การผลิตและลักษณะสมบัติของมอโนโคลนอลแอนติบอดีต่อเชื้อก่อโรค *Leptospira* spp.

นางสาวใจญา สถิตย์เสมากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AGAINST PATHOGENIC *Leptospira* spp.

Miss Jaiya Sathitsemakul

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AGAINST PATHOGENIC *Leptospira* spp.
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ใจญา สถิตย์เสมากุล : การผลิตและลักษณะสมบัติของมอโนโคลนอลแอนติบอดีต่อเชื้อก่อโรค *Leptospira* spp.. (PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AGAINST PATHOGENIC *Leptospira* spp.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.กิตตินันท์ โกมลภิส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.ธนาภัทร ปาลกะ, ผศ. พญ. ดร. กนิษฐา ภัทรกุล, 83 หน้า.

โรคฉี่หนูเป็นโรคติดเชื้อจากสัตว์สู่คนที่แพร่กระจายในพื้นที่เขตร้อน มีสาเหตุมาจากแบคทีเรียแกรมลบสายพันธุ์เลปโตสไปราชนิดก่อโรค ซึ่งมีผลต่อทั้งมนุษย์และสัตว์ โรคนี้เกิดได้จากการสัมผัสถูกปัสสาวะของสัตว์ติดเชื้อ โดยเชื้อจะเข้าสู่ผู้ป่วยผ่านทางบริเวณเยื่อเมือก หรือปากแผลเปิดบริเวณผิวหนัง อาการของโรคฉี่หนูมีหลากหลาย อาทิเช่น อาการคล้ายไข้หวัด ปวดศีรษะ เป็นต้น หากผู้ป่วยได้รับการวินิจฉัยและรักษาที่ไม่ทันการณ์ อาจเกิดภาวะการติดเชื้อในกระแสเลือดที่รุนแรง และเกิดภาวะอวัยวะภายในต่างๆ ล้มเหลว วัตถุประสงค์ของการวิจัยนี้ คือ การผลิตมอโนโคลนอลแอนติบอดีต่อเชื้อเลปโตสไปราสายพันธุ์ก่อโรค เพื่อใช้ในการพัฒนาวิธีวินิจฉัยโรคฉี่หนูเบื้องต้นทางภูมิคุ้มกันวิทยา โดยทำการฉีดกระตุ้นหนูไมซ์ด้วยเชื้อ *Leptospira interrogans* ซีโรวาร์ Manilae และสายพันธุ์กลาย M1352 ในรูปคงสภาพ และรูปผสมระหว่างคงสภาพกับเสียสภาพ หลังจากสร้างเซลล์ลูกผสมด้วยวิธีหลอมรวมเซลล์ และคัดเลือกมอโนโคลนอลแอนติบอดีที่ผลิตได้ ด้วยวิธี enzyme-linked immunosorbent assay (ELISA) โดยใช้เชื้อเลปโตสไปราซีโรวาร์ต่างๆ และแบคทีเรียอื่นๆ ที่ถูกทำให้อยู่ในรูปเสียสภาพเป็นแอนติเจนคัดเลือก ผลการทดลองพบว่า ได้มอโนโคลนอลแอนติบอดี 14 โคลนที่สามารถจำแนกได้เป็น 6 กลุ่มตามความจำเพาะต่อแบคทีเรียที่ใช้ทดสอบ มอโนโคลนอลแอนติบอดีกลุ่มที่ 1, 2, 3 และ 4 มีความจำเพาะต่อเลปโตสไปราสายพันธุ์ก่อโรคเพียงบางสายพันธุ์ที่นำมาทดสอบ มอโนโคลนอลแอนติบอดีกลุ่มที่ 5 และ 6 มีความจำเพาะต่อเลปโตสไปราสายพันธุ์ก่อโรคที่นำมาทดสอบที่หลากหลายกว่าสี่กลุ่มแรก แต่แสดงปฏิกิริยาข้ามกับ *Enterobacter aerogenes* มอโนโคลนอลแอนติบอดีเหล่านี้มีความสามารถในการตรวจหา *L. interrogans* ซีโรวาร์ Manilae โดยมีค่าความเข้มข้นของเชื้อที่ทำให้ค่าการดูดกลืนแสงเปลี่ยนแปลงไปครึ่งหนึ่ง (Half maximal effective concentration; EC50) อยู่ในช่วงพิสัย 2×10^{6} ถึง 1×10^{7} เซลล์ต่อมิลลิลิตร และค่าความเข้มข้นของเชื้อที่น้อยที่สุดที่แอนติบอดีตรวจจับได้ (Limit of detection; LOD) ของแต่ละโคลนอยู่ในช่วงพิสัย 4.7×10^{5} ถึง 3.5×10^{5} เซลล์ต่อมิลลิลิตร มอโนโคลนอลแอนติบอดีทั้ง 14 โคลนมีไอโซไทป์เป็น IgM และจากการตรวจจับด้วยวิธี Western blot มีมอโนโคลนอลแอนติบอดีที่จำเพาะต่อโมเลกุลคล้ายโปรตีนของ *L. interrogans* ซีโรวาร์ Manilae ที่น้ำหนักโมเลกุล 41 กิโลดาลตัน ดังนั้น จากการศึกษาเบื้องต้นดังกล่าวชี้ให้เห็นว่า มอโนโคลนอลแอนติบอดีที่ผลิตได้เหล่านี้ บางโคลนมีศักยภาพพอในการนำไปประยุกต์ใช้ในการตรวจสอบทางภูมิคุ้มกันวิทยา สำหรับการตรวจเชื้อเลปโตสไปราสายพันธุ์ก่อโรคนี้ต่อไปได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS: MONOCLONAL ANTIBODY / LEPTOSPIRES / ELISA

JAIYA SATHITSEMAKUL: PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AGAINST PATHOGENIC *Leptospira* spp.. ADVISOR: KITTINAN KOMOLPIS, Ph.D., CO-ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., ASST. PROF. KANITHA PATARAKUL, 83 pp.

Leptospirosis is a widespread zoonotic disease in tropical areas caused by pathogenic gram-negative spirochetes *Leptospira* spp., which affect both human and animals. The disease is transmitted by contacting urine of the infected animals. *Leptospira* penetrates through mucosa or open wound skin of infected individuals. Symptoms of leptospirosis are extremely broad such as flu-like illness, headache. If patients are not diagnosed or treated in time, symptoms can become severe sepsis with multi-organ failure. This study aimed to generate monoclonal antibodies (MAbs) against pathogenic *Leptospira* spp. that can be used in a development of immunological based assay for early diagnosis of leptospirosis. Mice were immunized with whole cells of fixed or mixed of fixed and sonicated form of *Leptospira interrogans* serovar Manilae and the mutant M1352. After the conventional cell hybridization technique, all MAbs were screened by enzyme-linked immunosorbent assay (ELISA) with sonicated cell lysates of various serovars of leptospira and other bacteria as antigen. The results showed that 14 clones of MAbs were obtained in this study could be divided into 6 groups based on the specificity against bacteria that were tested. MAbs group 1, 2, 3 and 4 were specific to some serovars of pathogenic *Leptospira* spp. MAbs group 5 and 6 were specific to various serovars of pathogenic leptospires, and also shown cross reactivity with *Enterobacter aerogenes*. These MAbs could detect *L. interrogans* serovar Manilae with the half maximal effective concentration (EC50) in the concentration range of 2×10^6 to 1×10^7 cells/ml and the limit of detection (LOD) was in the concentration range of 4.7×10^5 to 3.5×10^5 cells/ml. All 14 MAbs were isotyped as IgM. By Western blotting, there were some of the obtained MAbs recognizing the protein-like antigen of *L. interrogans* serovar Manilae with the molecular weight of 41 kDa. Therefore, these preliminary studies indicated that some of the obtained MAbs could be used for application in an immunological detection of pathogenic *Leptospira* spp.

Field of Study: Biotechnology
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	=	percent
A	=	ampere
APS	=	ammoniumpersulfate
ATCC	=	American type culture collection
BSA	=	bovine serum albumin
°C	=	degree Celsius
CAAT	=	cross-agglutinating absorption test
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
dot-ELISA	=	dot blot-enzyme-linked immunosorbent assay
EC50	=	half maximal effective concentration
EDTA	=	ethylenediaminetetraacetic acid
EIA	=	enzyme immunoassay
ELISA	=	enzyme-linked immunosorbent assay
EMJH	=	Ellinghausen-McCullough-Johnson-Harris
et al.	=	et alii
Fab	=	antigen-binding fragment
Fc	=	crystallizable fragment
FCA	=	Freund's complete adjuvant
FCS	=	fetal calf serum
FIA	=	Freund's incomplete adjuvant
g	=	gram
g	=	earth's gravitational force
GAM	=	goat anti mouse
h	=	hour
HAT	=	hypoxanthine-aminopterin-thymidine
HGPRT	=	hypoxanthine-guanine-phosphor-ribozyl-transferase

HRP	=	horseradish peroxidase
ICG-based LFA	=	immunochromatography based lateral flow assay
Ig	=	immunoglobulin
i.p.	=	interperitoneal
kDa	=	kilo Dalton
kHz	=	kilo Hertz
L	=	liter
LB	=	Luria-Bertani
LipL32	=	*Leptospira* lipoprotein 32 kDa
LOD	=	limit of detection
LPS	=	lipopolysaccharide
M	=	molar
MAb	=	monoclonal antibody
MAT	=	microscopic agglutination test
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
µm	=	micrometer
MW	=	molecular weight
N	=	normal
nm	=	nanometer
no.	=	number
OD	=	optical density
OE	=	outer envelope
OMP	=	outer membrane protein
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
PEG	=	polyethylene glycol
RIA	=	radioimmunoassay

SD	=	standard deviation
SDS	=	sodium dodecyl sulphate
SDS-PAGE	=	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	=	second
sg.	=	serogroup
SLS	=	sodium lauryl sulfate
spp.	=	species
sv.	=	serovar
TEMED	=	tetramethylethylenediamine
TMB	=	tetramethylbenzidine
V	=	voltage
v/v	=	volume by volume
WT	=	wild type
w/v	=	weight by volume

CHAPTER I
INTRODUCTION

Thailand is located in tropical zone, near the equator. The climate of Thailand is mainly tropical-hot and humid all year round with plentiful rainfall that is suitable for agricultural industry and farming. Hot humid environments provide ideal conditions for a number of diseases to survive and spread. One of the infectious tropical diseases is leptospirosis.

Leptospirosis is a widespread zoonosis and is considered as an emerging global public health disease. This disease is caused by pathogenic gram-negative spirochetes *Leptospira* spp., which affects both human and animals. The rodents are the main reservoirs of the disease causing pathogens. Leptospirosis is transmitted by direct or indirect contact with the urine of infected animals or contaminated water and soil. The bacteria can enter the body through mucous membranes and open wound skin. Although, leptospirosis can be cured easily with antibiotic treatment such as cefotaxime, doxycycline and penicillin, but the clinical presentation of leptospirosis is similar to other febrile illnesses, thus complicating the diagnosis. Symptoms of leptospirosis are high fever, headache, muscle aches, hemorrhage, vomiting, diarrhea, red eyes, jaundice, abdominal pain or a rash. If patients are not treated in time, they may develop meningitis, renal damage, liver failure and respiratory distress. So, early and accurate diagnosis is useful for proper treatment.

There are various methods for diagnosis of leptospirosis, such as isolation of leptospires from patient's samples, dark field microscopic observation, polymerase chain reaction (PCR) and immunological assays. Detection of leptospires by culture and isolation from patient's samples may require more than a month and thus does not suitable for early diagnosis. Direct observation of leptospires by dark-field microscopy is unreliable. In addition, polymerase chain reaction (PCR) assays need well-trained specialist for operating advance equipment. Currently, the standard method for diagnosis of leptospirosis is the microscopic agglutination test (MAT) which detects agglutinating antibodies in patient sera against leptospiral antigens.

Although, it has an advantage of being specific for serovars, it needs various antibodies specific for different serovars of viable leptospires in the detection. Enzyme-linked immunosorbent assays (ELISA) have also been developed using several antigen preparations such as leptospiral sonicates or recombinant outer membrane proteins to obviate the need of live cultures maintenance. Several immunological assays have been developed for detection of leptospiral antigens by monoclonal antibodies. However, no tests are sensitive and specific enough to be used in routine laboratories.

Because immunological methods are considered to be a suitable method for screening patient's infectious samples and MAbs specific for *Leptospira* spp. are still in need. This study aimed to produce and characterize monoclonal antibodies against pathogenic *Leptospira interrogans* serovar Manilae, one of the pathogenic *Leptospira* spp.

Scope of the study

1. Immunization of mice with *L. interrogans* serovar Manilae and the mutant M1352.
2. Production of hybridoma clones.
3. Characterization of monoclonal antibodies obtained in this study.

CHAPTER II
LITERATURE REVIEWS

2.1 *Leptospira* organism

Leptospira spp. is a member of the genus *Leptospira*, family *Leptospiracae* and order *Spirochaetales*. Leptospires are gram-negative and obligate aerobic bacteria with an optimum growth temperature at 28 to 30 °C. They are spiral shaped bacteria and have distinctive hooked ends, Figure 2.1. Bacteria range in size from 6 to 20 μm in length and 0.1 μm in diameter. The helical amplitude is approximately 0.1 to 0.15 μm, and the wavelength is approximately 0.5 μm (Faine, Adler, Bolin, & Perolat, 1999).

