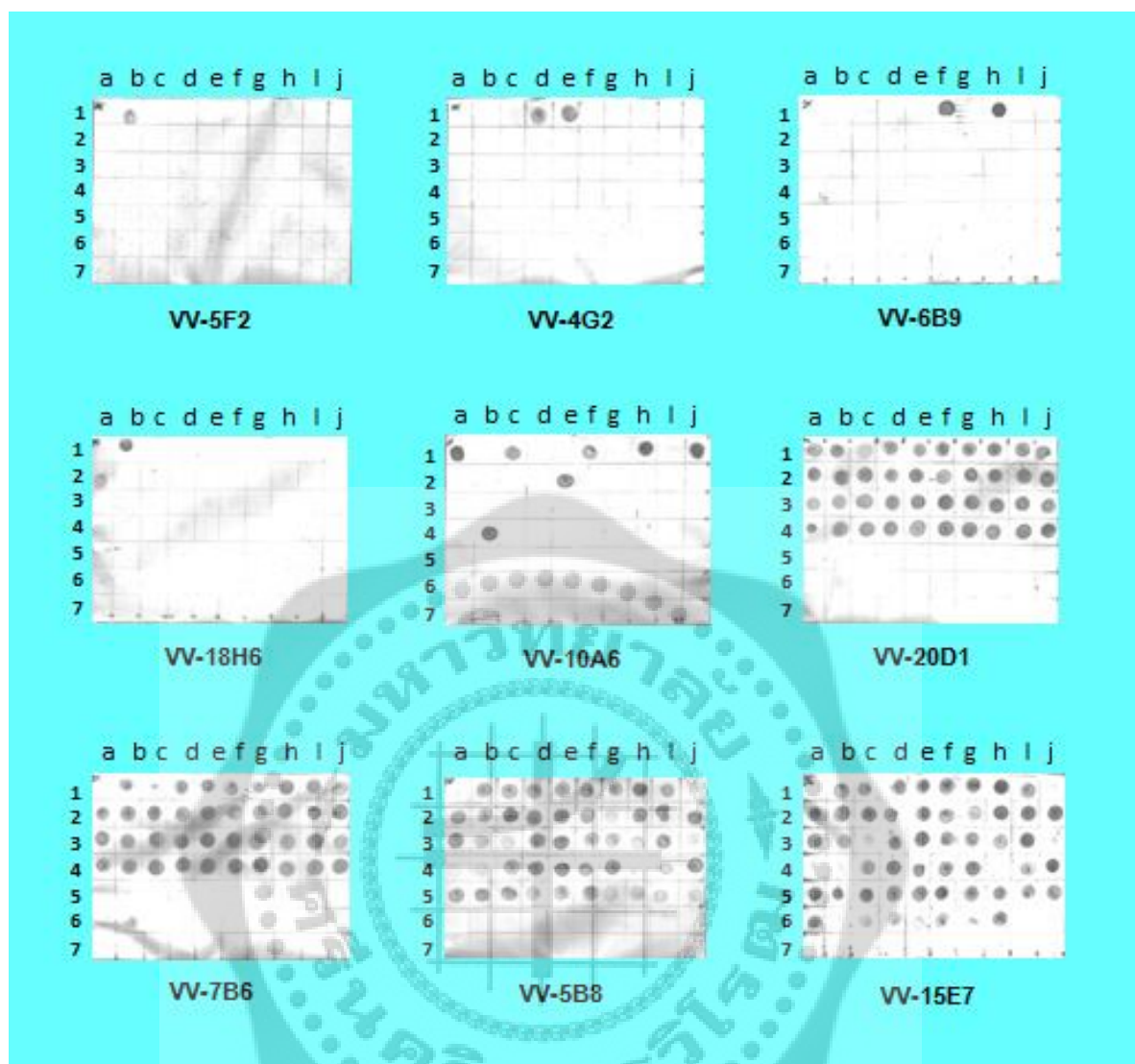


Figure 7 Cross-reactivity of MABs determined by dot blotting.

In figure 7, heat killed *V. vulnificus* (VV), *Vibrio* spp., and other bacteria were spotted onto each square of a nitrocellulose membrane (1 μ l per spot) and treated with various MABs from each group: (1) VV-5F2, (2) VV-4G2, (3) VV-6B9, (4) VV-18H6, (5) VV-10A6, (6) VV-20D1, (7) VV-7B6, (8) VV-5B8, and (9) VV-15E7. The bacteria on the membrane are as follows:

Row 1. (a) VV1, (b) VV2, (c) VV3, (d) VV4, (e) VV5, (f) VV6, (g) VV7, (h) VV8, (i) VV9 (j) VV10

Row 2. (a) VV11, (b) VV12, (c) VV13, (d) VV14, (e) VV15, (f) VV16, (g) VV17, (h) VV18, (i) VV19 (j) VV20

Row 3. (a) VV21, (b) VV22, (c) VV23, (d) VV24, (e) VV25, (f) VV26, (g) VV27, (h) VV28, (i) VV29 (j) VV30

Row 4. (a) VV31, (b) VV32, (c) VV33, (d) VV34, (e) VV35, (f) VV36, (g) VV37, (h) VV38, (i) VV39 (j) VV40

Row 5. (a) VF1, (b) VF2, (c) VF3, (d) VF4, (e) VF5, (f) VF6, (g) VF7, (h) VF8, (i) VF9 (j) VF10

Row 6. (a) VP, (b) VA, (c) VH, (d) Vcamp, (e) VC, (f) VM, (g) Vang, (h) Vpen, (i) AH (j) AC

Row 7. (a) AS, (b) AJ, (c) AV, (d) , (e) *Enterobacter cloacae*, (f) *Escherichia coli*, (g) *Klebsiella pneumonia*, (h) *Pseudomonas aeruginosa*, (i) *Shigella flexneri* (j) *Yersinia ruckeri*

MAbs in group one and three did not recognize the denatured form of antigen by Western blotting. MAbs (VV-4G2) in group two bound to a band of antigen at molecular mass approximately around 65 kDa. MAb in group four (VV-18H6) bound to a smear band of antigen with molecular masses between 8 to 15 kDa. MAb in group five (VV-10A6) bound to a thick band of antigens with molecular masses between 8 kDa. MAbs in groups six (VV-20D1) demonstrated a very weak reactivity to antigens with molecular masses of 10 and 13 kDa. MAbs in group seven (VV-7B6) recognized the same antigens with stronger immunoreactivity. MAbs in group eight (VV-5B8) and nine (VV-15E7) strongly bound to a thick band of antigen with molecular masses between 20 (Figure 8).

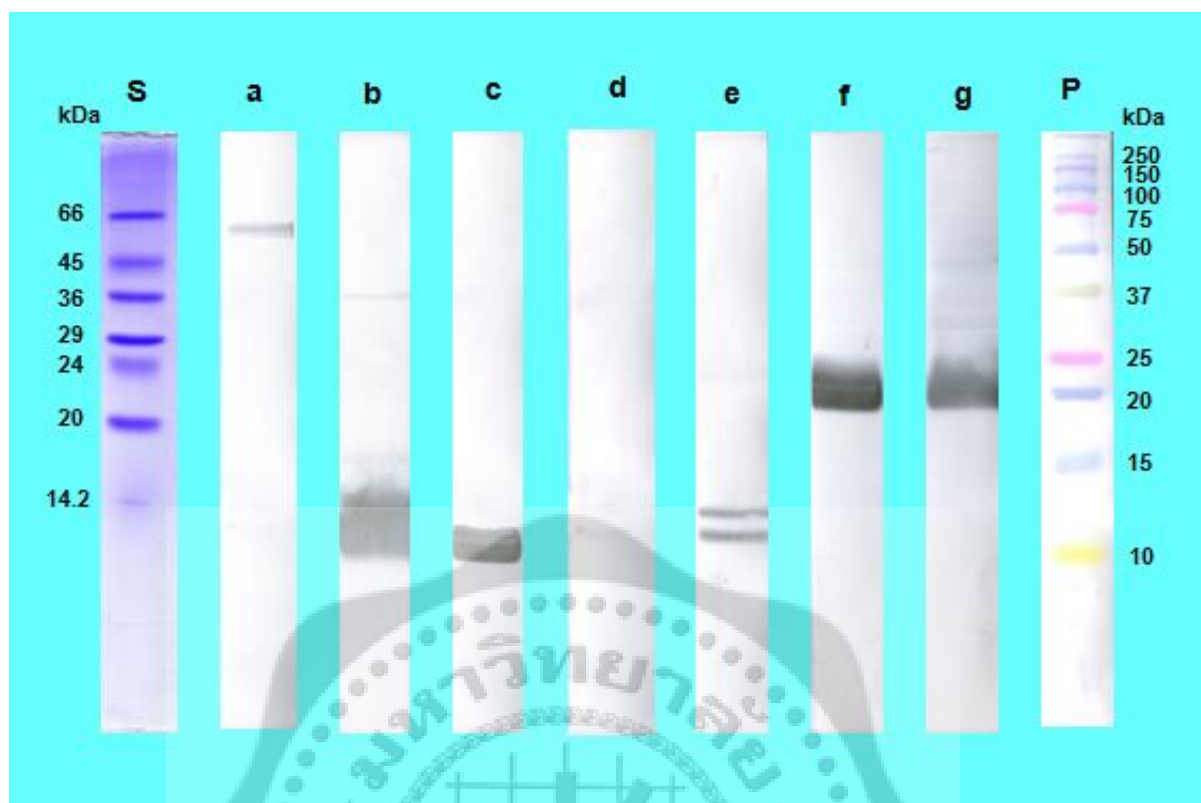


Figure 8 Western blot analyses of *V. vulnificus* using MAbs from each group.

The Figure 8 showed the Western blot analyses of *V. vulnificus* using MAbs from each group, (a) VV-4G2, (b) VV-18H6, (c) VV-10A6, (d) VV-20D1, (e) VV-7B6, (f) VV-5B8, (g) VV-15E7. S = standard marker proteins, P = Pre-stained standard proteins.

After processing for immunohistochemistry, MAbs in group two, four, five and nine were able to detect *V. vulnificus* in hepatopancreas, lymphoid organ and gill tissues of infected shrimp but MAbs in group one, three, six, seven and eight did not recognize the bacteria in shrimp tissues by immunohistochemistry (Table 5 and Figure 9).