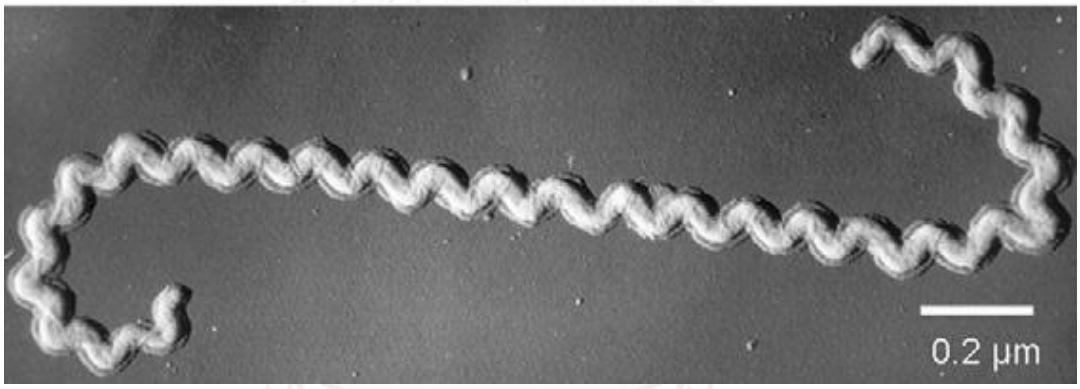


Figure 2.1 Photomicrographs of *Leptospira* spp.

(Adler & de la Peña Moctezuma, 2010)

Leptospires have a typical double membrane structure; inner membrane and outer membrane, shown in Figure 2.2. The peptidoglycan cell wall is closely associated with the inner membrane and they are overlaid with the outer membrane (Cullen, Haake, & Adler, 2004; Haake, 2000; Ko et al., 2009). The outer membrane contains lipopolysaccharide (LPS), the transmembrane porin outer membrane protein L1 (OmpL1) and the lipoproteins; LipL32, LigA and LigB. The periplasmic space is a location of two polar periplasmic flagella which are responsible for motility of leptospires (Levett, 2001; Swain, 1957).

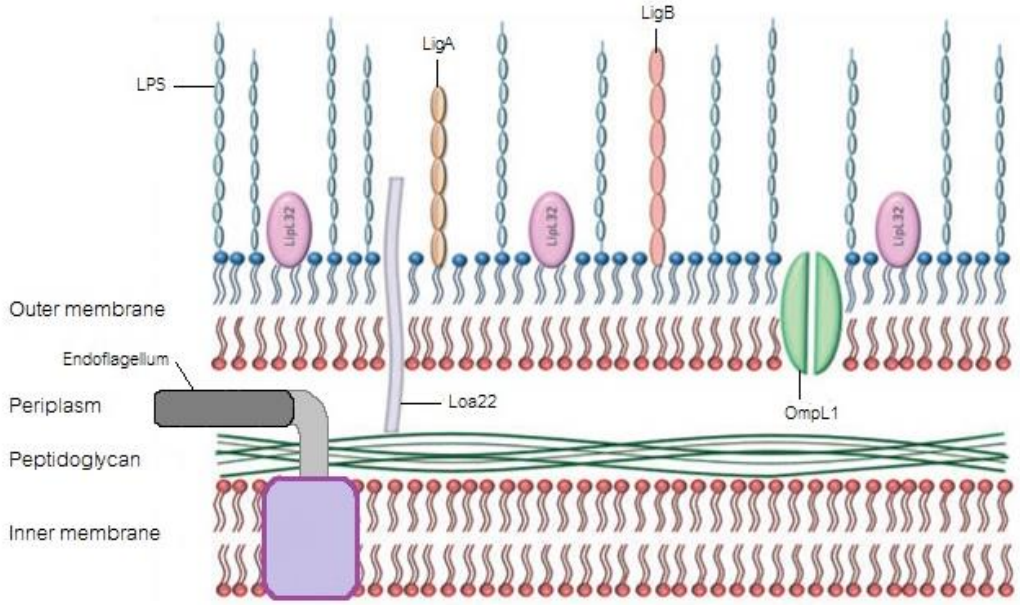


Figure 2.2 The cell wall structure of Leptospires

Modified from (Fraga, Barbosa, & Isaac, 2011; Ko, Goarant, & Picardeau, 2009)

Example of leptospiral outer membrane antigens:

1. Lipopolysaccharide (LPS) – The leptospiral LPS is a protective immunogen that is generally specific for each serovar or serogroup (Faine et al., 1999).
2. LipL32 – A lipoprotein, that is the most abundant protein on the cell surface of leptospiral membrane when is expressed in vivo (Cullen, Cordwell, Bulach, Haake, & Adler, 2002; Nally, Whitelegge, Bassilian, Blanco, & Lovett, 2007). This protein is highly conserved among pathogenic *Leptospira* spp. The function is adhesion to extracellular matrix components (Hauk et al., 2008; Hoke, Egan, Cullen, & Adler, 2008).
3. LipL41 – A lipoprotein that is among one of the 3 most abundant surface-exposed protein, is expressed in vivo (Nally et al., 2007). It is a protein of unknown function.

4. Loa22 – A surface-exposed protein that contains an OmpA domain (Koizumi & Watanabe, 2003). This protein is essential for virulence in infection model of hamster (Ristow et al., 2007).
5. LigA, LigB – The leptospiral immunoglobulin-like proteins that are considered to play a role in adhesion of leptospires to host tissues (Ko et al., 2009; Srikram et al., 2011).

2.2 Leptospire taxonomy and classification

The genus *Leptospira* is divided into two groups; *Leptospira interrogans* sensu lato (pathogenic strains) and *Leptospira biflexa* sensu lato (saprophytic strains) (Faine & Stallman, 1982). The differences of these two groups are their nutritional requirements, optimum temperature and generation time (Adler & de la Peña Moctezuma, 2010; Cerqueira & Picardeau, 2009; Levett, 2001), show in Table 2.1.

Table 2.1 The differences of pathogenic and saprophytic leptospires

Strain	8-azaguanine in culture media	Optimum temperature	Generation time
Pathogenic strains	No growth	28-30 °C	20 h
Saprophytic strains	Growth	13 °C	5 h

2.2.1. Serological classification

The serological classification of both *L. interrogans* and *L. biflexa* are defined by cross agglutinating absorption test (CAAT) (Cerqueira & Picardeau, 2009; Dikken & Kmety, 1978) based on the expression of the surface – exposed epitopes in a mosaic of the lipopolysaccharide (LPS) antigens, while the specificity of epitopes depends on their sugar composition and orientation (Adler & de la Peña Moctezuma, 2010). There are more than 260 serovars and 60 serovars in pathogenic and saprophytic strains, respectively. Serovars have traditionally been grouped into serogroups that have proved useful for epidemiological understanding. The serogroups of *L. interrogans* and some common serovars are shown in Table 2.2.

Table 2.2 Serogroups and some serovars of *L. interrogans* sensu lato

Serogroup	Serovar(s)
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai, Zimbabwe
Hebdomadis	Hebdomadis, Jules, Kremastos
Autumnalis	Autumnalis, Fortbragg, Bim, Weerasinghe
Pyrogenes	Pyrogenes, Manilae
Bataviae	Bataviae
Grippotyphosa	Grippotyphosa, Canalzonae, Ratnapura
Canicola	Canicola
Australis	Australis, Bratislava, Lora
Pomona	Pomona
Javanica	Javanica
Sejroe	Sejroe, Saxkoebing, Hardjo
Panama	Panama, Mangus
Cynopteri	Cynopteri
Djasiman	Djasiman
Sarmin	Sarmin
Mini	Mini, Georgia
Tatassovi	Tarassovi
Ballum	Ballum, Aroborea
Celledoni	Celledoni
Louisiana	Louisiana, Lanka
Ranarum	Ranarum
Manhao	Manhao
Shermani	Shermani
Hurstbridge	Hurstbridge

Modified from (Levett, 2001; Murray et al., 2010)

2.2.2. Genotypic classification

The phenotypic classification has been replaced by a genotypic classification. Based on DNA hybridization methods, the genus *Leptospira* is divided into 20 genomospecies are shown in Table 2.3 (Cerqueira & Picardeau, 2009). A number of genomospecies can include all serovars of both *L. interrogans* and *L. biflexa*.

Table 2.3 Distribution of serogroups among the several *Leptospira* species

Genomospecies	Serogroups
L. interrogans	Australis, Autumnalis, Bataviae, Canicola, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Louisiana, Manhao, Mini, Pyrogenes, Pomona, Ranarum, Sarmin, Sejroe
L. kirschneri	Australis, Autumnalis, Bataviae, Canicola, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Pomona
L. noguchii	Australis, Autumnalis, Bataviae, Djasiman, Louisiana, Panama, Pyrogenes, Pomona, Shermani, Tarassovi
L. borgpetersenii	Australis, Autumnalis, Ballum, Bataviae, Celledoni, Hebdomadis, Javanica, Mini, Pyrogenes, Sejroe, Tarassovi
L. weilii	Celledoni, Hebdomadis, Icterohaemorrhagiae, Javanica, Manhao, Mini, Pyrogenes, Sarmin, Sejroe, Tarassovi
L. santarosai	Autumnalis, Bataviae, Cynopteri, Grippotyphosa, Hebdomadis, Javanica, Mini, Pyrogenes, Pomona, Sarmin, Sejroe, Shermani, Tarassovi
L. alexanderi	Hebdomadis, Javanica, Manhao, Mini
L. alstonii	Ranarum, Undesignated
L. wolffii	Undesignated
L. licerasiae	Hurstbridge
L. inadai	Canicola, Icterohaemorrhagiae, Javanica, Lyme, Manhao, Shermani, Tarassovi
L. fainei	Hurstbridge
L. broomii	Undesignated
L. kmetyi	Tarassovi
L. wolbachii	Codice
L. meyeri	Javanica, Mini, Ranarum, Sejroe, Semaranga
L. biflexa	Andaman, Semaranga
L. vanthielii	Holland
L. terpstrae	Icterohaemorrhagiae
L. yanagawae	Semaranga

Modified from (Levett, 2001)

2.3 *Leptospira interrogans* serovar Manilae and the mutant M1352

2.3.1 *L. interrogans* serovar Manilae

Leptospira interrogans serovar Manilae is a serovar of serogroup Pyrogenes that were isolated from rat kidney in the Philippines (Murray et al., 2010; Villanueva et al., 2010). They were lethal in golden Syrian hamsters when after experimental infection (Villanueva et al., 2010). The phylogenic tree is shown in Figure 2.3.

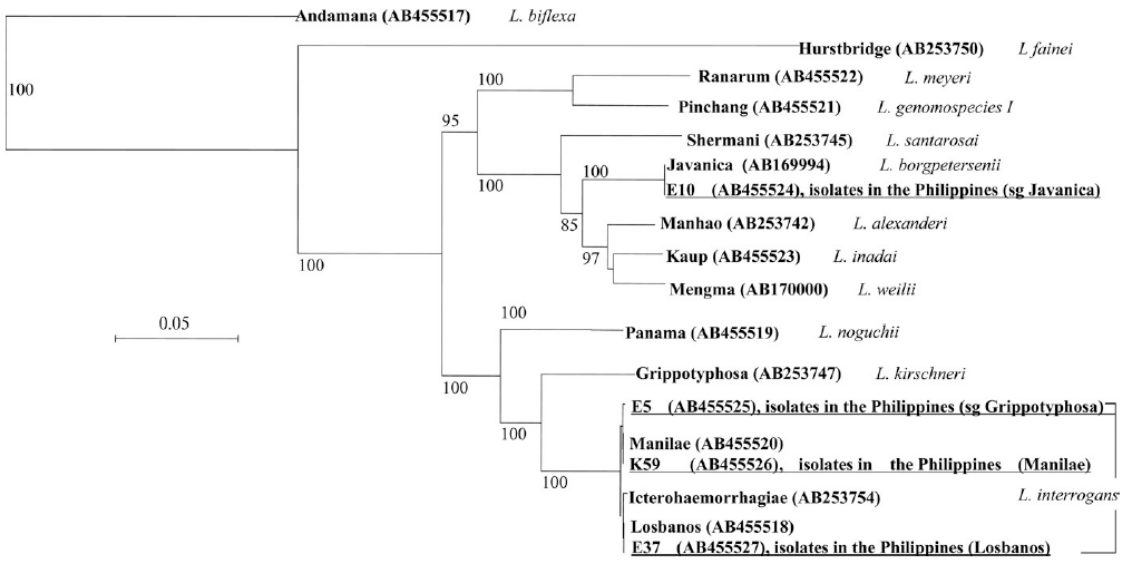


Figure 2.3 Phylogenic tree of *Leptospira* spp.

Bar = sequence divergence of 5%, sg = serogroup (Villanueva et al., 2010).

2.3.2 *L. interrgans* serovar M1352

Leptospira interrogans serovar M1352 is a Lipopolysaccharide Mutant bacterium that was conducted in *L. interrogans* serovar Manilae strain L495 by transposon mutagenesis. The transposon had inserted into the LPS biosynthesis locus in a chromosomal region unique to serovar Manilae at the 3’ end of a gene name Lman_1408 encoding a protein of unknown function (Figure 2.4). The mutant showed normal growth rates *in vitro*, and showed normal morphology and motility when observed under dark-field microscopy. (Murray et al., 2010).