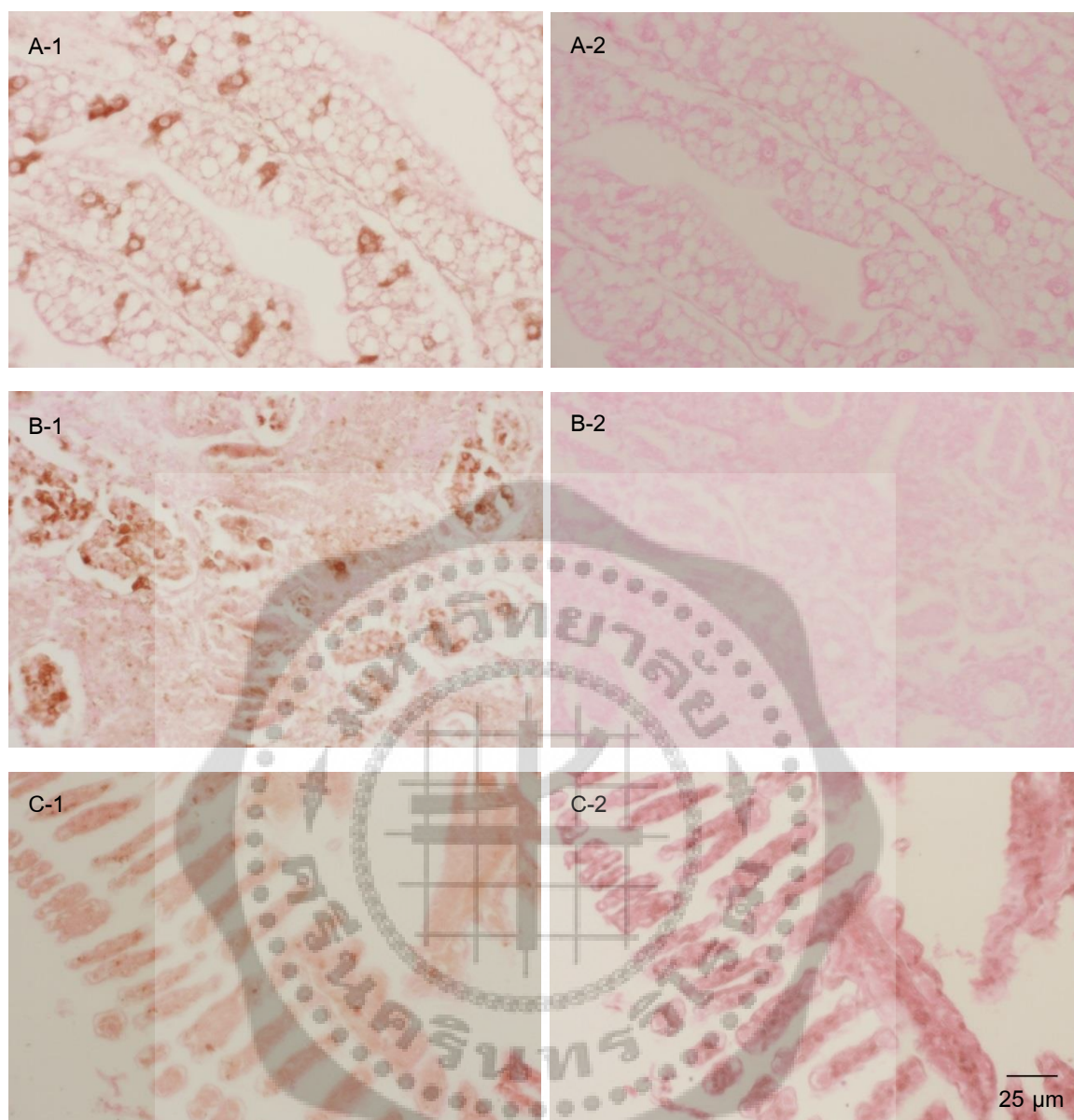


Figure 9 Immunohistochemistry.

In Figure 9, *L. vannamei* was experimentally infected with *V. vulnificus* VV2 (A-1), (B-1), or VV8 (C-1) and processed for immunohistochemistry using Mab (A-1) and (B-1) VV-18H6, (C-1) VV-10A6 compared with normal tissues (A-2, B-2 and C-2). The bacterial immunoreactivity appeared as brown coloration in various tissues: A= hepatopancreas, B = lymphoid organ and C = gill tissue.

1.4 Detection of *V. vulnificus* after pre-incubation in alkaline peptone water

The detection sensitivities of the MAbs ranged from 10^6 to 10^7 CFU ml⁻¹ (10^3 to 10^4 CFU μ l⁻¹) as determined by dot blotting using the heat-killed bacteria diluted in PBS (Figure 10). For the spiking experiment, APW was inoculated with a pure culture of *V. vulnificus* (VV3). At the beginning of the experiment (0 h), the detection sensitivities were reduced by one log phase from the pure culture diluted in PBS due to competition between the high protein content of the APW and the *V. vulnificus* antigens on binding to the membrane. After incubation, the detection sensitivity of MAb VV-20D1 increased to 10^5 CFU ml⁻¹ after 3 h incubation periods, and 10^4 CFU ml⁻¹ after 6, 12 and 24 h incubation periods. The detection sensitivity of MAb VV-7B6, VV-5B8 and VV-15E7 increased to 10^4 CFU ml⁻¹ after 3 h incubation periods, and 10^3 CFU ml⁻¹ after 6, 12 and 24 h incubation periods. (Figure 11).

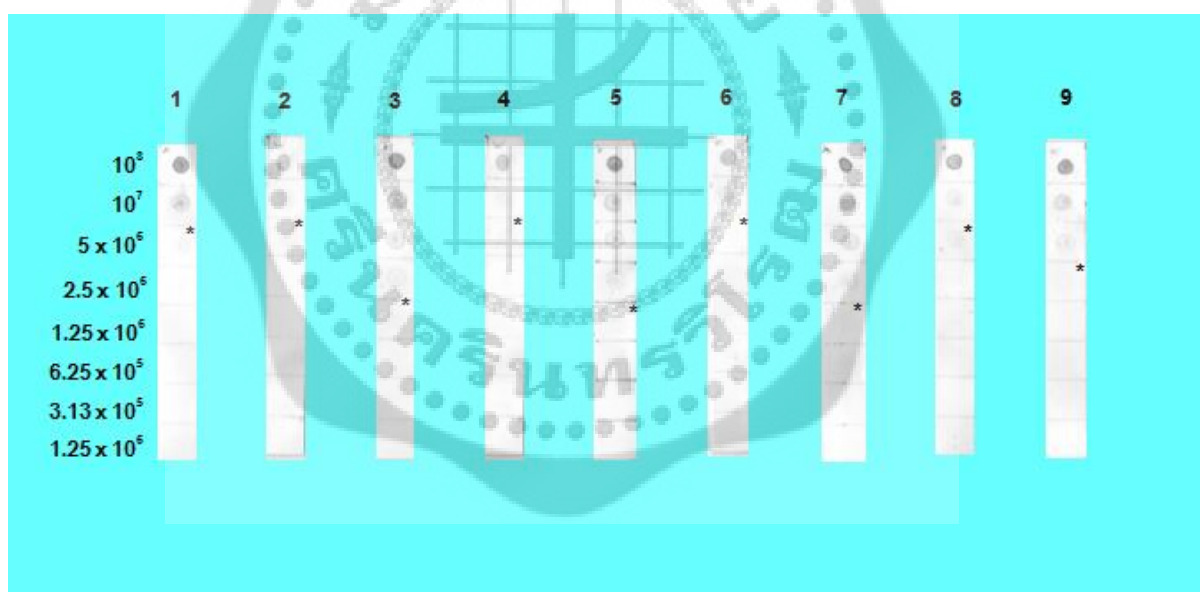


Figure 10 Sensitivity testing of MAbs for *V. vulnificus* as detected by dot blotting.

In Figure 10, diluted bacteria were spotted on a nitrocellulose membrane (1 μ l per spot) and probed with MAbs (1) VV-5F2, (2) VV-4G2, (3) VV-6B9, (4) VV-18H6, (5) VV-10A6, (6) VV-20D1, (7) VV-7B6, (8) VV-5B8 and (9) VV-15E7. * = lowest detectable immunoreactivity.

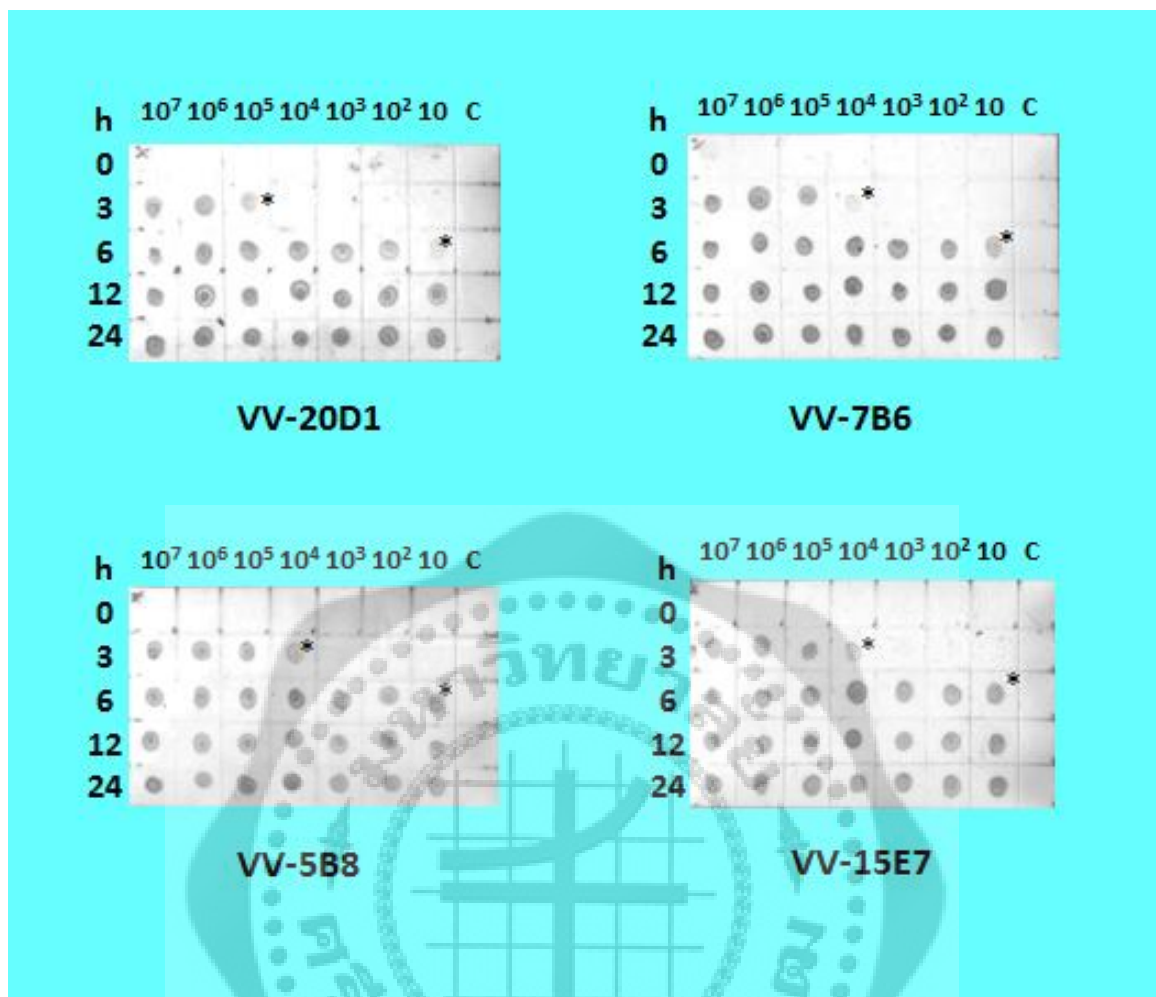


Figure 11 Sensitivity test after pre-incubating in APW.

In Figure 11, *V. vulnificus* VV3 was spiked into APW at 0-10⁷ CFU ml⁻¹, incubated at 37 °C, and sampled at 0, 3, 6, 12 and 24 h after inoculation. All samples were heat-treated to killed bacteria. One microliter of each samples were spotted onto a nitrocellulose membrane and probed with MAbs (A) VV-20D1(specific to *V. vulnificus*), (B) VV-7B6 (specific to *V. vulnificus* and *V. alginolyticus*), (C) VV-5B8 (specific to *V. vulnificus* and *V. fluvialis*) and (D) VV-15E7 (specific to *Vibrio* spp.). * = lowest detectable immunoreactivity.

1.5 Detection of *V. vulnificus* after pre-incubation in oyster homogenate-APW

When *V. vulnificus* (VV3) was spiked into oyster homogenates in APW, the detection sensitivity of MAb VV-20D1 was higher than 10^7 CFU ml⁻¹ (10^8 CFU g⁻¹ of oyster tissue) because the reactivity was not obtained at 0 h. However, bacteria at initial concentrations of 10^6 CFU ml⁻¹ (10^7 CFU g⁻¹) and 10^2 CFU ml⁻¹ (10^3 CFU g⁻¹) could be propagated to easily detectable levels following incubation for 3 h and 6 h, respectively. The detection sensitivity of MAb VV-7B6 increased to 10^6 CFU ml⁻¹ after 3 h after incubation periods, 10^2 CFU ml⁻¹ after 6 h, and 10 CFU ml⁻¹ after 12 and 24 h after incubation periods. The detection sensitivity of MAb VV-5B8 increased to 10^6 CFU ml⁻¹ after 3 h after incubation periods, 10^2 CFU ml⁻¹ after 6 h, and 10 CFU ml⁻¹ after 12 and 24 h after incubation periods. The detection sensitivity of MAb VV-15E7 increased to 10^4 CFU ml⁻¹ after 3 h after incubation periods, and 10 CFU ml⁻¹ after 6, 12 and 24 h after incubation periods. The control sample gave a negative immunoreactivity for all MAb tested in this study (Figure 12).

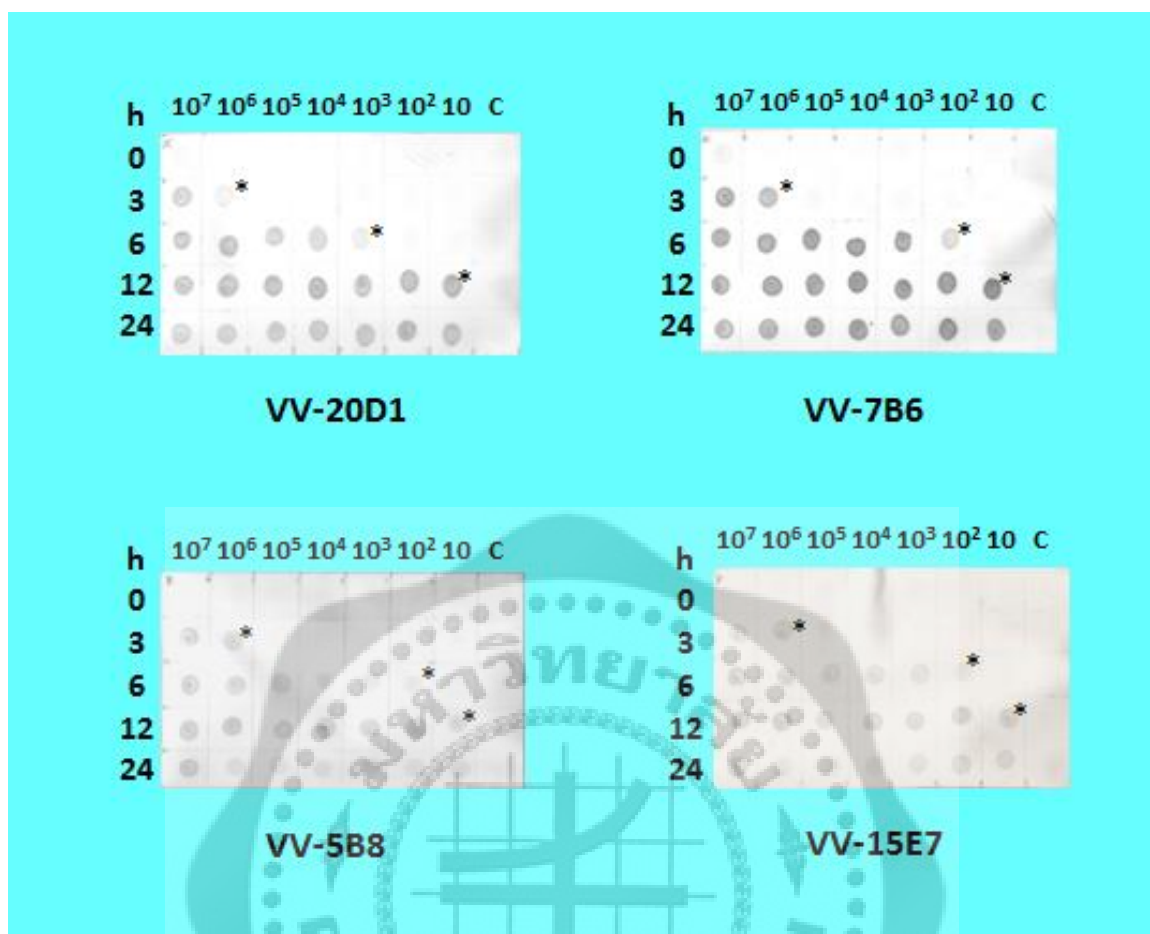


Figure 12 Sensitivity test after pre-incubating oyster homogenate in APW.

In Figure 12, *V. vulnificus* VV3 was spiked into oyster homogenate at $0-10^7$ CFU ml⁻¹, incubated at 37 °C, and sampled at 0, 3, 6, 12 and 24 h after inoculation. All samples were heat-treated to killed bacteria. One microliter of each samples were spotted onto a nitrocellulose membrane and probed with MAbs (A) VV-20D1 (specific to *V. vulnificus*), (B) VV-7B6 (specific to *V. vulnificus* and *V. alginolyticus*), (C) VV-5B8 (specific to *V. vulnificus* and *V. fluvialis*) and (D) VV-15E7 (specific to *Vibrio* spp.). * = lowest detectable immunoreactivity.

2. Detection of *Vibrio vulnificus* by loop-mediated isothermal amplification combined with lateral flow dipstick (LAMP-LFD) targeted to *rpoS* gene

2.1 LAMP primer design

A set of six primers for LAMP was designed according to the published sequence of *rpoS* coding region (GenBank ID: AY187681) using Primer Explorer ver. 4 (<http://primerexplorer.jp/elamp4.0.0/index.html>). A forward inner primer (FIP), a backward inner primer (BIP), two outer primers (F3 and B3) and two loop primers (LF and LB) were used for LAMP method. The sequences of primers and the locations were shown in Table 4.

2.2 Optimization of the temperature and time for *V. vulnificus* detection by LAMP

To determine the optimum temperature for LAMP assay, three different temperatures including 60, 63 and 65 °C were used in the reaction for 60 min. At all tested temperatures, the LAMP products displayed the ladder-like pattern characteristic of LAMP amplicons in agarose gels. However, at 65 °C, the LAMP products were the clearest (Figure 13). Therefore, the temperature at 65 °C was selected for the subsequent LAMP assays. To determine the optimum time for LAMP assay, three different reaction times including 30, 60 and 90 min were used in the LAMP reaction. The LAMP amplicons could be observed at 60 min. However, at 90 min, the intensity of LAMP amplicons was stronger and clearly detected (Figure 14). Therefore, the reaction time of 90 min was chosen as an optimal reaction time for LAMP assay.

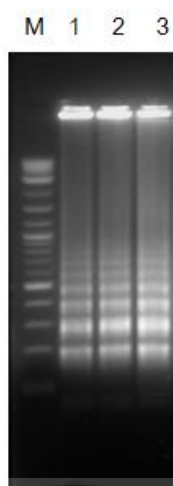


Figure 13 Determination of the optimal temperature for LAMP assay targeted to *rpoS* gene of *V. vulnificus*.

The Figure 13 showed the determination of the optimal temperature for LAMP assay targeted to *rpoS* gene of *V. vulnificus*. Lane M : 2-Log DNA Ladder, 1 : 60 °C, 2 : 63 °C, 3 : 65°C.



Figure 14 Determination of the optimal time for LAMP assay targeted to *rpoS* gene of *V. vulnificus*.

The Figure 14 showed the determination of the optimal time for LAMP assay targeted to *rpoS* gene of *V. vulnificus*. Lane M : 2-Log DNA Ladder, 1 : 30 min, 2 : 60 min, 3 : 90 min.

2.3 The optimal amount of FITC-labeled probe for lateral flow dipstick assay

To estimate the appropriate amount of FITC-labeled probe in the hybridization reaction, 200, 20 and 2 pmol of DNA probe were tested. The result showed that the 20 pmol of FITC-labeled DNA probe gave the highest intensity of purple color at the test line (Figure 15). Thus, the amount of 20 pmol was used for all subsequent assays.

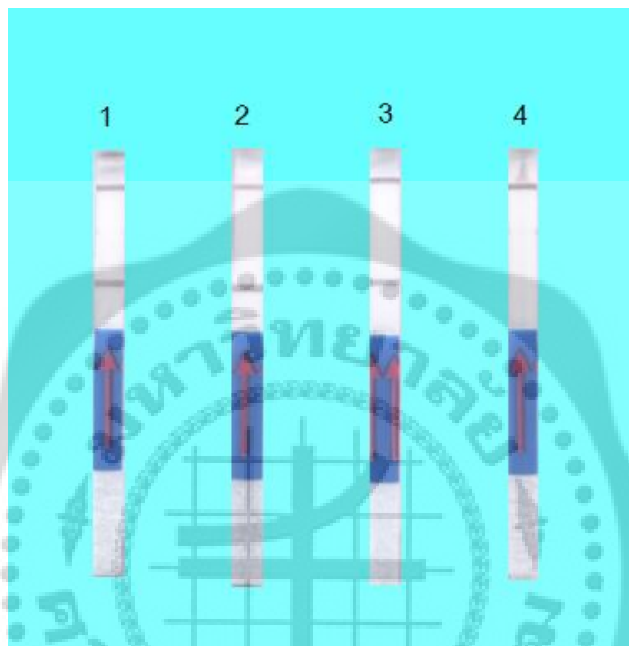


Figure 15 The various amount of FITC-labeled probe used in this study for lateral flow dipstick assay.