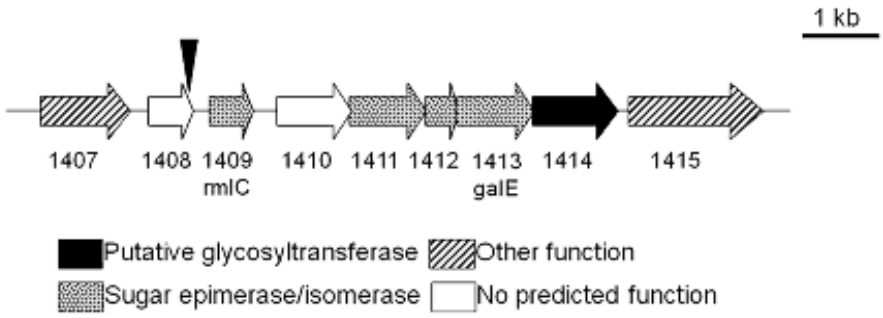


Figure 2.4 The location of transposon insertion of M1352 in *L. interrogans* serovar Manilae. (Murray et al., 2010), Insertion points are indicated by a vertical arrow head. Open reading frames predicted to encode; 1407, sugar pyridoxal-phosphate-dependent aminotransferase; 1408, hypothetical; 1409, RmlC, dTDP-4-dehydrohamnose 3, 5-epimerase; 1410, hypothetical protein; 1411, nucleoside-diphosphate-sugar epimerase; 1412, sugar isomerase; 1413, GalE, UDP-glucose 4-epimerase; 1414, glycosyltransferase; 1415, ATP-binding protein of an ABC transporter complex.

The LPS mutant M1352 is attenuated for virulence which failed to produce any symptoms of leptospirosis when injected into hamsters, in contrast, hamsters died when infected with the parent strain at the same dose (Murray et al., 2010). For protective immunity against leptospirosis, Live M1352 vaccine induced better protection than heat killed Manilae WT when inject single dose in hamsters and challenge with virulent Manilae WT (Srikram et al., 2011).

2.4 Leptospirosis

Leptospirosis was first described by Adolf Weil in 1886 as Weil's disease (Heringman & Phillips, 1947; Musso & La Scola, 2013). There are rodents as the main reservoir of the disease. The disease may range from asymptomatic infection to severe illness and sometimes fatal disease.

The symptoms of leptospirosis usually develop in 1 to 2 weeks after exposure to the *Leptospira* bacteria. The clinical presentation of this disease is biphasic (Figure 2.5). In the acute phase, lasting about one week is followed by an immune phase characterized by antibody and excretion of leptospires in the urine.

The most common mild symptoms (Anicteric leptospirosis) are febrile illness, chills, headache, abdominal pain, muscle pain, rash and conjunctivitis that usually resolve within 5 to 7 days. However, a small number of patients will go on to severe symptoms (Icteric leptospirosis) when the organs have become infected. The serious symptoms such as jaundice, pulmonary hemorrhage, meningitis, hepatic or renal dysfunction can be fatal (Adler & de la Peña Moctezuma, 2010; Levett, 2001; Musso & La Scola, 2013).

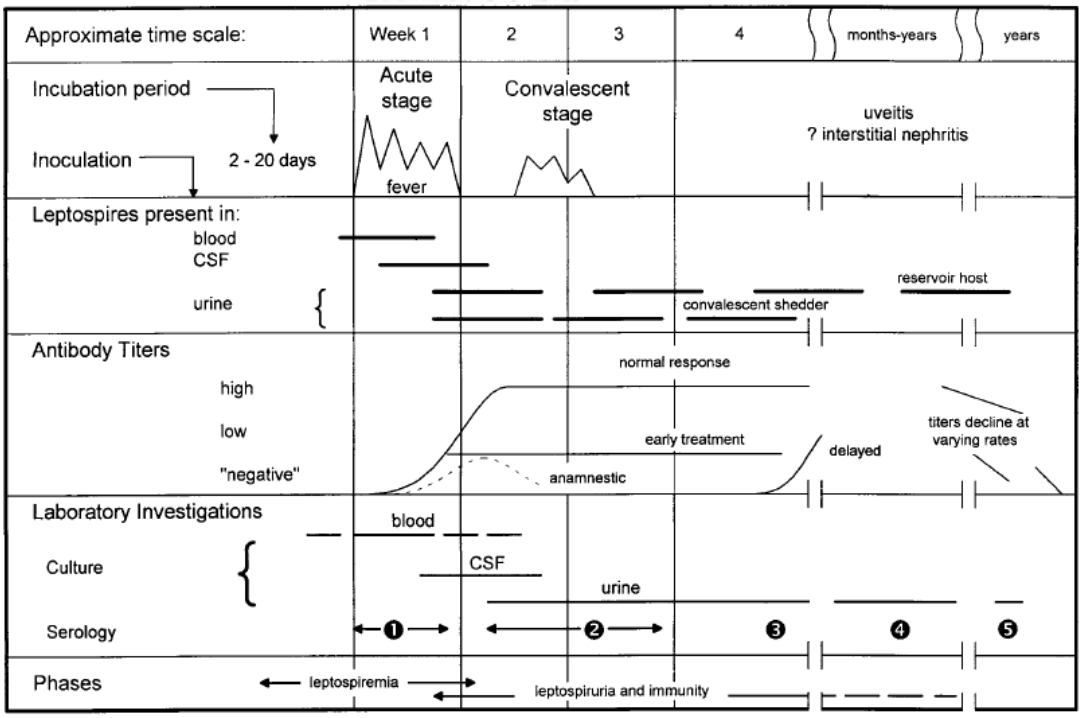


Figure 2.5 Biphasic nature of leptospirosis and relevant investigations at different stage of disease (Levett, 2001)

Treatment of leptospirosis is depending on presentation time of the severity and symptoms duration. In acute leptospirosis can be successfully treated with an antibiotic such as doxycycline, cefotaxime and penicillin (Chakraborty, Miyahara, Villanueva, Gloriani, & Yoshida, 2010; Sehgal, Sugunan, Murhekar, Sharma, & Vijayachari, 2000; Suputtamongkol et al., 2004). But in cases of severe leptospirosis, patients will require hospital admission and close observation. For example, patients in acute renal failure require dialysis as an artificial kidney for removing waste materials from blood (Kobayashi, 2001).

For diagnosis, leptospirosis can be confirmed by blood and urine tests to detect the presence of the leptospires in patients' blood and urine.

1. Nonspecific laboratory findings

The report of clinical specimen analysis in Table 2.4 had shown various non-diagnostic abnormalities that can suggest leptospirosis. But for the confirmation, specific microbiological tests are required.

Table 2.4 Nonspecific laboratory findings (Musso & La Scola, 2013)

Clinical specimen analysis	Results
1. Blood analysis	1.1 Leukocytosis with a shift to the left 1.2 Thrombocytopenia in >50% 1.3 Elevated 1.3.1 Erythrocyte sedimentation rate 1.3.2 Creatinine (usually <20-80 mg/L 1.3.3 Urea (usually <1000 mg/L) 1.3.4 Aminotransterases (rarely >200 IU/L) 1.3.5 Bilirubin (may rise to 800 mg/L) 1.3.6 Alkaline phosphatase
2. Urine analysis	2.1 Proteinuria, pyuria, microscopic hematuria, hyaline, and granular casts
3. Cerebrospinal fluid analysis	3.1 Normal or slightly elevated cerebrospinal fluid pressure 3.2 Initially a predominance of polymorphs or lymphocytes (total cell counts generally <500×10^6/L) and lymphocytes predominance later. Pleocytosis can persist for weeks 3.3 Elevated protein (50-100g/L) 3.4 Glucose is usually normal 3.5 Xanthochromia may occur

2. Microscopic observation

Leptospires can be observed under dark - field microscope as thin, coiled and rapidly moving microorganism. Sensitivity of dark-field microscopy is approximately 10^4 leptospires/ml. They are not stained by conventional Gram staining, but can be a stained with immunofluorescence, immunoperoxidase, silver staining and immunohistochemistry to increase the sensitivity of direct examination. Nevertheless, there are high risk of false-negative and false-negative results (Musso & La Scola, 2013).

3. Isolation of leptospires

Isolation of leptospires will be successful when samples for culture are collected from fresh blood, urine or tissue, prior to the administration of antibiotics treatment. Cultures are incubated at 28 to 30 °C for up to 13 weeks in commercially medium, Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, and examined weekly by dark-field microscopy (Adler & de la Peña Moctezuma, 2010; Musso & La Scola, 2013).

4. Antibody detection

Antibodies are detectable in blood approximately 5 to 7 days after the onset of symptoms. The microscopic agglutination test (MAT) is the serological reference test. The agglutination is the reaction between live antigens of each serogroup and serum samples from leptospirosis patients which is examined by dark-field microscopy. This test cannot differentiate between current, recent or past infections. Therefore, to confirm leptospirosis, a fourfold or greater rise in titer between paired sera is required (Levett, 2001; Levett & Haake, 2010).

The enzyme-linked immunosorbent assay (ELISA) can detect antibodies that reacting with genus specific antigen. But is not suitable for identify the causative serovar or serogroup. ELISA is usually positive, earlier than the MAT, from Day 6 to 8 (Musso & La Scola, 2013).

5. Antigen detection

Antigen can be detected earlier than antibody after infection, and can be detected in urine (Saengjaruk et al., 2002; Widiyanti et al., 2013). In present, different antigen detection tests have been developed to be used in routine (Musso & La Scola, 2013). The monoclonal antibodies are an important agent which has to be produced and developed for specificity and sensitivity of pathogenic leptospires detection.

6. Molecular diagnosis

In a leptospiremia, the first 2 weeks of illness, results of human infection may reach 10^7 leptospires/ml of blood. The polymerase chain reaction (PCR) can detect leptospires in this stage before the appearance of antibodies (Segura et al., 2005; Truccolo, Serais, Merien, & Perolat, 2001). However, PCR techniques are not generally used in the clinical setting because of the cost-limiting of the test and the trained personals are required. Moreover this method can identify only to species level of leptospires, therefore they are not useful for epidemiological tool(Levett & Haake, 2010).

2.5 Epidemiology

Leptospirosis is a zoonotic disease with a worldwide distribution. The spirochetes that cause this disease are shed in the urine of infected animals. Human infections are transmitted by direct contact with that urine or contaminated water and soil. The pathogenic leptospires can enter the body of patients through the conjunctiva, mucous membranes and opened-wound skin. Therefore, people who risk contact with leptospirosis are veterinary, farmer, sewage workers and people who live in flood disaster crisis etc. (Picardeau, 2013) that shown in Figure 2.6.

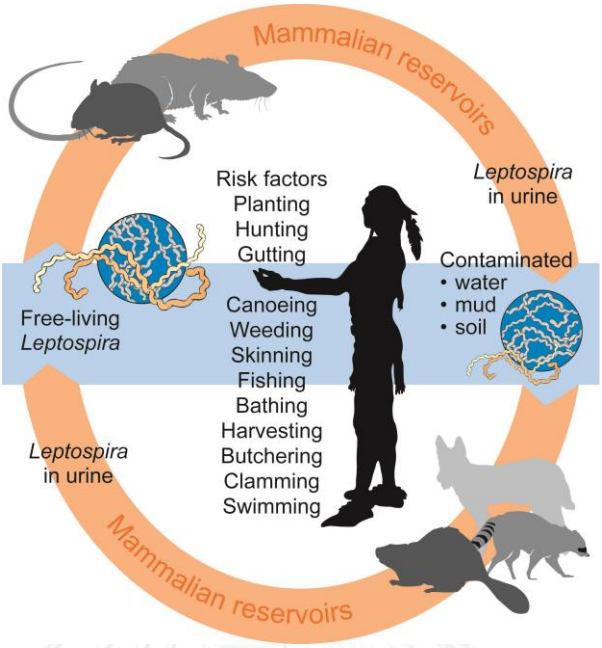


Figure 2.6 Epidemiology of leptospirosis in humans and animals

Available from: http://upload.wikimedia.org/wikipedia/en/thumb/2/2b/EID_ Leptospirosis_AcsB_1.jpg/719px-EID_Leptospirosis_AcsB_1.jpg [2014, April 3]

Leptospirosis is found throughout the world, it is most common in tropical and subtropical areas (Pappas, Papadimitriou, Siozopoulou, Christou, & Akritidis, 2008). Because of the pathogenic leptospires are able to survive longest in hot and humid conditions (Trueba, Zapata, Madrid, Cullen, & Haake, 2010). The International Livestock Research Institute publish the map (Figure 2.7) displays the distribution of leptospirosis in human and several other mammalian hosts in the geographic areas with the greatest burden of disease, 2012.

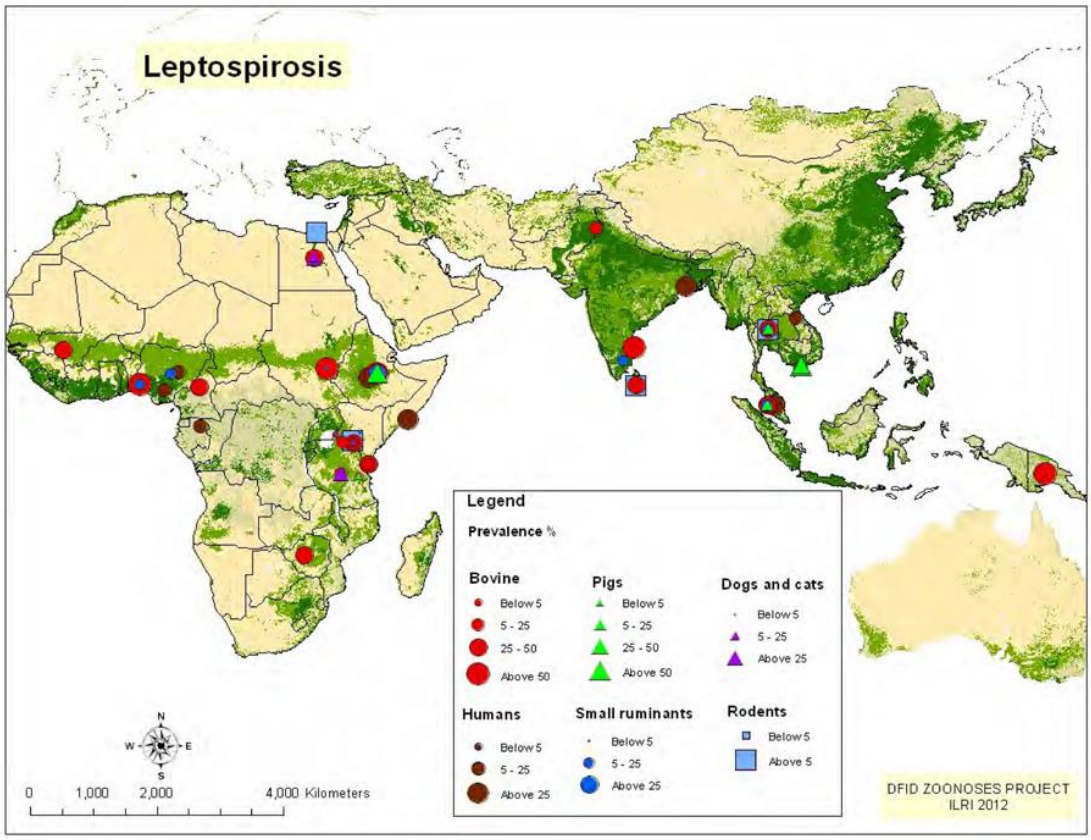
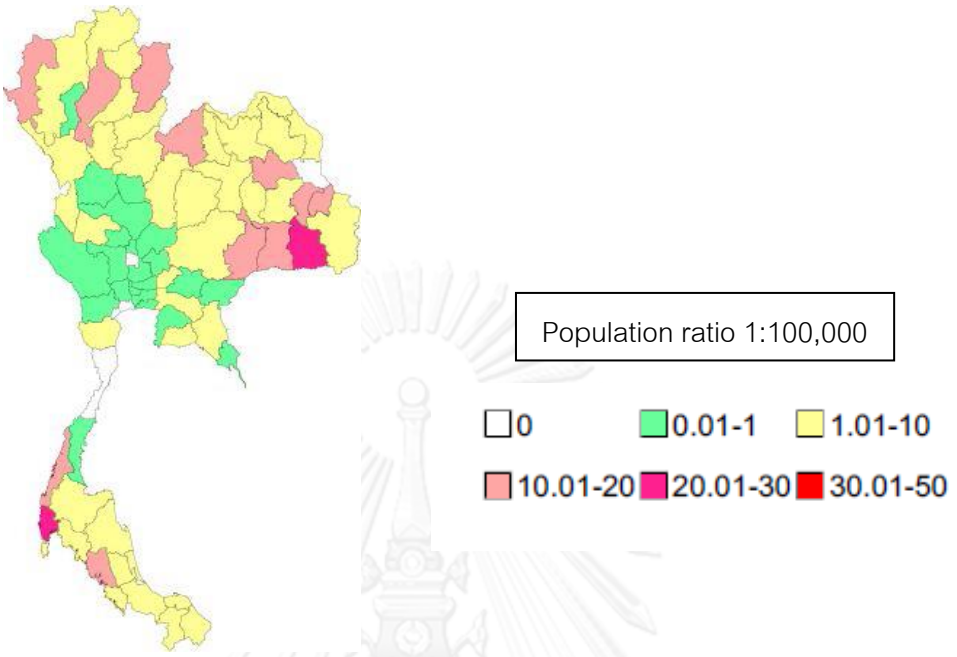


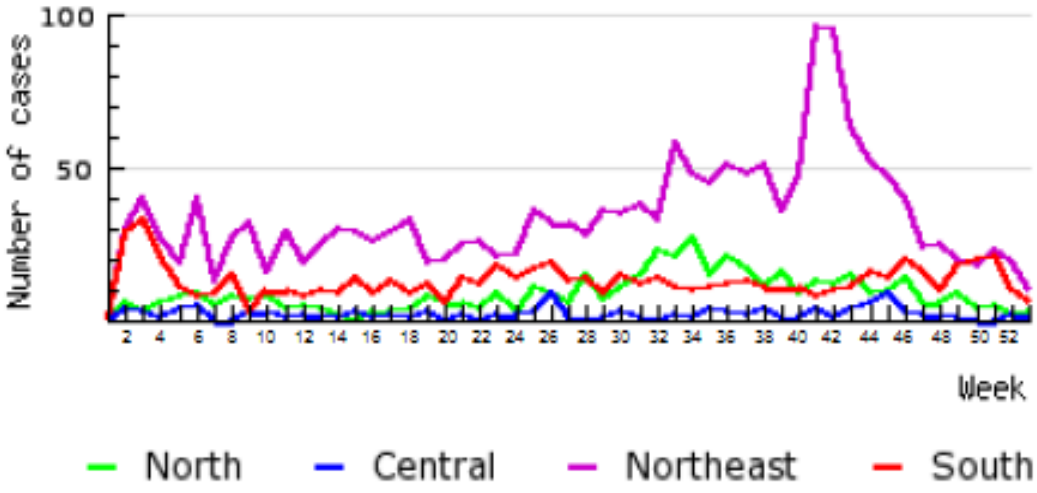
Figure 2.7 The distribution of leptospirosis in human and several other mammalian hosts in the geographic areas

Available from: http://www.infectionlandscapes.org/2013/06/leptospirosis.html [2014, April 3]

In Thailand, Leptospirosis is most found in late rainy season, October to November. The leptospirosis surveillance summarized in 2013 by the Bureau of epidemiology (Figure 2.8A) reported that they found morbidity (Figure 8A) and mortality rate per 100,000 populations are approximately 4.80 and 0.05 respectively from 72 provinces and found in male more than female. The most regions that found leptospirosis are Northeast, South, North and Central, respectively (Figure 2.8B).



A. Morbidity ratio in Thailand



B. Number of leptospirosis cases by week of onset and region

Figure 2.8 Leptospirosis patient that found in Thailand, 2013

Available from: http://www.boe.moph.go.th/boedb/d506_1/ds_wk2pdf.php?ds=43&yr=56 [2014, 20 March]

From the study reported in 2013, serovars which were most commonly found in livestock in Thailand are Ranarum, Sejroe, Mini, Pomona, Bratislava and Shermani. However, the previously reported serovars distributions in human and other species in Thailand are shown in Table 2.5 (Doungchawee et al., 2005; Kositanont, Naigowit, Imvithaya, Singchai, & Puthavathana, 2003; Oni, Sujit, Kasemsuwan, Sakpuaram, & Pfeiffer, 2007; Panaphut, Domrongkitchaiporn, & Thinkamrop, 2002; Suwancharoen, Chaisakdanugull, Thanapongtharm, & Yoshida, 2013).

Table 2.5 Serovars of *Leptospira* spp. that found in Thailand, detected by the MAT

Serovars of *Leptospira* spp.	
1. Bratislava	13. Louisiana
2. Autumnalis	14. Manhao
3. Ballum	15. Mini
4. Bataviae	16. Panama
5. Canicola	17. Pomona
6. Cellidoni	18. Pyrogenes
7. Cynopteri	19. Ranarum
8. Djasiman	20. Sarmin
9. Grippotyphosa	21. Sejroe
10. Hebdomadis	22. Shermani
11. Icterhaemorrhagiae	23. Tarrassovi
12. Javanica	

2.6 Antibodies

Antibodies or immunoglobulins (Igs) are glycoproteins secreted by B lymphocytes or B cells (known as plasma cells) which are induced by specific foreign molecule called an antigen, and present in the serum or tissue fluids of all mammals. The basic structure of antibodies consist of four polypeptide chains, there are two identical light (L) chains; each containing molecular weight about 25 kDa and

two identical heavy (H) chains; each containing molecular weight about 55 kDa. All four chains are held together by disulfide bond (Figure 2.9). Light chains and heavy chains are consisting of two distinct regions; variable region and constant region. The variable region is in the amino-terminal half of the chain which shows highly amino acid variability, and called hypervariable regions where the antigen binds to antibody (antigen binding sites). The constant region is the carboxyl-terminal half of the chain and shows constant sequence. Antibodies were divided into three fragments by papain (a proteinase present in papaya); one is crystallizable fragment (Fc), a fragment that do not binds to antigens and others is antigen – binding fragment (Fab), a region that binds to antigens (Lipman, Jackson, Trudel, & Weis-Garcia, 2005).

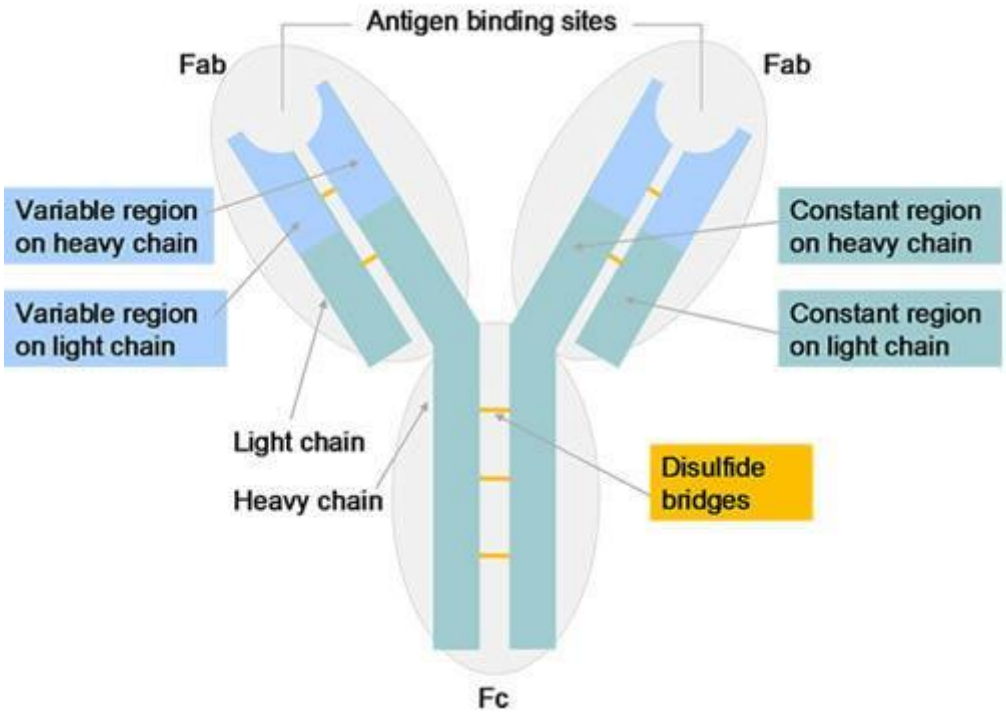


Figure 2.9 The structure of antibody

Available from: http://www.ptglab.com/Support/TechnicalSupport/Learning Center/AntibodyBasics.aspx [2014, March 26]

The five types of immunoglobulin heavy chains are γ, α, μ, δ and ε which defines the classes of the antibody as IgG, IgA, IgM, IgD and IgE, respectively (Figure 2.10); they differ in structural and antigenic properties. And the light chains are divided into kappa (κ) and lambda (λ) chains that either differs in Ig classes and subclasses (Khan, 2014).

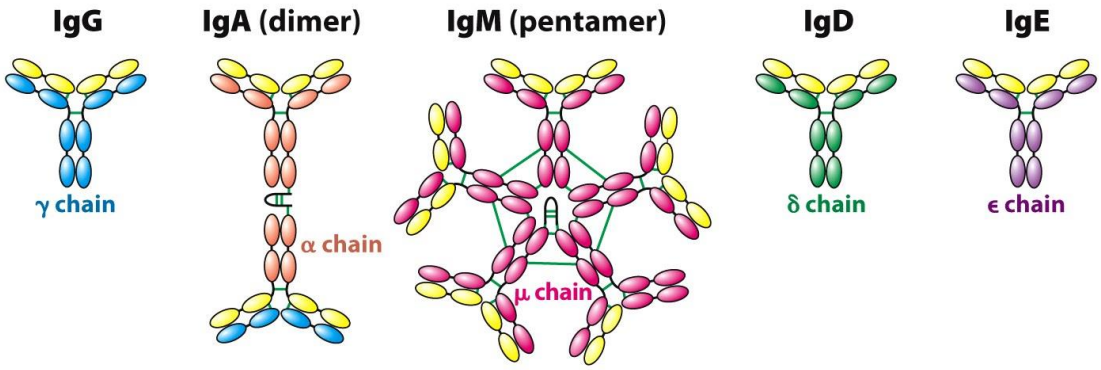


Figure 2.10 Types and structure of antibody

Available from: http://oregonstate.edu/instruct/bb451/spring13/stryer7/CH34/figure_34_08.jpg [2014, March, 26]

The classes and subclasses of antibodies are IgG (IgG1, IgG2, IgG3 and IgG4, in human and IgG1, IgG2a, IgG2b and IgG3, in mice), IgM, IgA, IgD and IgE. The properties and functions are shown in Table 2.6.