The Figure 15 showed various amount of FITC-labeled probe used in this study for lateral flow dipstick (LFD) assay, (1) 200 pmol, (2) 20 pmol, (3) 2 pmol, (4) negative control.

2.4 Specificity for *V. vulnificus* detection by LAMP-LFD

To determine the specificity of LAMP-LFD assay for detection of total *V. vulnificus*, various bacterial species were tested. As shown in Table 6, LAMP products were detected in all 14 *V. vulnificus* isolates, whereas no LAMP amplicons were revealed in 25 other *Vibrio* spp. isolates and 37 non-*Vibrio* isolates. All the results of LAMP assay agreed with that of *rpoS* PCR. However, two isolates of *V. vulnificus* designated as VVS 4907006 and VVS 4907011 yielded negative result for *vvhA* PCR amplifications as shown in Table 6.

Table 6 Specificity for *V. vulnificus* detection by PCR and LAMP

Bacterial isolates	Origin	Amplification			Source
		<i>rpoS</i>		<i>vvhA</i>	
		LAMP	PCR	PCR	
<i>Vibrio vulnificus</i> (n=14)					
DABU	Sea bass	+	+	+	DABU
MT 1506	Unknown	+	+	+	DABU
VVS 4907001	<i>Litopenaeus vannamei</i>	+	+	+	DBSWU
VVS 4907002	<i>L. vannamei</i>	+	+	+	DBSWU
VVS 4907006	<i>L. vannamei</i>	+	+	-	DBSWU
VVS 4907011	<i>L. vannamei</i>	+	+	-	DBSWU
DMST 5852	Hemoculture	+	+	+	DMST
DMST 19346	Hemoculture	+	+	+	DMST
DMST 22441	Hemoculture	+	+	+	DMST
DMST 26351	Hemoculture	+	+	+	DMST
DMST 27426	Hemoculture	+	+	+	DMST
DMST 27427	Blood	+	+	+	DMST
DMST 27428	Hemoculture	+	+	+	DMST
DMST 27429	Hemoculture	+	+	+	DMST
<i>V. fluvialis</i> (n=4)					
DMST 22086	Stool	-	-	-	DMST
DMST 22087	Stool	-	-	-	DMST
DMST 24049	Stool	-	-	-	DMST
DMST 24050	Stool	-	-	-	DMST
<i>V. cholerae</i> (n=3)					
DMST 22140	Food	-	-	-	DMST
DMST 22142	Food	-	-	-	DMST
DMST 22143	Stool	-	-	-	DMST
<i>V. mimicus</i> (n=3)					
DMST 22088	Food	-	-	-	DMST
DMST 22089	Food	-	-	-	DMST
DMST 22090	Food	-	-	-	DMST

Table 6 (continued)

Bacterial isolates	Origin	Amplification			Source
		<i>rpoS</i>		<i>vvhA</i>	
		LAMP	PCR	PCR	
<i>V. alginolyticus</i> (n= 3)					
DMST 22082	Stool	-	-	-	DMST
DMST 22084	Food	-	-	-	DMST
DMSC 14800	Seafood	-	-	-	DMSC
<i>V. parahaemolyticus</i> (n=3)					
DMST 15285	Food	-	-	-	DMST
DMST 21330 (O3:KUT)	Shrimp	-	-	-	DMST
DABU (O10:KUT)	<i>P. monodon</i>	-	-	-	DABU
<i>V. campbellii</i> (n=3)					
LMG 21361	Unknown	-	-	-	GB
LMG 21362	Unknown	-	-	-	GB
LMG 21363	Unknown	-	-	-	GB
<i>V. harveyi</i> (n=3)					
Centex 639	Unknown	-	-	-	Centex
Centex 1526	Unknown	-	-	-	Centex
LMG 22888	Unknown	-	-	-	GB
<i>V. shilonii</i> VS 4907012 (n=1)	<i>L. vannamei</i>	-	-	-	DBSWU
<i>V. anguillarum</i> AV L01 (n=1)	Unknown	-	-	-	DABU
<i>V. ordalii</i> Vib02 (n=1)	Unknown	-	-	-	DABU
Other non- <i>Vibrio</i> bacteria (n=37)		-	-	-	

2.5 Sensitivity for *V. vulnificus* detection by LAMP-LFD and PCR methods

To determine the sensitivity of LAMP-LFD methods, the DNA templates extracted from pure culture and spiked oyster samples were used in the LAMP reaction. Based on initial inoculums of *V. vulnificus* (1.5×10^8 CFU ml⁻¹), the sensitivity of LAMP for the detection of *V. vulnificus* in pure culture was 1.5×10^3 CFU ml⁻¹ or equivalent to 2.8 CFU per reaction, while that of PCR was 280 CFU per reaction (Figure 16a and 17a). For spiked oyster samples, the sensitivity of LAMP was 1.2×10^4 CFU g⁻¹ or equivalent to 11 CFU per reaction, while that of PCR was 1.1×10^3 CFU per reaction (Figure 16b and 17b).



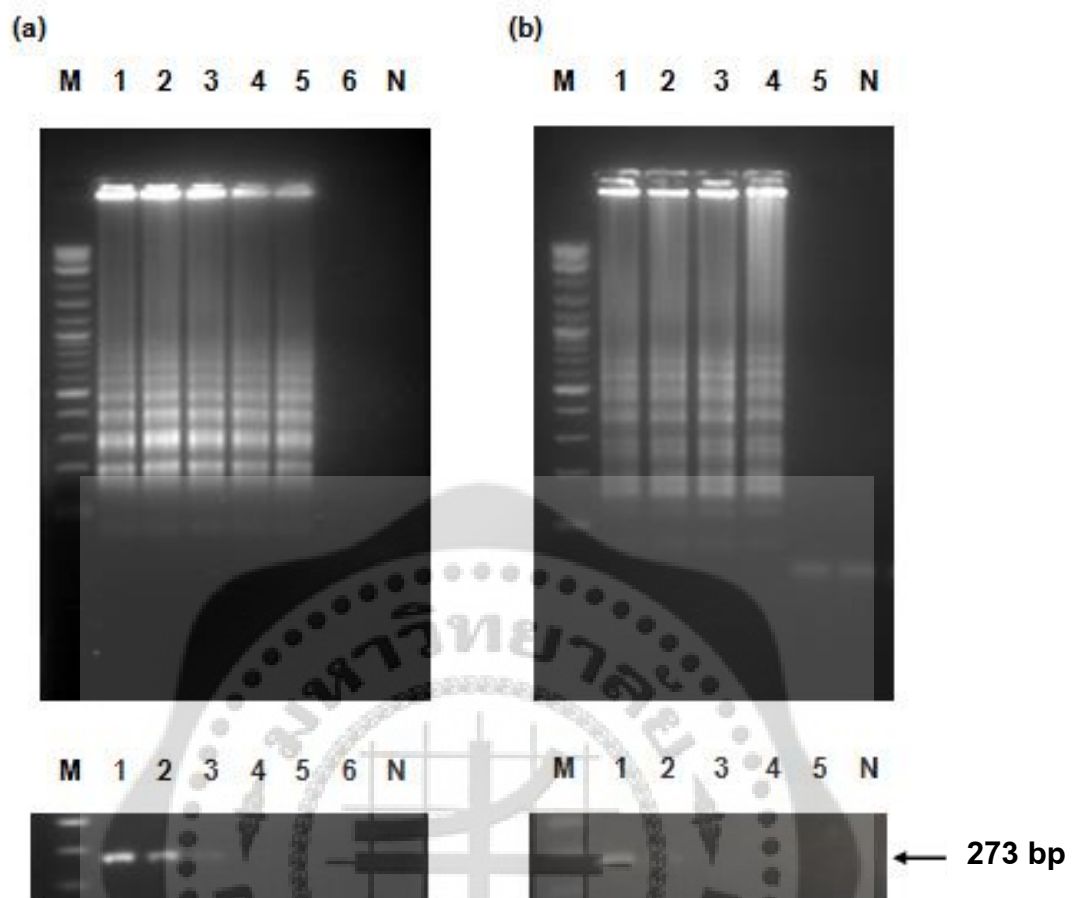


Figure 16 Sensitivity comparison of loop-mediated isothermal amplification and PCR assays for the *V. vulnificus* detection in pure cultures.

The Figure 16 showed sensitivity comparison of loop-mediated isothermal amplification (LAMP) (top panel) and PCR (bottom panel) assays for the *V. vulnificus* detection in pure cultures (a), lanes 1-6 represent agarose gel electrophoresis of LAMP and PCR amplicons from 2.8×10^4 to 0.28 CFU per reaction. The sensitivity of LAMP and PCR assays for the *V. vulnificus* detection in spiked oyster samples (b), lanes 1-5 represent agarose gel electrophoresis of LAMP and PCR amplicons from 1.1×10^4 to 1.1 CFU per reaction. Lane M: 2-Log DNA Ladder and lane N: no template control (negative control). LAMP amplicons of 2.5 μ l out of 25 μ l were loaded per lane, whereas PCR amplicons of 5 μ l out of 50 μ l were loaded per lane.

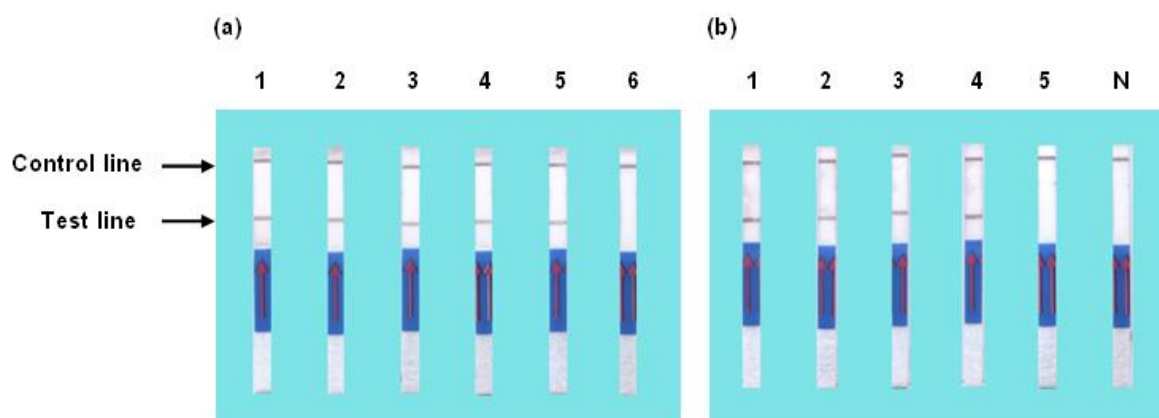


Figure 17 Sensitivity of LAMP combined with LFD for the detection of *V. vulnificus*.