Table 2.6 Properties and functions of the five immunoglobulin (Ig) classes

Class	MW (kDa)	No. of subunits	Heavy chain	Subclass in human	Function
IgG	150	1	γ	4	Major Ig in serum. Provides the majority of antibody based immunity against invading pathogens. Moderate complement fixer (IgG3) can cross placenta.
IgA	300	2-3	α	2	Most produced Ig. Found in mucosal areas, such as the gut, respiratory and urogenital tract, and prevents their colonization by pathogens. Resistant to digestion and is secreated in milk.
IgM	900	5	μ	-	First response antibody. Expressed on the surface of B cells and in a secreated form with very high avidity. Eliminates pathogens in the early stages of B cell mediated immunity before there is sufficient IgG.
IgE	190	1	ε	-	Binds to allergens and triggers histamine release from mast cells and is involved in allergy. Also protects against parasitic worms.
IgD	150	1	δ	-	Function uncleae. Works with IgM in B-cell development; mostly B cell bound.

Modified and available from:

http://medicaltextbooksrevealed.s3.amazonaws.com/files/11245-53.pdf

http://www.abcam.com/index.html?pageconfig=resource&rid=11258&pid=10996

2.6.1 Monoclonal antibody

Monoclonal antibodies (MAbs) are a type of antibodies that produced by a single cell of B lymphocyte (B cell) in immune system. They are specific against antigenic determinant of the antigen, homogeneity and able to unlimited quantities production. This has become an important tool in biochemistry, molecular biology, and medicine. The technology for MAbs generation was introduced by Kölher and Milstein in 1975, which is involves fusing the normal antibody-secreting B cells (derived from a mouse that has been immunized with the intended antigen) with immortal myeloma cells (a class of malignant B cell tumors) to produce a hybrid cell that called hybridoma. These hybridoma cells are immortal growth and secreting the specific antibody, called monoclonal antibody, Figure 2.11 (Marx et al., 1997).

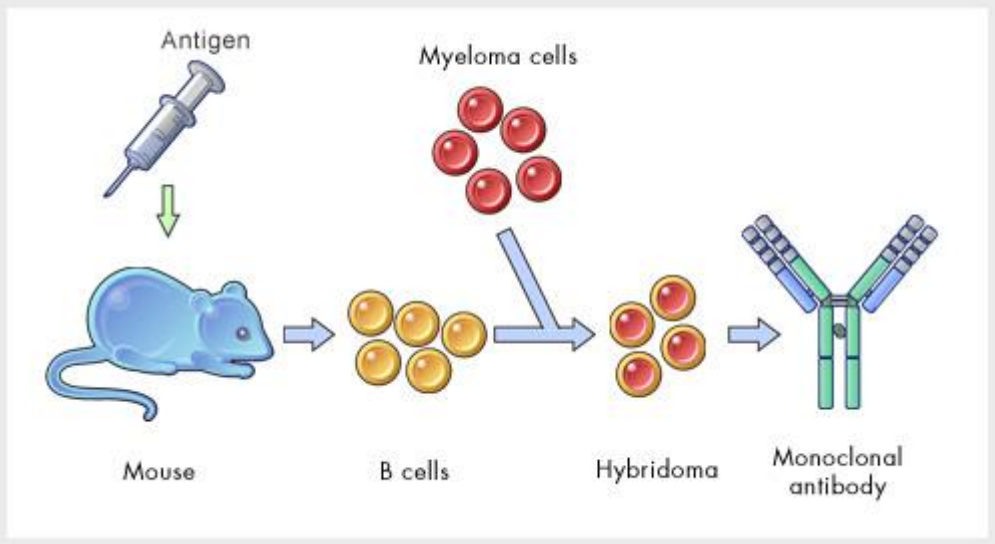


Figure 2.11 Monoclonal antibodies production
Available from: http://www.kyowa-kirin.co.jp/antibody/english/img/about/production_illust.jpg [2014, March 26]

2.6.1.1 Monoclonal antibodies production

The production of MAbs, mice is immunized with specific antigen and removed spleen (the source for lymphocytes) for fusion. The fusion technique is performed under aseptic condition. B cells are fused with myeloma cells by fusogen, polyethylene glycol (PEG), and then a mixture of fused cells (myeloma-myeloma,

myeloma-B cell and B cell-B cell) is cultured in Hypoxanthine, Aminopterin and Thymidine (HAT) medium, as a selective medium.

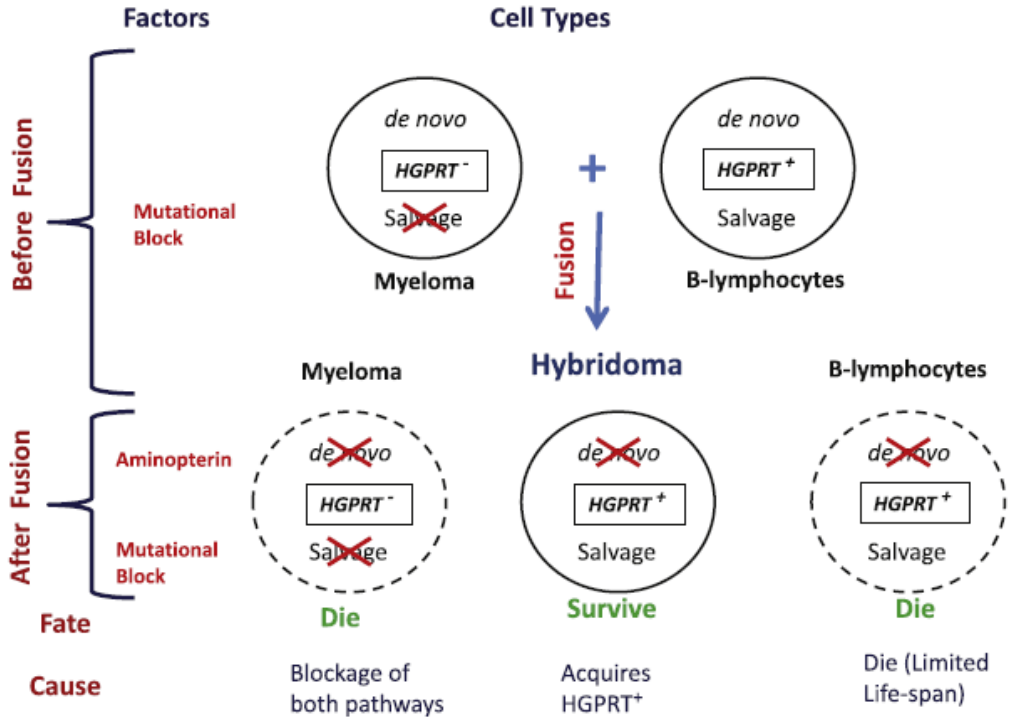


Figure 2.12 Biochemical pathways in hybridomas selection (Anchal Singh, Chaudhary, Agarwal, & Verma, 2014)

After that primary B cells will die by limited lifespan and myeloma cells that do not hybridized will die because of nonfunctional salvage pathway (lack of hypoxanthine-guanine-phosphor-ribosyl-transferase, $HGPRT^-$) and the *de novo* pathway is blocked by aminopterin, a drug that act as a folate antagonist, in HAT medium. The *de novo* and salvage pathways are the synthesis of the nucleotide; purines and pyrimidines. In the *de novo* pathway, nucleotides are synthesized from simple precursors that cells can procure from outside sources. And in the salvage pathway, nucleotides are synthesized by using intermediates found in degradation pathway of nucleotides such as nucleotides and nitrogenous bases. Therefore, only hybridized cells that acquire the $HGPRT^+$ gene from B cells can survive in HAT medium by synthesizing nucleotides via the salvage pathway while the *de novo* is

blocked (Figure 2.12). After selection of hybridoma cells, the next steps are the screening for specific clone and reclone to obtain monoclone (the clone of hybridoma cells which secreting monoclonal antibodies), respectively. (Nelson et al., 2000; Anchal Singh et al., 2014)

2.7 Monoclonal antibodies against *Leptospira* spp.

In 1985, monoclonal antibodies against *L. interrogans* serovar Pomona were produced by immunizing BALB/cJ mice with heat-killed *L. interrogans* serovar Pomona. All three MAbs reacted only with *L. interrogans* serovar Pomona but not reacted with serovars Grippotyphosa, Canicola, Icterohaemorrhagiae and Hardjo in the enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Serovars Pomona could easily be detected at 100 organisms per mL (Ainsworth, Lester, & Capley, 1985).

Monoclonal antibodies were produced against an outer envelope of *L. interrogans* serovar Copenhageni in 1988 (Jost, Adler, & Faine, 1988). The antigen for immunized mice was treated with salt and sodium lauryl sulfate (SLS) solution to isolate the outer sheath of *Leptospira* (Auran, Johnson, & Ritzi, 1972). All twenty-four MAbs were the IgG1 or IgG2a subclass and reacted with species-specific determinants of an antigen in the leptospiral outer envelope (OE) of pathogenic but not of saprophytic species of leptospire in enzyme immunoassay (EIA), but did not agglutinate live leptospires. In Western blotting, these MAbs reacted with a 35 kDa band that found in only serovars of pathogenic leptospires tested and also reacted with other bands to a lesser extents in protein species of 51 and 62 kDa in OE separated on SDS-polyacrylamide gels.

In 1996, the ethylenediaminetetraacetic acid (EDTA) extraction of *L. interrogans* serovar Icterohaemorrhagiae was an immunized antigen for producing MAbs. Fourteen MAbs are class of IgM (1), IgG1 (3), IgG2b (7) and three were not tested. One of IgG1 MAbs reacted with the EDTA extract bands around 20 kDa in the Western blot. The EDTA extract was suggested that the determinant is carbohydrate nature when was oxidized by periodate and was not digested by pronase. And the MAbs against the EDTA extract of serovar Icterohaemorrhagiae could not protect

hamsters from lethal challenge with virulent leptospires (Leite, Resende, Souza, Camargos, & Koury, 1996).

Saengjaruk et al. immunized mice with sonicated *L. interrogans* serovar Icterrohaemorrhagiae to produce MAbs. One of three MAbs which were IgG1 reacted to a 38 kDa of whole-cell lysates of all *Leptospira* spp. and the others which were IgG1 and IgG2a reacted to the 35 to 36 kDa components of all serogroups of the pathogenic *Leptospira*. They chose the IgG1 MAb which reacted to all pathogenic leptospires for used in a dot blot-enzyme-linked immunosorbent assay (dot-ELISA) for detecting *Leptospira* antigen in urine samples. The MAb-based dot-ELISA was positive for 75.0, 89.9, 97.2, 97.2 and 100% of patients on day 1, 2, 3, 7 and 14 of hospitalization, respectively (Saengjaruk et al., 2002).

Six MAbs against leptospires were produced by immunizing BALB/C mice with formalin killed whole cell antigens. There were three serogroup-specific MAbs against *L. interrogans* serovars Autumnalis, Bataviae and Pyrogenes and three broadly reactive MAbs. One of all MAbs was specific to the 38 kDa protein and the others recognized specific epitopes which were carbohydrate on LPS. The MAb which specific to the 38 kDa showed the most effective antibody to neutralize all tested leptospires (Gaudart et al., 2005).

Triton X-100 extract containing outer envelope membrane of virulent strains of hamster challenged leptospires was used as antigen for production of MAbs produced by immunizes mice. The MAbs were used in ELISA and were shown to be serogroup-specific that recognized lipopolysaccharide-like epitopes on the surface of the whole cell and agglutinated only the homologous serovars leptospires. Passive transter of the MAbs protected hamsters against a homologous challenge but failed to protect against a heterologous challenge (Ruby & Srinivas, 2013).

The heat-killed *L. interrogans serovar* Icterohaemorrhagiae was used to immunize mice for production of MAbs. One MAb of class IgG3 was specific and reactive to the 12 kDa LPS of *Leptospira* spp. This MAb was conjugated with colloidal gold for the dipstick assay. The detection limit of the assays, when disrupted whole

bacterial cells were used, was 10^6 cells/ml. The assay can detect both pathogenic and saprophytic strains of *Leptospira* (*L. interrogans* serovars Autumnalis, Canicola, Manilae, Grippothyphosa and Icteroharmorrhagiae, *L. borgpetersenii* serovars Poi, Tarassovi and Javanica and *L. biflexa* serovar Patoc) but did not bind to other bacterial species that are excreted in urine such as *L. pneumophila* and *S. pneumonia*. The sensitivity and specificity of the dipstick assay were 80% and 74%, respectively, but lower than that of the immunochromatography-based lateral flow assays (ICG-based LFA) which were 89% and 87%, respectively (Widiyanti et al., 2013).

Productions of MAbs against an antigen of the outer membrane protein of pathogenic *Leptospira* spp. were investigated. Two MAbs was generated with an isotype of IgG2b and IgM, by immunization of mice with a recombinant LipL32 (rLipL32)(Lüdtke et al., 2003). They reacted with most of the pathogenic serovars tested (Australis, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Grippotyphosa, Javanica, Icterphaemorrhagiae, Panama, Pomona, Pyrogenes, Shermani, Djasiman and Louisiana) except for Sejroe and none reacted with non-pathogenic *Leptospira* in both indirect ELISA and immunoblotting technique.

rLipL32 protein was also used to immunization of mice for MAbs production (Coutinho et al., 2007). All MAbs were of the IgG2b isotype and specifically reacted with native LipL32 in pathogenic serovars. Their affinity constant was between 5×10^7 M^{-1} and 6×10^6 M^{-1}. These results, suggest that these MAbs are suited for diagnosis tests of leptospirosis but cannot be used together.

Production and characterization of MAbs against a recombinant fragment of LigB (rLigBrep) of approximately 54 kDa that comprise the portions of LigA and LigB were studied. Two MAbs and three MAbs were of the IgG1 and IgG2b isotypes, respectively. Their affinity constants for rLigBrep ranged from 7×10^7 M^{-1} to 4×10^8 M^{-1}. The MAbs were able to react with the native antigen on the surfaces of *L. interrogans*, *L. borgpetersneii* and *L. noguchii* by indirect immunofluorescence, immunoblotting and immunoelectron microscopy (Monte et al., 2011)

CHAPTER III
MATERIAL AND METHODS

3.1 Animals and Cell lines

- Female ICR mice (outbred strain) 8 weeks old were purchased from the National Laboratory Animal Centre, Mahidol University, Nakorn Pathom, Thailand.

- Female BALB/c mice (inbred strain) 8 weeks old were purchased from the National Laboratory Animal Centre, Mahidol University, Nakorn Pathom, Thailand.

- Myeloma cells P3-X63Ag8 (P3X) were purchased from the American Type Culture Collection (ATCC; TIB-9).

- Myeloma cells P3/NSI/1-4A4-1 (NSI) were purchased from the American Type Culture Collection (ATCC; TIB-18).

3.2 Bacterials strains

3.2.1 *Leptospira* spp.

Leptospira interrogans serovar Manilae, M1352 and Pomona were obtained from Khon Kaen University, Khon Kaen, Thailand with the approval of Professor Ben Adler's lab (Monash University. Melbourne. Australia).

L. interrogans serovar Bratislava, Autumnalis, Ballum, Bataviae, Canicola, Celledoni, Grippotyphosa, Javanica, Louisiana, Panama, Pomona, Pyrogenes, Ranarum, Sejroe and Shermani were obtained from Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand.

3.2.2 Other bacteria species

Enterobacter aerogenes, *Enterrococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Staphylococcus epidermidis* were obtained from Department of Medical Science, Ministry of Public Health, Thailand.

3.3 Chemicals, Antibodies and Kits

- Acetic acid	Sigma-Aldrich, St. Louis, Missouri
- 40% Acrylamide and Bis-acrylamide solution	Bio-Rad, Hercules, USA
- Aminopterin	Sigma-Aldrich, St. Louis, Missouri
- Ammoniumpersulfate (APS)	Bio-Rad, Hercules, USA
- Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, Missouri
- Bromophenol blue	Labchem, Australia
- Citric acid monohydrate	Sigma-Aldrich, St. Louis, Missouri
- Coomassie brilliant blue R-250	USB Corporation, USA
- Developer rapid process	J.Nasen, Co., Ltd., Thailand
- Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
- di-Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt, Germany
- Ethanol	Merck, Darmstadt, Germany
- Fetal calf serum (FCS)	PAA lab, Pasching, Austria
- Freund's complete adjuvant (FCA)	Sigma-Aldrich, St. Louis, Missouri

- Freund's incomplete adjuvant (FIA) — Sigma-Aldrich, St. Louis, Missouri
- Fixer rapid process — J.Nasen, Co., Ltd., Thailand
- 40% formaldehyde — Carlo Erba, Milan, Italy
- Gentamycin — T.P. drug laboratories (1969) Co., Ltd., Thailand
- Glycerol — Merck, Darmstadt, Germany
- Glycine — Bio-Rad, Hercules, USA
- Goat anti mouse IgG-Horseradish peroxidase conjugates (GAM-HRP) — Jackson Immuno, USA
- Goat anti mouse IgM-Horseradish peroxidase conjugates (GAM-HRP) — Abcam, Cambridge, USA
- Hydrochloric acid (HCl) — Merck, Darmstadt, Germany
- 30% Hydrogen peroxide (H_2O_2) — Merck, Darmstadt, Germany
- Hypoxanthine — Sigma-Aldrich, St. Louis, Missouri
- Isoflurane — Abbott Laboratories, Illinois, USA
- L-glutamine — Sigma-Aldrich, St. Louis, Missouri
- Luria Broth (LB) — Difco Laboratories, USA

- Methanol — Merck, Darmstadt, Germany
- Mouse monoclonal antibody isotyping Kit — Sigma-Aldrich, St. Louis, Missouri
- Penicillin G — Sigma-Aldrich, St. Louis, Missouri
- Peroxidase labeled Goat Anti-Mouse IgG (Fab specific) — Sigma-Aldrich, St. Louis, Missouri
- Polyethylene glycol (PEG) — Sigma-Aldrich, St. Louis, Missouri
- Potassium chloride (KCl) — Merck, Darmstadt, Germany
- Potassium citrate — Sigma-Aldrich, St. Louis, Missouri
- Potassium dihydrogen phosphate (KH_2PO_4) — Merck, Darmstadt, Germany
- Prestained molecular weight markers — Bio-Rad, Hercules, USA
- RPMI 1640 medium — Biochrom Berlin, Germany
- Skim milk — Anline, Bangkok, Thailand
- Skim milk — Difco Laboratories, USA
- Sodium chloride (NaCl) — Merck, Darmstadt, Germany
- Sodium dihydrogen phosphate (NaH_2PO_4) — Carlo Erba, Milan, Italy
- Sodium dodecyl sulfate (SDS) — Sigma-Aldrich, St. Louis,

	Missouri
- Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
- Sodium pyruvate ($C_3H_3O_3Na$)	Sigma-Aldrich, St. Louis, Missouri
- Streptomycin	Sigma-Aldrich, St. Louis, Missouri
- Sulfuric acid (H_2SO_4)	Merck, Darmstadt, Germany
- 3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma-Aldrich, St. Louis, Missouri
- N, N, N',N'-Tetramethyl ethylenediamine (TEMED)	Pierce, Rockford, Illinois
- Thymidine	Sigma-Aldrich, St. Louis, Missouri
- Tris [hydroxymethyl] aminomethane (Tris base)	Bio-Rad, Hercules, USA
- Tween 20	Sigma-Aldrich, Gillinyham, UK

3.4 Equipments

- -20 °C Freezer	Sanyo, Chachoeng Sao, Thailand
- -70 °C Freezer	Sanyo, Osaka, Japan
- 4 °C Refrigerator	Toshiba, Nonthaburi, Thailand
- 37 °C 5%CO_2 Incubator	Yamato, Tokyo, Japan

- 96-well EIA/RIA plate	Corning Incorporation, New York, USA
- Autoclave (high pressure steam sterilizer)	Udono, Tokyo, Japan
- Autopipette, P2.5, P10, P20, P200, P1000 and P5000	Eppendorf, Hamburg, Germany
- Autopipette tip, 10, 200, 300, 1000	Axygen, Union City, California
- Cell culture dish, 60 and 90 mm	Bibby Sterilin Ltd., UK
- Centrifuge tube, 15 and 50 ml	Axygen, Union City, California
- Centrifuge, model: universal 320, swing out rotor 1619	Hettich, Tuttlingen, Germany
- Compact rocker, model: CR300	FINEPCR, Seoul, Korea
- Cryotube, 2 ml	Nunc, Roskilde, Denmark
- Dialysis membrane, Cellu Sep, MWCO: 12,000-14,000	Membrane Filtration Products, Seguin, Texas
- Dark-field microscope	Olympus, USA
- Disposable syringe, 1 and 5 ml	Nipro, Ayutthaya, Thailand
- Examination gloves	Magaglove, Chon Buri, Thailand
- Filter paper #1	Whatman, Kent, UK
- Heat block, model: Thermomixer Compact	Eppendorf, Hamburg, Germany

- High Intensity Ultrasonic Processor, model: VC/VCX 130,500, 750)	Becthai, Thailand
- High speed refrigerator centrifuge, model: J2-21	Beckman, USA
- Hot air oven, model: D06063	Memmert, Schwabach, Germany
- Hot plate stirrer, model: C-MAG HS 10	Becthai, Thailand
- Hypodermic needle, 18G and 21G	Nipro, Ayutthaya, Thailand
- Inverted microscope, model: TMS	Nikon, Tokyo, Japan
- Laminar flow, model: 'Clean' model V6	Lab Survice L.td., Bangkok, Thailand
- Liquid Nitrogen Tank , model: 34 HC Taylor Wharton Cryogenic	Harsco Corp., Camp Hill, Pennsylvania
- Microcentrifuge, model: WiseSpin® CF-10	Wisd Laboratory Instruments, Irland
- Microcentrifuge tube, 1.5 ml	Axygen, Union City, California
- Microplate reader, model: MCC/340	Titertek multiskan, Helsinki, Finland
- Mini-PROTEAN® II Multiscreen Apparatus	Bio-rad, Hercules, USA
- Mini-Protein® Tetra System for SDS-PAGE	Bio-Rad, Hercules, USA
- Multichannel autopipette	HTL, Warsaw, Poland
- Multi-detection microplate reader, Synergy HT	BIO-TEK, Richmond, Virginia

- Nitrocellulose transfer membrane	Bio-Rad, Hercules, USA
- Orbital shaker	Fisher Sciencetific, Illkirch-Graffenstadam, France
- Petri Dish, 90 mm	Hycon, Germany
- pH meter, model: AB15	Fisher Sciencetific, UE Tech Park, Singapore
- Pipettes, 10 ml	HBG, Luetzelinden, Germany
- Precision weighting balance, model: AG204 and PG402S	Mettler Toledo, Greifensee, Switzerlan
- Refrigerated Microcentrifuge 6500	Hettich Zentrifugen, Germany
- Semi-dry Electrophoretic Transfer Cell, model: Trans-Blot® SD	Bio-Rad, Hercules, USA
- Tissue cell culture plate, 24-, 48- and 96-well	Corning Incorporation, USA
- Ultra-Pure Water Purification System, model: LA534 BOOST PUMP	Elga, England
- Vacuum pump	Iwaki pump, Fukushima, Japan
- Vacuum pump	Edwards, Crawley, England
- Vortex mixer, model: G560E	Scientific Industries, Boulder, Colorado

- Water bath — Memmert, Schwabach, Germany
- X-ray film, model: Amersham Hyperfilm™ ECL — Amersham Biosciene, UK
- X-ray film cassette, model: Hyper cassette™ — Amersham Biosciene, UK

3.5 Experimental procedures

3.5.1 Preparation of bacteria

Leptospira spp. were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, and incubated at 30 °C for 7-10 days while other bacterial strains were cultured in Luria broth (LB) with agitation at 37 °C for 24 h. Number of viable cells was counted under a dark-field microscope. All bacteria were harvested by centrifugation at 10,000 g for 10 min at 4 °C, washed twice with 0.01 M Phosphate buffer saline (PBS), pH 7.4 and resuspended in PBS.

3.5.2 Preparation of antigen

For immunization, *L. interrogans* serovar Manilae and the mutant M1352 were fixed overnight by 4% paraformaldehyde or sonicated at 20 kHz for 2 min and used as the immunogen. For detection, the other pathogenic *Leptospira* spp. and other bacteria cells were sonicated at 20 kHz for 2 min and used in ELISA and Western blotting.

3.5.3 Immunization of mice

Six 8-weeks-old, female ICR mice and two 6-weeks-old, female BALB/c mice were immunized by intraperitoneal (i.p.) injection with 100 µl of fixed or sonicated suspension (10^7 cells/ml, for ICR and 10^6 cell/ml, for BALB/c) of immunogen in complete Freund's adjuvant (Sigma) at 1:1 (v/v). Booster injections at two week interval were performed using the same antigen mixed in incomplete Freund's adjuvant. One week after three boosters, mice antiserums were collected by tail bleeding, blood from each mouse was centrifuged at 8,000 g for 10 min for collected mouse antisera and determined antiserum titer using indirect ELISA. Three days prior

to cell fusion, final boost of the same antigen in normal saline solution was performed. The serum which was collected before first immunization was used as negative control.

3.5.4 Production of monoclonal antibodies

3.5.4.1 Myeloma cell lines

Myeloma cell P3-X63Ag8 (P3X) and P3/NSI/1-4A4-1 (NSI) were used for fusions. Myeloma cells were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) as a supplement and incubated at 37 °C with humidified 5% CO_2. Before fusion, the myelomas were subcultured for 3 to 4 days, approximate 10^7 cells. For use in hybridoma production, they were centrifuged at 380 g for 5 min, the pellet was resuspended in 30 ml of RPMI 1640 with 0.2 mg/ml gentamicin and placed in humidified incubator.

3.5.4.2 Spleen cells

Immunized mice were anesthetized with isoflurane before blood drawn by cardiac puncture. Their serum were collected and stored at -20 °C for used as positive control. The spleen were collected from immunized mice with aseptic technique and gentle washed in sterile Petri dishes which containing RPMI 1640 medium supplemented with 0.2 mg/ml gentamicin for reduced contamination. The connective tissues were removed from spleen. And the spleen was cut into small pieces by sterile scissors. Then, small pieces of spleen were crushed by 10 ml syringe plunger through a sterile grid into RPMI 1640 medium supplemented with 0.2 mg/ml gentamicin. The spleen cells were collected by centrifugation at 380 g for 5 min and resuspended the pellet into 30 ml of RPMI 1640 medium for used in hybridoma production.