The Figure 17 showed sensitivity of LAMP combined with LFD for the detection of *V. vulnificus* in pure cultures (a), lanes 1-6 represent LFD of LAMP amplicon from 2.8×10^4 to 0.28 CFU per reaction. The sensitivity of LAMP combined with LFD for the detection of *V. vulnificus* in spiked oyster samples (b), lanes 1-5 represent LFD of LAMP amplicon from 1.1×10^4 to 1.1 CFU per reaction. Lane N: no template control (negative control).

CHAPTER 5

DISCUSSIONS

Vibrio vulnificus is a part of the natural flora of the coastal marine environment worldwide and has been isolated from water, sediment and seafood, especially in raw oyster. This bacterium causes two distinct disease syndromes such as primary septicemia and necrotizing wound infections. It is the leading cause of food-related mortality reported in many cases (Jones; & Oliver; 2009: 1723-1733). Therefore, the problem of *V. vulnificus* contaminated food is a concern and an effective method to detect this bacterium is required.

The identification of *V. vulnificus* played important roles but have incorporated many techniques and required microbiological and immunological skills of laboratory staff. However, the simple method for identification of species-specific of *V. vulnificus* was not successfully developed. In this study, MAbs specific to *V. vulnificus* and LAMP-LFD targeted to *rpoS* gene of *V. vulnificus* have been successfully developed.

In the case of confirmation of *V. vulnificus* used in this study, polymerase chain reaction (PCR) targeted to *rpoS* gene gave positive results for detection of all *V. vulnificus* and did not show any cross reactivity to the other bacteria. However, the commonly used *vhA* PCR amplification for *V. vulnificus* identification yielded negative results with two isolates of *V. vulnificus*. It has been shown that *rpoS* gene was suitable for detection of *V. vulnificus* (Kim et al; 2008: 1841-1847). These results showed that *V. vulnificus* used in this study is actually *V. vulnificus*.

In the case of production of MAbs against *V. vulnificus*, heat-killed and formalin-fixed form of *V. vulnificus* were used for immunization, which different from previous studies that used heat-killed plus SDS-mercaptoethanol-treated (Rengpipat et al: 2008: 398-404), extracted cellular components from bacteria including lipopolysaccharide (Martin and Siebeling, 1991: 1684-1688; Biosca et al, 1996: 918-927 ; Amaro et al, 1997: 1454-1457), capsular polysaccharide (Wright et al, 1990: 1769-1773) and flagella (Simonson and Siebeling, 1986: 1299-1304). The different forms of antigen using heat-killed and formalin-fixed form could increase the ability of B-cell to response and produce MAbs specific to various epitopes of *V. vulnificus*.

After fusion, nine groups of MAbs specific to *V. vulnificus* and *Vibrio* spp. were generated. This results indicate some variation among the *V. vulnificus* isolates used in this

study. The first five groups (VV-5F2, VV-4G2, VV-19G12, VV-6B9, VV-18H6 and VV-10A6) were isolates specific that recognized one to seven *V. vulnificus* isolates without any cross reactivity to the other tested *V. vulnificus* isolates. Therefore, they can be used for isolate-specific identification but did not for species-specific identification. Interestingly, the MAbs in group 6 (VV-20D1, VV-14B) recognized all tested isolates almost of *V. vulnificus* but did not show cross reactivity with other *Vibrio* species. MAbs in this group were more specific to *V. vulnificus* than previous reports (Simonson; & Siebeling. 1986: 1299-1304; Martin; & Siebeling. 1991: 1684-1688; Rengpipat; Pusiririt; & Rukpratanporn. 2008: 398-404; Jadeja; Janes; & Simonson. 2010: 1288-1293) and can be used to identify *V. vulnificus*. The MAbs in group 7 (VV-6D2, VV-7B6, VV-16A11 and VV-18B11) recognized all tested isolates of *V. vulnificus* and *V. alginolyticus* but did not show cross reactivity with other *Vibrio* species. Therefore, these MAbs can be used for differentiation of *V. vulnificus* and *V. alginolyticus* from the other *Vibrio* species. The MAbs in group 8 (VV-5B8 and VV-13B4) recognized all tested isolates of *V. vulnificus* and *V. fluvialis* but did not show cross reactivity with other *Vibrio* species. Thus, these MAbs can be used for identification of *V. vulnificus* and *V. fluvialis* from the other *Vibrio* species. The MAb in group 9 (VV15E7) bound to all tested isolates of *Vibrio* spp. and did not show cross reactivity to non-*Vibrio* isolates used in this study. MAb in this group was not similar to MAb (VVB-100) in previous experiment (Rengpipat et al. 2008: 398-404). MAb VV-15E7 bound to a range of antigens with molecular masses of 20-25 kDa in Western blot analysis while VVB-100 bound to antigens with molecular masses of 10 and 13 kDa. Furthermore, both VV-15E7 and VVB-100 can be used for detection of *Vibrio* spp. with different epitope. Further developments in using MAbs (VV-20D1 combined with VV-15E7 and VVB-100) in a simple immunochromatographic strip test would reduce the detection time to 15 min with similar sensitivity to dot blotting and without the requirement for skilled personnel, additional supplies, or instruments (Sithigorngul et al. 2007: 256-264)

The detection sensitivities of MAbs range from 10^6 - 10^7 CFU ml⁻¹, which is less sensitive than that of the molecular techniques, such as PCR, real time PCR and LAMP. However, after pre-incubation enrichment in APW for 3 to 6 h, the detection limit were comparable to that of molecular techniques and could detect up to 10 CFU ml⁻¹ after incubation for 24 h. In the case of pre-incubation of oyster homogenate-APW, the sensitivity of MAbs was one log phase lower than that of pre-incubation enrichment in APW. It may be due to competition between the high protein content of the oyster samples and the *V. vulnificus* antigens on binding to the membrane

MAbs in group two, four, five and nine obtained from this study could be used to determine *V. vulnificus* infection in shrimp tissues by immunohistochemistry. Although the MAbs in group six, seven and eight bound denatured forms of antigens in Western blotting, these MAbs did not recognize modified antigen in immunohistochemical process. It may be due to some modifications of antigens during sample preparation, such as heat treatment during paraffin impregnation, and formalin fixation (Pengsuk et al. 2010: 17-24).

In the case of LAMP-LFD method targeted to the *rpoS* gene of *V. vulnificus*, the optimum temperature and time for LAMP reaction are 65 °C and 90 min, respectively. This is faster than the typical methods of PCR and more suitable for low equipment setting laboratory. The use of the four specific primers and two loop primers targeting to *rpoS* gene of *V. vulnificus* ensured high specificity of nucleic acid amplification. The LAMP reaction was positive for all *V. vulnificus* isolates and negative for the other species tested, demonstrating that these primers were specific for identification of *V. vulnificus*. However, the commonly used *vvhA* PCR amplification for *V. vulnificus* identification yielded negative results with two isolates of *V. vulnificus*. These two isolates were isolated from infected shrimp by our laboratory. It has been shown that in certain cases *vvhA* gene was not suitable for detection of *V. vulnificus* because of rearrangement or mutation within this gene (Brauns; Hudson; & Oliver. 1991: 2651-2655; Maugeri et al. 2006: 194-200). Therefore, *rpoS* gene used in this study appears to be useful for detection of *V. vulnificus*.

In this study, the sensitivity of LAMP-LFD assay with loop primers in pure culture was 2.8 CFU per reaction (1.5×10^3 CFU ml⁻¹), was 100 times higher than that of *rpoS* PCR and was 6 times higher than that of previous LAMP assays without loop primers for *V. vulnificus* detection (16 CFU per reaction (Yongjum et al. 2010: 93-97), and 20 CFU per reaction (Han; & Ge, 2010: 60-66)). In addition, the sensitivity of LAMP-LFD assay in pure culture in this study was comparable to that of previous LAMP assay with loop primers for *V. cholerae* detection (2.9 CFU per reaction (Yamazaki et al. 2008b: 94), and 8 CFU per reaction (Srisuk et al. 2010: 36-42), *V. parahaemolyticus* detection (2.0 CFU per reaction (Yamazaki; et al. 2008a: 163) and *Salmonella* detection (2.2 CFU per reaction (Hara-Kudo; et al. 2005: 155-161). These results demonstrate that the LAMP assay using loop primers have higher sensitivity than LAMP assay without loop primers because loop primers accelerate LAMP reaction by hybridizing to stem-loops formed during LAMP reaction and facilitating strand displacement and amplification (Yano; Ishimaru; & Hujikata. 2007: 414-420; Hara-Kudo; et al. 2005: 155-161; Jaroenram; Kiatpathomchai; & Flegel. 2009: 65-70; Nagamine; Hase; & Notomi. 2002: 223-229).

In the case of spiked oyster samples, the sensitivity of LAMP-LFD in this study (11 CFU per reaction) was 100 times higher than that of PCR assay (1.1×10^3 CFU per reaction) and was 10 times higher than that of previous LAMP assays for *V. vulnificus* detection in spiked oyster without enrichment (116 CFU per reaction (Han; & Ge. 2010: 60-66)), 2 times higher than *V. cholerae* detection in spiked shrimp samples (20 CFU per reaction (Srisuk; et al. 2010: 36-42)), but 5 times lower than *V. parahaemolyticus* detection in spiked shrimp samples (2.0 CFU per reaction (Yamazaki; et al. 2008a: 163)) and *Salmonella* detection in spiked egg samples (2.2 CFU per reaction (Hara-Kudo; et al. 2005: 155-160)). In previous work on LAMP-LFD for *V. parahaemolyticus* detection in spiked shrimp samples demonstrated the detection limit of 3 CFU per reaction, approximately 3 times higher than that of LAMP-LFD for *V. vulnificus* detection in spiked oyster samples in this study (Prompamorn; et al. 2011: 344-351). The discrepancy of detection limit may be due to the differences of target gene or the food matrix that contain different degree of LAMP inhibitors.