3.5.4.2 Production of hybridoma

Myeloma and spleen cells suspension were individual transferred into a polypropylene tube and mixing. The mixed suspension was centrifuged at 380 g, 5 min, the cell pellet was kept and supernatant was removed. After that, the tube was gently taped and gradually added 1 ml of pre-warmed (37 °C) 50% (w/v)

polyethylene glycol (PEG) by using sterile Pasteur pipette, following with 20 ml RPMI 1640 medium supplemented with 0.2 mg/ml gentamicin within 2 min. Then, the fused cell suspension was centrifuged and washed twice with the same medium for removed 50% (w/v) PEG. The fused cell pellet was suspended in hypoxanthine-aminopterin-thymidine (HAT) selective medium containing 20% FCS and 200 microliter of this suspension were added into each well of 96-wells cell culture plate and cultured in incubator at 37 °C with humidified 5% CO_2. After seven days, half of hybridoma cultured medium were removed and were replaced with fresh HAT medium with 20% FCS supplement. And seven days later, the cultured mediums were removed and were replaced with 200 microliter fresh HAT medium with 20% FCS supplement. When the cultured cells grew to half of well and cultured media was yellow, the culture supernatants were collected for screened by indirect ELISA. The positive clones that against *L. interrogans* serovar Manilae or mutant M1352 were selected and were maintained their growth in HT medium (HAT medium without aminopterine) with 20% FCS supplement for 3^{rd} to 4^{th} week after fusion and RPMI 1640 medium for several next weeks.

3.5.5 Screening of hybridoma cells

Culture supernatants from hybridoma cells were tested for monoclonal antibodies against *Leptospira* spp. using indirect ELISA procedure. Plates were coated with sonicated bacteria samples (*L. interrogans* serovar Manilae or other bacteria) at a concentration of 1×10^7 cells/ml in a volume of 50 µl per well and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween20 (PBST) and blocked with 5% skim milk in PBS at 37 °C for 1 h. After washing, hybridoma cell culture supernatants were added and plates were incubated at 37 °C for 2 h. Plates were washed and added secondary antibody (horseradish peroxidase-conjugates goat anti mouse IgG; GAM-HRP) diluted 1:10,000 with PBS, incubated at 37 °C for 1 h and washed. Plates were added with substrate solution (3, 3', 5, 5'-tetramethylbenzidine; TMB and H_2O_2 in 205 mM citrate buffer, pH 4.0). After 10 min, the reaction was stopped with 1 M H_2SO_4 and was measured absorption at 450 nm using microtiter plate reader. Then the positive clones were selected by a cut-off value of 0.3.

3.5.6 Limiting dilution for single cloning of hybridoma cells

Hybridoma cells were recloned to obtain monoclone by a limiting dilution method. The cells suspension of each positive well was serial diluted in HT medium with 10% FCS supplement, volume of cells were counted under Inverted microscope. The wells, there was 70 to 80 cells, were selected and mixed with 8 ml HT medium supplemented with 10% FCS. The mixture 80 microliter was dispensed into each well of 96-wells cell culture plate which there is 120 microliter HT medium supplemented with 10% FCS in a wells, for obtained one cell per well. After 10 to 12 days of limiting dilution, the well containing a single colony was tested by ELISA. At least 3 times, the selected hybridoma cells were cloned by limiting dilution for achieves stable clone producing monoclone. The obtained monoclones were stored in liquid nitrogen.

3.5.7 Cell storage

The obtained stable monoclones were cultured in RPMI 1640 medium supplemented with 10% FCS and expanded from wells to plates. The stable monoclones were incubated at 37 °C in humidified 5% CO_2 for 7 days and were collected by centrifugation at 380 g, 5 min. The cell pellet was suspended with 1 ml freezing medium which kept at 4 °C by using sterile Pasteur pipette and transferred to sterile cryotube. Then, the lid was closed and the cryotube was placed in a foam box at -70 °C for 24 h before transferred to liquid nitrogen (-196 °C).

3.5.8 Cell thawing

The cell suspension in cryotube from liquid nitrogen was placed in a water bath at 37 °C for thawing. The suspension was transferred into a tube that contains 8 ml of RPMI 1640 medium immediately and centrifuged at 380 g for 5 min. The cell pellet was resuspended in RPMI 1640 medium with 10% FCS supplement, transferred into sterile cell culture plate and cultured at 37 °C in humidified 5% CO_2 incubator.

3.5.9 Characterization of monoclonal antibodies

3.5.9.1 Specificity test

Specificity of the MAbs, was determined by ELISA based on the reactivity with *L. interrogans* serovar Manilae and the mutant M1352. They were assessed for cross-reactivity with other pathogenic *Leptospira* spp, gram-negative bacteria and gram-positive bacteria that found in urinary tract infection at a concentration of 1×10^{7} cells/ml.

3.5.9.2 Sensitivity test

Sensitivity test was determined by an indirect ELISA. Plates were coated with 50 µl per well of two-fold serial dilutions of sonicated *L. interrogans* serovar Manilae from 3×10^{7} to 1×10^{5} cells/ml dilute in PBS and incubated overnight at 4 °C.

The sensitivity of the obtained MAbs was justified based on the half maximal effective concentration (EC50) which was defined as the concentration at which 50% B/B_0 was obtained, where B and B_0 is the average of absorbance obtained from indirect ELISA with or without *L. interrogans* serovar Manilae, respectively (Oaew, Charlermroj, Pattarakankul, & Karoonuthaisiri, 2012).

$$\%\frac{B}{B_0} = \left[B_0 + \frac{B\text{-}B_0}{1+10^{\log EC50-x}}\right]\times100$$

The limit of detection (LOD) was determined by titration with serial dilution of *L. interrogans* serovar Manilae and calculated by subtracting the concentration at B_0 values with three times of its standard deviation (SD) values, LOD = [3SD].

3.5.9.3 Isotype determination

Isotype of MAbs was determined for class and subclass using Sigma-Aldrich's mouse monoclonal antibody isotyping kit based on a sandwich ELISA. Plates were coated with isotyping specific antibodies: IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, incubated at 37 °C for 1 h. After washing, culture supernatant was added and plates were incubated at 37 °C for 1 h. After washing, HRP goat anti-mouse IgG (Fab specific) was added and incubated at 37 °C for 30 min. After another washing, the assay was performed as described previously.

3.5.9.4 Antigen recognition by Western blotting

3.5.9.4.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE method followed by Laemmli (1970), a 1.0 mm thick of slab gel with 5% stacking gel and 12% separating gel were used. Protein marker was used as the stand molecular weight of protein, ranging from 10 to 230 kDa. For 10-wells comb, 30 microliter of sonicated antigen, concentration at 5×10^{9} cells/ml, was denatured with 7.5 microliter of 5X concentrations of SDS strain dye for adjusted to be 1X SDS strain dye, and boil for 5 min. Then load into each well of gels. Samples were electrophoresed in running buffer at 100 V for 105 min.

For 1-well comb which used in Mini-PROTEAN® II Multiscreen Apparatus, the volume of antigen and solution were multiplied tenfold from volume that use in 10-wells comb.

3.5.9.4.2 Western blot

When running of electrophoresis completely, the stacking gel was removed from separating gel and measured the size of separating gel. The separating gel was equilibrated in transfer buffer for 5 min. Nitrocellulose membrane and 6 pieces of filter paper were cut as the same size of separating gel and were soaked in transfer buffer before use. After that, 3 pieces of filter paper, nitrocellulose membrane, separating gel and 3 pieces of filter paper were placed on to semi-dry apparatus, respectively and operated at 85 Amp, 90 min for transferred protein from separating gel to nitrocellulose membrane. After that, the membrane was blocked in 3% skim milk for 1 h and was incubated with supernatant of MAbs (Primary antibody) for 1 h with rocking at RT and kept overnight at 4 °C. In next day, this membrane was continuing rocked at RT for 1 h, then, was wash two times for 5 min and three times for 15 min with PBST. The membrane was incubated 1 h with Goat Anti-Mouse IgM HRP (Secondary antibody) at dilution of 1:8000 and was washed two times, 5 min and three times, 15 min with PBST. After that the membrane were probed with chemiluminescence solution and detected antibody-reaction of on X-ray film.

For primary antibody incubation with Mini-PROTEAN® II Multiscreen Apparatus, in Figure 3.1 the blocked membrane in 3% skim milk was laid on the

sealing gasket, which was placed onto the base plate, with the antigen side facing up. The sample template was placed on top of the membrane with the channels of the sample template covered the length of the blocked membrane. When the screws were tightened, be used a diagonal crossing pattern in Figure 3.2 to insure even pressure on the membrane surface. Then, 600 microliter of MAbs were loaded into channel of the sample template, during the base plate was tilted up ~30°. And assays were performed as described previously excepted when MAbs were washed with PBST before removed the multiscreen apparatus. MAb samples were removed by vacuum aspiration and loaded 1 ml PBST, continuously for three times.

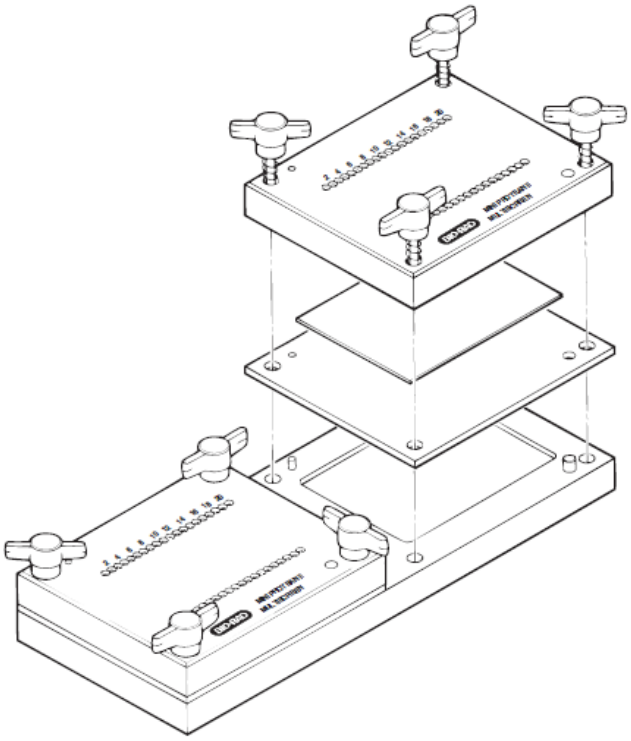


Figure 3.1 Assembly of the multiscreen apparatus
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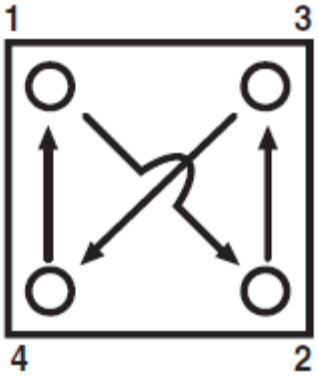

Figure 3.2 Diagonal crossing pattern for tightening screws in the multiscreen apparatus
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CHAPTER IV
RESULTS

4.1 Immunization of mice

Two ICR mice were immunized with fixed form of *L. interrogans* serovar M1352, four ICR and two BALB/c female mice were immunized with fixed and sonicated antigen forms of *L. interrogans* serovar Manilae, that shown in Table 4.1. After immunization, highest antiserum titer of each immunized mice was determined by the indirect ELISA. The result shown in Figure 4.1 suggested that the end point titers of the eight immunized mice were 1:8,000 and 1:256,000 respectively. Immune-reactivity of the pre-immunized mice serum was used as the negative control.

Table 4.1 Information of mice immunization

Fusion no.	Type of mouse	Immunization								
		Serovars of leptospire	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
F1	ICR	M1352	Fixed form, 10^6 cells				X	X	X	X	X
F2	ICR	Manilae	Fixed form, 10^6 cells				X	X	X	X	X
F3	ICR	Manilae	Fixed form, 10^6 cells				X	X	X	X	X
F4	ICR	M1352	Fixed form, 10^6 cells				X	X	X	X	X
F5	ICR	Manilae	Fixed form, 10^6 cells				X	X	X	X	X
F6	ICR	Manilae	Fixed form, 10^6 cells							Sonicated form, 10^6 cells	
F7	BALB/c	Manilae	Fixed form, 10^5 cells				Sonicated form, 10^5 cells				X
F8	BALB/c	Manilae	Fixed form, 10^5 cells				Sonicated form, 10^5 cells				

X = No immunization

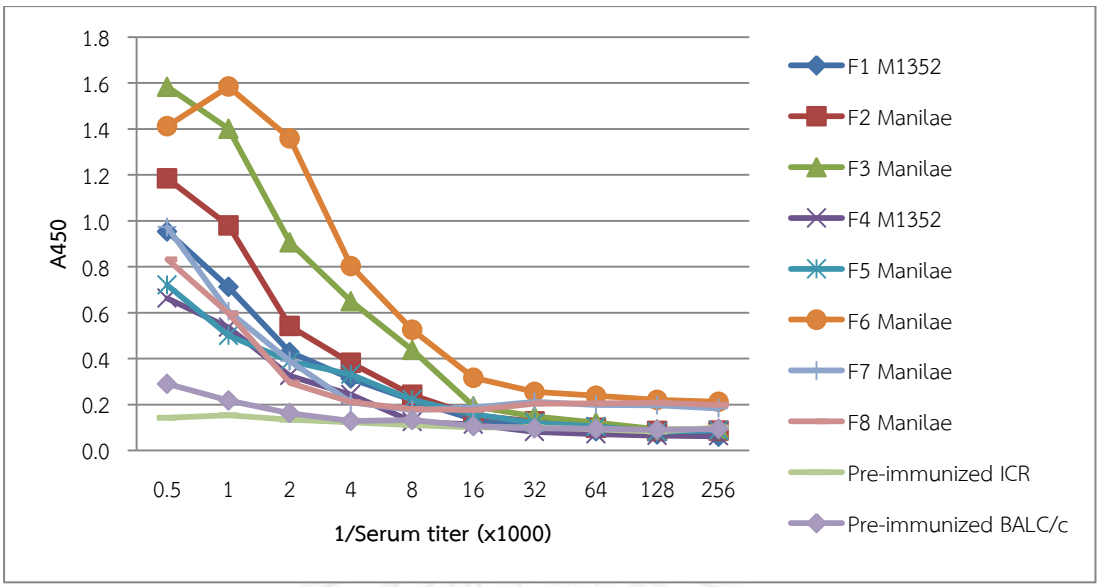

Figure 4.1 Antiserum titers of eight immunized mice and pre-immunized ICR and BALB/C mice against sonicated *L. interrogans* serovars Manilae and M1352 were determined by indirect ELISA.