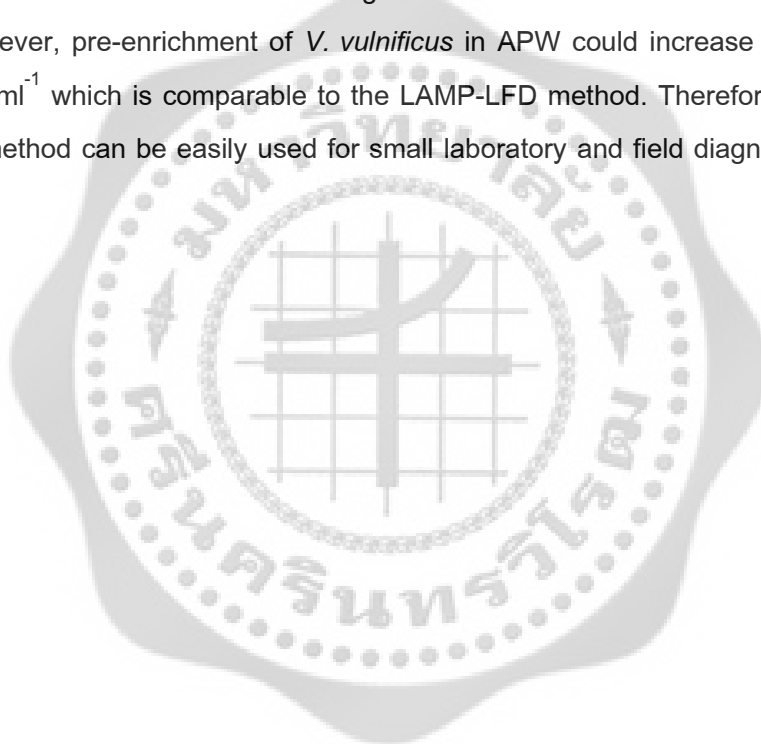
In this study, a detection limit of *V. vulnificus* at 1.2×10^4 CFU g⁻¹ of oyster samples without enrichment was demonstrated by LAMP-LFD. However, the regulatory guideline of less than 30 MPN g⁻¹ of *V. vulnificus* in postharvest-processed oysters is recommended by U.S. Food and Drug Administration (2007: online). Therefore, to achieve the needed sensitivity the pre-enrichment step may be required. In a previous report, after 5-h enrichment LAMP assay was capable of detecting 7 CFU of *V. vulnificus* per gram of spiked oyster tissue, whereas without enrichment the detection limit was 4×10^7 CFU g⁻¹ of oyster samples (Han; & Ge. 2008: 311-320).

The sensitivity of LAMP-LFD assay in both pure culture and spiked oyster samples was 100 times higher than that of PCR assay. This result agreed with previous studies demonstrated higher sensitivity of LAMP compared to that of PCR (Yamazaki; et al. 2008b: 94; Yongjum; et al. 2010: 93-97; Prompamorn; et al. 2011: 344-351). The lower sensitivity of PCR in spiked oyster may be due to inhibitors in oyster samples. The previous studies suggested that LAMP assay was less vulnerable to certain inhibitors present in the food contexts (Yamazaki; et al. 2008a: 163; Yamazaki; et al. 2008b: 94; Han; & Ge. 2008: 311-320 ; Kaneko; et al. 2007: 499-501).

Although a more rapid, sensitive and specific technique called LAMP has been developed for detection of several pathogenic bacteria including *V. vulnificus* (Han; & Ge. 2008: 311-320; Yongjum; et al. 2010: 93-97), *V. cholerae* (Yamazaki; et al. 2008b: 94; Srisuk; et al. 2010: 36-42), *V. parahaemolyticus* (Yamazaki; et al. 2008a: 163) and

Salmonella (Hara-Kudo; et al. 2005: 155-161) but LAMP amplicons in all these reports were detected by gel electrophoresis followed by staining with carcinogenic ethidium bromide which is not suitable for field applications. In this study, the LAMP-LFD products can be easily detected by dipping the membrane into the assay buffer which can detect LAMP amplicons within 10 min. Furthermore, the specificity of the LAMP-LFD assay can be increased since the hybridization with specific probe to LAMP amplicons is employed.

In summary, the methods for detection of *V. vulnificus* using both immunological based assay and LAMP-LFD targeted *rpoS* gene of *V. vulnificus* are sensitive, rapid, simple and valuable tool for detection of *V. vulnificus* for both research and diagnostic purposes. Although the detection limit of immunological based method was lower than LAMP-LFD method. However, pre-enrichment of *V. vulnificus* in APW could increase the detection limit to 10 CFU ml⁻¹ which is comparable to the LAMP-LFD method. Therefore, both MAbs and LAMP-LFD method can be easily used for small laboratory and field diagnosis.





BIBLIOGRAPHY

- Amaro, C.; & Biosca, E. G. (1996). *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. *Applied and environmental microbiology*. 62(4): 1454-1457.
- Bang, W.; & Drake, M. A. (2002). Resistance of cold- and starvation-stressed *Vibrio vulnificus* to heat and freeze-thaw exposure. *Journal of food protection*. 65(6): 975-980.
- Baumann, P; Furniss, A. L.; & Lee, J.V. (1984). Genus *Vibrio*. In: (9th edition ed.), *Bergeys's Manual of Systematic Bacteriology*, Williams and Wilkins, Baltimore, MD. 518-538.
- Biosca, E. G.; Oliver, J. D.; Amaro, C. (1996). Phenotypic characterization of *Vibrio vulnificus* biotype 2, a lipopolysaccharide-based homogeneous O serogroup within *Vibrio vulnificus*. *Applied and Environmental Microbiology*. 62(3): 918.
- Bisharat, N.; et al. (1999). Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Israel *Vibrio* Study Group. *Lancet*. 354(9188): 1421-1424.
- Blackwell, K. D.; & Oliver, J. D. (2008). The ecology of *Vibrio vulnificus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* in North Carolina estuaries. *Journal of microbiology*. 46(2): 146-153.
- Brasher, Cynthia W.; et al. (1998). Detection of microbial pathogens in shellfish with multiplex PCR. *Current Microbiology*. 37(2): 101-107.
- Brauns, L. A.; Hudson, M. C.; & Oliver, J. D. (1991). Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl. Environ. Microbiol.* 57(9): 2651-2655.
- Brayton, P. R.; et al. (1983). New selective plating medium for isolation of *Vibrio vulnificus* biogroup 1. *Journal of clinical microbiology*. 17(6): 1039-1044.
- Bryan, Patrick J.; et al. (1999). Adaptive response to cold temperatures in *Vibrio vulnificus*. *Current Microbiology*. 38(3): 168-175.
- Campbell, M. S.; & Wright, A. C. (2003). Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Applied and Environmental Microbiology*. 69(12): 7137-7144.

- Cao, Y. T.; et al. (2010). Evaluation of a loop-mediated isothermal amplification method for the rapid detection of *Vibrio harveyi* in cultured marine shellfish. *Letters in applied microbiology*. 51(1): 24-29.
- CDC. (1996). *Vibrio vulnificus* infections associated with eating raw oysters. *MMWR*. 45(29): 621-624.
- Cerda-Cuellar, M.; Jofre, J.; & Blanch, A. R. (2000). A selective medium and a specific probe for detection of *Vibrio vulnificus*. *Applied and environmental microbiology*. 66(2): 855-859.
- Cerda-Cuellar, M.; et al. (2001). Comparison of selective media for the detection of *Vibrio vulnificus* in environmental samples. *Journal of applied microbiology*. 91(2): 322-327.
- Chen, C. Y.; et al. (1996). Expression of *Salmonella typhimurium rpoS* and *rpoS*-dependent genes in the intracellular environment of eukaryotic cells. *Infection and immunity*. 64(11): 4739-4743.
- Chen, S.; & Ge, B. (2010). Development of a *toxR*-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. *BMC microbiology*. 10: 41.
- Chiang, S-R ; & Chuang, Y-C. (2003). *Vibrio vulnificus* infection: clinical manifestations, pathogenesis, and antimicrobial therapy. *Journal of Microbiology, Immunology and Infection* 36(2): 81-88.
- Christman, M. F.; et al. (1985). Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell*. 41(3): 753-762.
- Dalmasso, A.; Civera, T.; & Bottero, M. T. (2009). Multiplex primer-extension assay for identification of six pathogenic Vibrios. *International Journal of Food Microbiology*. 129(1): 21-25.
- Daniels, N. A.; et al. (2000). A foodborne outbreak of gastroenteritis associated with Norwalk-like viruses: first molecular traceback to deli sandwiches contaminated during preparation. *The Journal of infectious diseases*. 181(4): 1467-1470.
- Davies, G. E.; & Stark, G. R. (1970). Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 66(3): 651-656.

- del Camen Vargas, M. ; et al. (2003). Only one catalase, *katG*, is detectable in *Rhizobium etli*, and is encoded along with the regulator *OxyR* on a plasmid replicon. *Microbiology*. 149(5): 1165-1176.
- Drake, Stephenie L.; DePaola, Angelo; & Jaykus, Lee-Ann. (2007). An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *Comprehensive Reviews in Food Science and Food Safety*. 6(4): 120-144.
- Edwards, D. C.; et al. (1982). A comparison of the *in vitro* and *in vivo* activities of conjugates of anti-mouse lymphocyte globulin and abrin. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 717(2): 272-277.
- Elmore, S. P.; et al. (1992). Reversal of hypotension induced by *Vibrio vulnificus* lipopolysaccharide in the rat by inhibition of nitric oxide synthase. *Microbial pathogenesis*. 13(5): 391-397.
- Farmer, J. J. (1980). Revival of the name *Vibrio vulnificus*. *International Journal of Systematic Bacteriology*. 30(4): 656.
- Foire, A.; et al. (1992). Infection with *Vibrio vulnificus* elicits an antibody response to capsular polysaccharide. *Clinical Research*. 40: 428-431.
- Goding, J. W. (1980). Antibody production by hybridomas. *Journal of immunological methods*. 39(4): 285-308.
- Gonzalez-Flecha, B; & Demple, B. (1997). Transcriptional regulation of the *Escherichia coli oxyR* gene as a function of cell growth. *Journal of Bacteriology*. 179(19): 6181-6186.
- Gopal, S.; et al. (2005). The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *International Journal of Food Microbiology*. 102(2): 151-159.
- Han, Feifei; & Ge, Beilei. (2008). Evaluation of a loop-mediated isothermal amplification assay for detecting *Vibrio vulnificus* in raw oysters. *Foodborne Pathogens and Disease*. 5(3): 311-320.
- (2010). Quantitative detection of *Vibrio vulnificus* in raw oysters by real-time loop-mediated isothermal amplification. *International Journal of Food Microbiology*. 142(1-2): 60-66.
- Han, Feifei; Wang, Fei; & Ge, Beilei. (2011). Detecting potentially virulent *Vibrio vulnificus* strains in raw oysters by quantitative loop-mediated isothermal amplification. *Applied and environmental microbiology*. 77(8): 2589-2595.

- Hara-Kudo, Y.; et al. (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. *FEMS microbiology letters*. 253(1): 155-161.
- Harwood, V. J.; Gandhi, J. P.; & Wright, A. C. (2004). Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. *Journal of microbiological methods*. 59(3): 301-316.
- Hill, W. E.; et al. (1991). Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Applied and Environmental Microbiology*. 57(3): 707-711.
- Hoi, L.; Dalsgaard, I.; & Dalsgaard, A. (1998). Improved isolation of *Vibrio vulnificus* from seawater and sediment with cellobiose-colistin agar. *Applied and environmental microbiology*. 64(5): 1721-1724.
- Jadeja, R.; Janes, M. E.; & Simonson, J. G. (2010). Immunomagnetic separation of *Vibrio vulnificus* with anti flagellar monoclonal antibody. *Journal of Food Protection*. 73: 1288-1293.
- Jamet, Alexandre; et al. (2005). The katA catalase gene is regulated by OxyR in both free-living and symbiotic sinorhizobium meliloti. *Journal of Bacteriology*. 187(1): 376-381.
- Jaroenram, W.; Kiatpathomchai, W.; & Flegel, T. W. (2009). Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with lateral flow dipstick. *Molecular and Cellular Probe*. 23: 65-70.
- Jishage, M; et al. (1996). Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of four species of sigma subunit under various growth conditions. *Journal of Bacteriology*. 178(18): 5447-5451.
- Johnston, J. M.; Becker, S. F.; & McFarland, L. M. (1986). Gastroenteritis in patients with stool isolates of *Vibrio vulnificus*. *The American journal of medicine*. 80(2): 336-338.
- Jones, M. K.; & Oliver, J. D. (2009). *Vibrio vulnificus*: disease and pathogenesis. *Infection and immunity*. 77(5): 1723-1733.
- Kaneko, H.; et al. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods*. 70: 499-501.
- Kelly, M. T. (1982). Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurrence in a Gulf Coast environment. *Applied and Environmental Microbiology*. 44(4): 820-824.

- Kennedy, R. C.; & Dreesman, G. R. (1983). Common idiotypic determinant associated with human antibodies to hepatitis B surface antigen. *The Journal of Immunology*. 130(1): 385-389.
- Kim, D. G.; et al. (2008). Application of the *rpoS* gene for species-specific detection of *Vibrio vulnificus* by real-time PCR. *Journal of microbiology and biotechnology*. 18(11): 1841-1847.
- Kim, H. R.; et al. (1993). Hemolytic mechanism of cytolysin produced from *V. vulnificus*. *Life sciences*. 53(7): 571-577.
- Kim, S. O.; et al. (2002). OxyR: A molecular code for redox-related signaling. *Cell*. 109(3): 383-396.
- Kitaura, T.; et al. (1983). Halo production by sulfatase activity of *V. vulnificus* and *V. cholerae* O1 on a new selective sodium dodecyl sulfate-containing agar medium: A screening marker in environmental surveillance. *FEMS microbiology letters*. 17(1-3): 205-209.
- Knutton, S.; & Pasternak, C. A. (1979). The mechanism of cell-cell fusion. *Trends in biochemical sciences*. 4(10): 220-223.
- Kohler, G.; & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 256(5517): 495-497.
- Kong, In-Soo; et al. (2004). Role of catalase and OxyR in the viable but nonculturable state of *Vibrio vulnificus*. *FEMS Microbiology Ecology*. 50(3): 133-142.
- Kumar, H. Sanath; et al. (2006). A *gyrB*-based PCR for the detection of *Vibrio vulnificus* and its application for direct detection of this pathogen in oyster enrichment broths. *International Journal of Food Microbiology*. 111(3): 216-220.
- Lange, R; & Hengge-Aronis, R. (1994). The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes & development*. 8(13): 1600-1612.
- Linkous, D. A.; & Oliver, J. D. (1999). Pathogenesis of *Vibrio vulnificus*. *FEMS microbiology letters*. 174(2): 207-214.
- Littlefield, John W. (1964). Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. *Science*. 145(3633): 709-710.
- Litwin, C. M.; Rayback, T. W.; & Skinner, J. (1996). Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. *Infection and Immunity*. 64(7): 2834-2838.
- Loewen, P. C.; et al. (1998). Regulation in the *rpoS* regulon of *Escherichia coli*. *Canadian journal of microbiology*. 44(8): 707-717.

- Martin, S. J.; & Siebeling, R. (1991). Identification of *Vibrio vulnificus* O serovars with antilipopolysaccharide monoclonal antibody. *Journal of Clinical Microbiology*. 29: 1684-1688.
- Massad, G.; & Oliver, J. D. (1987). New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. *Applied and environmental microbiology*. 53(9): 2262-2264.
- Maugeri, T. L.; et al. (2006). Detection and differentiation of *Vibrio vulnificus* in sea water and plankton of coastal zone of the Mediterranean sea. *Research in Microbiology*. 157: 194-200.
- McPherson, V. L.; et al. (1991). Physiological effects of the lipopolysaccharide of *Vibrio vulnificus* on mice and rats. *Microbios*. 67(272-273): 141-149.
- Merkel, S. M.; et al. (2001). Essential role for estrogen in protection against *Vibrio vulnificus*-induced endotoxic shock. *Infection and immunity*. 69(10): 6119-6122.
- Miceli, G. A.; Watkins, W. D.; & Rippey, S. R. (1993). Direct plating procedure for enumerating *Vibrio vulnificus* in oysters (*Crassostrea virginica*). *Applied and environmental microbiology*. 59(11): 3519-3524.
- Morgan, R. W.; et al. (1986). Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proceedings of the National Academy of Sciences*. 83(21): 8059-8063.
- Mori, Y.; Hirano, T.; & Notomi, T. (2006). Sequence specific visual detection of LAMP reactions by addition of cationic polymers. *BMC Biotechnology*. 6(1): 3.
- Morris, J. Glenn; et al. (1987). Virulence of *Vibrio vulnificus*: association with utilization of transferrin-bound iron, and lack of correlation with levels of cytotoxin or protease production. *FEMS microbiology letters*. 40(1): 55-59.
- Mosmann, T. R.; Baumal, R.; & Williamson, A. R. (1979). Mutations affecting immunoglobulin light chain secretion by myeloma cells. I. Functional analysis by cell fusion. *European journal of immunology*. 9(7): 511-516.
- Motes, M. L.; & DePaola, A. (1996). Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). *Applied and environmental microbiology*. 62(10): 3875-3877.
- Motes, M. L.; et al. (1998). Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). *Applied and environmental microbiology*. 64(4): 1459-1465.

- Nagamine, K.; Hase, T.; & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*. 16: 223-229.
- Nemoto, J.; et al. (2009). Rapid and specific detection of the thermostable direct hemolysin gene in *Vibrio parahaemolyticus* by loop-mediated isothermal amplification. *Journal of food protection*. 72(4): 748-754.
- Nimitphak, T.; et al. (2010). Rapid and sensitive detection of *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Molecular and cellular probes*. 24(1): 1-5.
- Notomi, T.; et al. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research*. 28(12): e63.
- O'Hara, C. M.; et al. (2003). Accuracy of six commercially available systems for identification of members of the family vibronaceae. *Journal of clinical microbiology*. 41(12): 5654-5659.
- Oliver, J. D. (2006). *Vibrio vulnificus*. In *The Biology of Vibrios*. ASM Press. American society of Microbiology. 1752 N St., N. W. Washington, DC.
- Oliver, J. D.; et al. (1995). Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. *Applied and environmental microbiology*. 61(7): 2624-2630.
- Oliver, J. D.; et al. (1986). Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. *Diagnostic microbiology and infectious disease*. 5(2): 99-111.
- Olsén, A.; et al. (1993). The *RpoS* Sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Molecular microbiology*. 7(4): 523-536.
- Olson, M. V. (1995). A time to sequence. *Science*. 270: 394-396.
- Panek, H. R.; & O'Brian, M. R. (2004). *KatG* Is the Primary Detoxifier of Hydrogen Peroxide Produced by Aerobic Metabolism in *Bradyrhizobium japonicum*. *Journal of Bacteriology*. 186(23): 7874-7880.
- Panicker, G.; et al. (2004). Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Applied and Environmental Microbiology*. 70(12): 7436-7444.
- Panicker, G.; & Bej, A. K. (2005). Real-time PCR detection of *Vibrio vulnificus* in oysters: Comparison of oligonucleotide primers and probes targeting *vwhA*. *Applied and Environmental Microbiology*. 71(10): 5702-5709.

- Parida, M.; et al. (2004). Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *Journal of clinical microbiology*. 42(1): 257-263.
- Pengsuk, C.; et al. (2010). Development of monoclonal antibodies for simple detection and differentiation of *Vibrio mimicus* from *V. Cholerae* and *Vibrio* spp. by dot blotting. *Aquaculture*. 300: 17-24.
- Pfeffer, C. S.; Hite, M. F.; & Oliver, J. D. (2003). Ecology of *Vibrio vulnificus* in estuarine waters of eastern North Carolina. *Applied and environmental microbiology*. 69(6): 3526-3531.
- Pontecorvo, G. (1975). Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic cell genetics*. 1(4): 397-400.
- Poste, G.; & Nicolson, G. L. (1978). *Membrane fusion*. Amsterdam ; New York: North-Holland Pub. Co. (Cell surface reviews)
- Prompamorn, P.; et al. (2011). The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Vibrio parahaemolyticus*. *Letters in applied microbiology*. 52(4): 344-351.
- Ren, Chun-Hua; et al. (2009). Sensitive and rapid identification of *Vibrio vulnificus* by loop-mediated isothermal amplification. *Microbiological Research*. 164(5): 514-521.
- Rengpipat, S.; Pusiririt, S.; & Rukpratanporn, S. (2008). Differentiating between isolates of *Vibrio vulnificus* with monoclonal antibodies. *Journal of microbiological methods*. 75(3): 398-404.
- Ringertz, N. R.; & Savage, R. E. (1976). *Cell hybrids*. New York: Academic Press.
- Roberts, N. C.; et al. (1982). Vibrios in the Louisiana gulf coast environment. *Microbial ecology*. 8(4): 299-312.
- Savan, R.; et al. (2005). Loop-mediated isothermal amplification: an emerging technology for detection of fish and shellfish pathogens. *Journal of fish diseases*. 28(10): 573-581.
- Simonson, J.; & Siebeling R. J. (1986). Rapid serological identification of *Vibrio vulnificus* by anti-H coagglutination. *Applied and Environmental Microbiology*. 52(6): 1299-1304.
- Simpson, L. M.; Engle, M. ; & Oliver, J. D. (1994). Effect of osmotic variation on the outer membrane proteins of *Vibrio vulnificus*: identification of a major heat-modifiable protein. *Microbios*. 80(325): 209-214.

- Simpson, L. M.; & Oliver, J. D. (1987). Ability of *Vibrio vulnificus* to obtain iron from transferrin and other iron-binding proteins. *Current microbiology*. 15(3): 155-157.
- Sithigorngul, P.; et al. (2000a). Diagnosis of YHV infection in *Penaeus monodon* using monoclonal antibody. *SWU Science Journal*. 16: 34-45.
- Sithigorngul, P.; et al. (2000b). Development of a monoclonal antibody specific to yellow head virus (YHV) from *Penaeus monodon*. *Diseases of aquatic organisms*. 42(1): 27-34.
- Sithigorngul, P.; et al. (2007). A simple and rapid immunochromatographic test strip for detection of pathogenic isolates of *Vibrio harveyi*. *Journal of microbiological methods*. 71(3): 256-264.
- Sithigorngul, W.; et al. (2006). Development of monoclonal antibodies for simple identification of *Vibrio alginolyticus*. *Letters in applied microbiology*. 43(4): 436-442.
- Small, P.; et al. (1994). Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. *Journal of bacteriology*. 176(6): 1729-1737.
- Srisuk, C.; et al. (2010). Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein *ompW*. *Letters in applied microbiology*. 50(1): 36-42.
- Stelma, G. N., Jr.; et al. (1992). Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. *Applied and environmental microbiology*. 58(9): 2776-2782.
- Strom, M. S.; & Paranjpye, R. N. (2000). Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and infection / Institut Pasteur*. 2(2): 177-188.
- Takahashi, H.; et al. (2005). Development of a quantitative real-time polymerase chain reaction targeted to the *toxR* for detection of *Vibrio vulnificus*. *Journal of microbiological methods*. 61(1): 77-85.
- Tamplin, M. L.; et al. (1991). Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. *Applied and environmental microbiology*. 57(4): 1235-1240.
- Triggs-Raine, B. L.; & Loewen, P. C. (1987). Physical characterization of *katG*, encoding catalase HPI of *Escherichia coli*. *Genes*. 52(2-3): 121-128.
- Twedt, R. M. (1989). *Vibrio parahaemolyticus* in foodborne bacterial pathogens. In: M.P. Doyle, Editor, Foodborne Bacterial Pathogens, Marcel Dekker, New York. 544-568.

- USFDA. (2001). Bacteriological analytical manual online: Chapter 24 Identification of foodborne bacterial pathogens by gene probes. [Online.]. online.
- Weber, K.; & Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *The Journal of biological chemistry*. 244(16): 4406-4412.
- Weisburg, W. G.; et al. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*. 173(2): 697-703.
- West, P. A. (1989). The human pathogenic vibrios – A public health update with environmental perspectives. *Epidemiology and Infection*. 103: 1-34.
- White, J.; & Helenius, A. (1980). pH-dependent fusion between the Semliki forest virus membrane and liposomes. *Proceedings of the National Academy of Sciences of the United States of America*. 77(6): 3273-3277.
- Wong, Hin-Chung; & Liu, Shu-Hui. (2008). Characterization of the Low-Salinity Stress in *Vibrio vulnificus*. *Journal of Food Protection*. 71(2): 416-419.
- Wright, A. C.; et al. (1993). Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Applied and environmental microbiology*. 59(2): 541-546.
- Wright, A. C.; & Morris, J. G., Jr. (1991). The extracellular cytotoxin of *Vibrio vulnificus*: inactivation and relationship to virulence in mice. *Infection and immunity*. 59(1): 192-197.
- Wright, A. C.; Simpson, L. M.; & Oliver, J. D. (1981). Role of iron in the pathogenesis of *Vibrio vulnificus* infections. *Infection and immunity*. 34(2): 503-507.
- Wright, A. C.; et al. (1990). Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. *Infection and immunity*. 58(6): 1769-1773.
- Yamazaki, W.; et al. (2008). Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Vibrio parahaemolyticus*. *BMC microbiology*. 8: 163.
- Yamazaki, W.; et al. (2010). Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of the *tdh* and *trh* genes of *Vibrio parahaemolyticus* and related *Vibrio* species. *Applied and environmental microbiology*. 76(3): 820-828.
- Yamazaki, W.; et al. (2008). Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. *BMC microbiology*. 8: 94.

- Yano, A.; Ishimaru, R.; & Hujikata, R. (2007). Rapid and sensitive detection of heat-labile I and heat stable I enterotoxin genes of enterotoxigenic *Escherichia coli* by loop-mediated isothermal amplification. *Journal of Microbiological Methods*. 68: 414-420.
- Yongjun, L.; et al. (2010). A culture-free method for detection of *Vibrio vulnificus* from coastal seawater based on loop-mediated isothermal amplification targeting *vcgC* gene. *Acta Oceanologica Sinica*. 29(2): 93-97.
- Zheng, M.; Åslund, F.; & Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science*. 279(5357): 1718-1722.







APPENDIX A

APPENDIX A

BUFFER AND REAGENT PREPARATION

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

NaCl	8.00	g
KCl	0.20	g
KH ₂ PO ₄	0.20	g
Na ₂ HPO ₄	1.15	g
or Na ₂ HPO ₄ · 7H ₂ O	2.15	g
Distilled water adjust volume to	1,000.00	ml

2. Bradford solution (Bradford. 1976)

Coomassie brilliant blue G-250	100.00	
mg		
Methanol	50.00	ml
85% Phosphoric acid	100.00	ml
Distilled water adjust volume to	1,000.00	ml

3. 1% Merthiolate

Thimerosal (Sigma)	1.00	g
Distilled water adjust volume to	100.00	ml

4. 5% Blotto

Skim milk	5.00	g
1% Merthiolate	1.00	ml
Triton X-100	0.10	ml
PBS adjust volume to	100.00	ml

5. Davidson's fixative

95 % Ethyl alcohol	300.00	ml
40 % Formaldehyde	100.00	ml
Glacial acetic acid	100.00	ml
Distilled water	300.00	ml

6. Gelatin coating solution

Chrome alum	0.05	g
Gelatin	1.00	g
Distilled water	100.00	ml

Dissolved chrome alum in distilled water followed by gelatin and warmed up the temperature to 50-70°C for gelatin melting. Filtered with filter paper no. 1. Kept the filtrate in 60°C until use. To coat the slide, glass slide were washed clearly with distilled water. Dipped the slide once into the warmed gelatin coating solution then air-dried and kept in slide box until use.





APPENDIX B

REAGENT PREPARATION FOR HYBRIDOMA PRODUCTION

1. RPMI medium

RPMI 1640 (Roswell Park Memorial Institute - Gibco BRL, USA)	10.40	g
D-glucose (Sigma)	3.60	g
L-glutamine (Sigma)	0.2923	g
Sodium pyruvate (C ₃ H ₃ O ₃ Na) (Sigma)	1.1005	g
NaHCO ₃	2.0160	g
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma)	5.9525	g
H ₂ O (Milli Q water)	1,000.00	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) filtered and stored at 4°C.

2. RPMI medium with serum

RPMI medium	80.00	ml
Fetal calf serum (FCS, Starrate, Australia)	20.00	ml
or Calf bovine serum (CBS, 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)

RPMI medium	80.00	ml
FCS	20.00	ml
HT supplement	1.00	ml
50 X Aminopterin (Sigma)	2.00	ml
1 % Mouse red blood cell		

4. Fusion solution (50 % polyethylene glycol)

Polyethylene glycol (PEG) 5.00 g

The solution was prepared by dissolving 5.0 g of polyethylene glycol in 5 ml of RPMI medium. The solution was incubated at 37 °C in CO₂ incubator before use.

5. Freezing medium (12 % DMSO)

Dimethylsulfoxide (DMSO, Sigma) 12.00 ml

RPMI medium 88.00 ml

The medium was stored in 4 °C before use.





APPENDIX C

BUFFER AND SOLUTION FOR SDS-PAGE AND WESTERN BLOT ANALYSIS

1. Stock solution :

1.1 Monomer solution (30 % T, 2.7 % CBis)

Acrylamide (BIO-RAD)	58.40	g
Bis (N,N'-methylene-bis-acrylamide, BIO-RAD)	1.60	g
Distilled water adjust volume to	200.00	ml
Stored at 4°C in the dark bottle.		

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

Tris (hydroxymethyl) aminomethane (BIO-RAD)	36.30	g
Distilled water adjust volume to	200.00	ml
adjusted pH with HCl		

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

Tris	3.00	g
Distilled water adjust volume to	50.00	ml
adjusted pH with HCl		

1.4 10 % SDS

SDS (sodium dodecyl sulfate, BIO-RAD)	50.00	g
Distilled water adjust volume to	500.00	ml

1.5 10 % Ammonium persulfate (freshly prepared)

Ammonium persulfate (BIO-RAD)	0.10	g
Distilled water	1.00	ml

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

1.5 M Tris	25.00	ml
10 % SDS	1.00	ml
Distilled water adjust to 100.0 ml		

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

0.5 M Tris	2.50	ml
10 % SDS	4.00	ml
Glycerol	2.00	ml
2-Mercaptoethanol	1.00	ml
Distilled water	0.50	ml

2 Preparation of separating gel and stacking gel

2.1 Stacking gel for SDS-PAGE 4 % gel (4 % T 2.7 % C Bis)

Monomer solution	2.66	ml
0.5 M tris-Cl pH 6.8	5.00	ml
10 % SDS	0.20	ml
Distilled water	12.22	ml
10 % Ammonium persulfate	100.00	μl
TEMED	10.00	μl

2.2 Separating gel for SDS-PAGE 7.5 % gel (7.5 % T 2.7 % C Bis)

Monomer solution	7.50	ml
1.5 M tris-Cl pH 8.8	7.50	ml
10 % SDS	0.30	ml
Distilled water	14.55	ml
10 % Ammonium persulfate	150.00	μl
TEMED	20.00	μl

3. Running buffer :

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

Tris	12.00	g
Glycine	57.60	g
10% SDS	40.00	ml
Distilled water	4,000.00	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	1.00	g
Distilled water	100.00	ml

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

Stain stock	50.00	ml
Methanol	250.00	ml
Acetic acid	50.00	ml
Distilled water	500.00	ml

4.2 Destaining solution for Coomassie blue

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

Methanol	500.00	ml
Acetic acid	100.00	ml
Distilled water	1,000.00	ml

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

Methanol	50.00	ml
Acetic acid	70.00	ml
Distilled water	1,000.00	ml

Method of protein staining

A gel was stained with 0.1 % Coomassie blue R-250, 50% methanol, 10% acetic acid for 5-6 hrs. The gel was then washed in destain I for 1 hr. with 1-2 changes and followed by destain II until the gel was cleared. After washing in distilled H₂O for a few times, the gel was dried in a gel air dryer (BIO-RAD)

5. Towbin transfer buffer pH 8.8

5.1 25 mM tris, 192 mM glycine, 20 % methanol pH 8.8

The buffer consisted of the following ingredients :

Tris	3.03	g
Glycine	14.40	g
Methanol	200.00	ml
Distilled water adjusted to	1,000.00	ml

The buffer was pre-chill before use





APPENDIX D

REAGENT FOR DETERMINATION OF ISOTYPES AND SUBISOTYPES OF MONOCLONAL ANTIBODIES

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

- A) Rabbit anti-Mouse IgG₁ (γ 1 chain specific)
- B) Rabbit anti-Mouse IgG_{2a} (γ 2a chain specific)
- C) Rabbit anti-Mouse IgG_{2b} (γ 2b chain specific)
- D) Rabbit anti-Mouse IgG₃ (γ 3 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 % H₂O₂)
- 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 % NaN₃)
- N) HRP-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% NaN₃)



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