4.2 Production of hybridoma

Hybridoma cell was produced by the somatic cell fusion, between NSI or P3X myeloma cells and spleen cells of the immunized mice with antigens, that previous described, of *L. interrogans* serovars Manilae and the mutant M1352. In each fusion, the fused cells were distributed into ten to fifteen 96-well cell culture plates. The culture mediums of hybridoma cells, which survive in selective medium, were screened by indirect ELISA. The secreted antibodies hybridoma cells against *L. interrogans* serovars Manilae and M1352 were selected and recloned until 1 cell per well by limiting dilution for obtained a monoclone. Total of monoclones that were obtained are shown in Table 4.2. The stable MAbs were produced for further characterization.

Table 4.2 Summary of hybridoma production

Fusion no.	Myeloma cells	Number of well cultured	Percent of hybridoma	Monoclone no.
F1	P3X	960	90	-
F2	NS1	864	100	F2-5/2E/1
F3	NS1	960	70	F3-10/1G/1, F3-10/1G/2
F4	P3X	960	100	F4-7/1B/1, F4-8/12D/1, F4-5/10A/1
F5	NS1	960	80	-
F6	NS1	960	54	F6-4/9E/1, F6-4/9E/2, F6-8/6C/1, F6-5/11H/1
F7	NS1	1,440	92	F7-15/1H/2
F8	NS1	1,440	100	F8-13/3B/3, F8-8/2C/3, F8-8/2C/4, F8-6/10H/2

- = No clone was obtained

4.3 Characterization of monoclonal antibodies

4.3.1 Specificity

Fifteen stable monoclones were obtained from eight fusions. The monoclones were divided into six groups depending on their specificity of MAbs against various sonicated bacteria by indirect ELISA, based on the optical density (OD) cut-off of 0.3. The results were shown in Figure 4.2 to 4.7 and Table 4.3.

Group 1 consisted of five monoclones were F2-5/2E/1, F4-8/12D/1, F4-5/10A/1, F6-5/11H/1 and F8-13/3B/3. The MAbs in this group were only specific against *L. interrogans* serovar M1352.

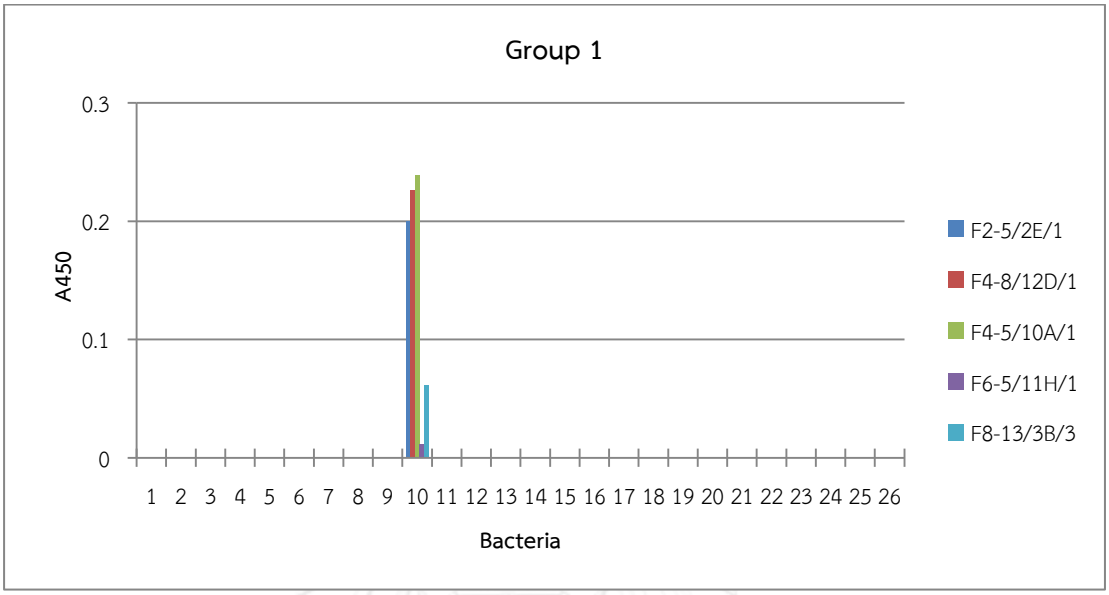

Figure 4.2 The specificity of MAbs (non-dilute) against various sonicated bacteria were determined by indirect ELISA, in group 1.

Gram-negative bacteria: (1) *Enterobacter aerogenes*; (2) *Enterrococcus faecalis*; (3) *Escherichia coli*; (4) *Klebsiella pneumoniae*; (5) *Proteus mirabilis*; (6) *Pseudomonas aeruginosa*; (7) *Serratia marcescens*; Gram-positive bacteria: (8) *Staphylococcus epidermidis*; Pathogenic *Leptospira* spp. (9) *L. interrogans* sv. Manilae; (10) *L. interrogans* sv. M1352; (11) *L. interrogans* sv. Bratislava; (12) *L. interrogans* sv. Autumnalis; (13) *L. interrogans* sv. Ballum; (14) *L. interrogans* sv. Bataviae; (15) *L. interrogans* sv. Canicola; (16) *L. interrogans* sv. Celledoni; (17) *L. interrogans* sv. Grippotyphosa; (18) *L. interrogans* sv. Javanica; (19) *L. interrogans* sv. Louisiana; (20) *L. interrogans* sv. Panama; (21) *L. interrogans* sv. Pomona; (22) *L. interrogans* sv. Pyrogenes; (23) *L. interrogans* sv. Ranarum; (24) *L. interrogans* sv. Sejroe; (25) *L. interrogans* sv. Shermani; (26) LipL32

Group 2 consisted of four monoclones were F3-10/1G/1, F6-4/9E/1, F6-4/9E/2 and F6-8/6C/1. All MAbs in this group almost reacted with *L. interrogans* serovar Manilae and serovar M1352 and weak reacted with *L. interrogans* serovar Pomona, except MAb no.9 F6-8/6C/1 which strongly reacted with serovar Manilae and weakly reacted with serovars M1352 and Pomona.

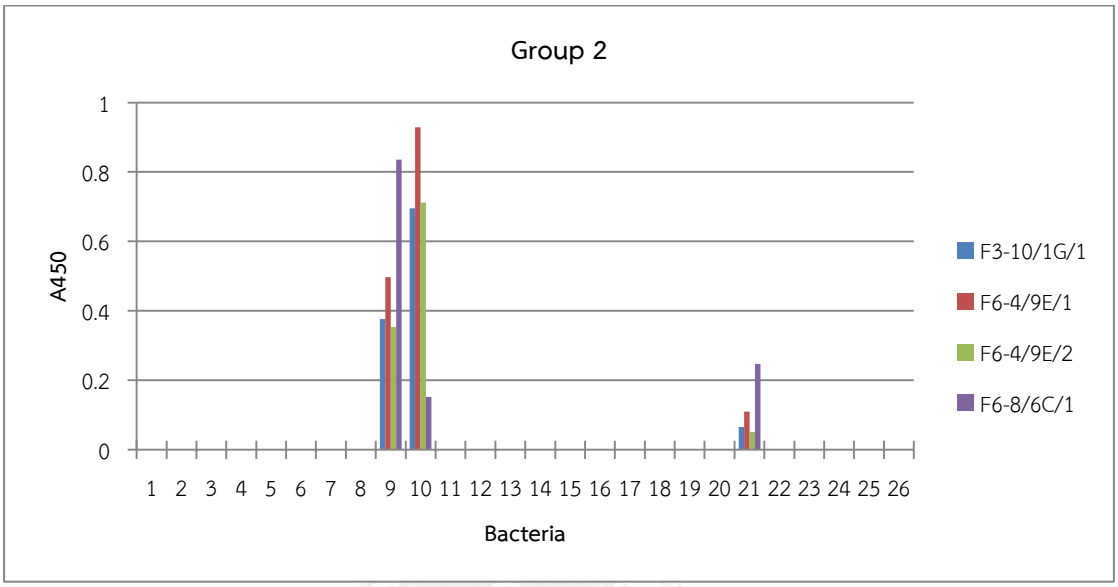

Figure 4.3 The specificity of MAbs (non-dilute) against various sonicated bacteria were determined by indirect ELISA, in group 2.

Gram-negative bacteria: (1) *Enterobacter aerogenes*; (2) *Enterrococcus faecalis*; (3) *Escherichia coli*; (4) *Klebsiella pneumoniae*; (5) *Proteus mirabilis*; (6) *Pseudomonas aeruginosa*; (7) *Serratia marcescens*; Gram-positive bacteria: (8) *Staphylococcus epidermidis*; Pathogenic *Leptospira* spp. (9) *L. interrogans* sv. Manilae; (10) *L. interrogans* sv. M1352; (11) *L. interrogans* sv. Bratislava; (12) *L. interrogans* sv. Autumnalis; (13) *L. interrogans* sv. Ballum; (14) *L. interrogans* sv. Bataviae; (15) *L. interrogans* sv. Canicola; (16) *L. interrogans* sv. Celledoni; (17) *L. interrogans* sv. Grippotyphosa; (18) *L. interrogans* sv. Javanica; (19) *L. interrogans* sv. Louisiana; (20) *L. interrogans* sv. Panama; (21) *L. interrogans* sv. Pomona; (22) *L. interrogans* sv. Pyrogenes; (23) *L. interrogans* sv. Ranarum; (24) *L. interrogans* sv. Sejroe; (25) *L. interrogans* sv. Shermani; (26) LipL32

Group 3 consisted of two monoclones were F3-10/1G/2 and F4-7/1B/1. Both MAbs reacted with *L. interrogans* serovars Manilae and the mutant M1352.

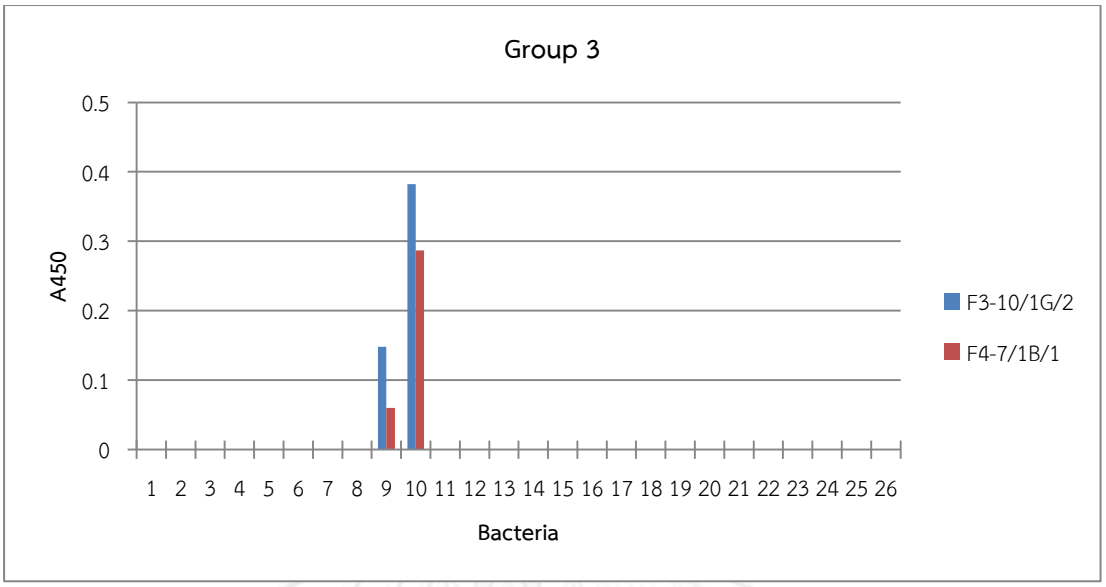

Figure 4.4 The specificity of MAbs (non-dilute) against various sonicated bacteria were determined by indirect ELISA, in group 3.

Gram-negative bacteria: (1) *Enterobacter aerogenes*; (2) *Enterrococcus faecalis*; (3) *Escherichia coli*; (4) *Klebsiella pneumoniae*; (5) *Proteus mirabilis*; (6) *Pseudomonas aeruginosa*; (7) *Serratia marcescens*; Gram-positive bacteria: (8) *Staphylococcus epidermidis*; Pathogenic *Leptospira* spp. (9) *L. interrogans* sv. Manilae; (10) *L. interrogans* sv. M1352; (11) *L. interrogans* sv. Bratislava; (12) *L. interrogans* sv. Autumnalis; (13) *L. interrogans* sv. Ballum; (14) *L. interrogans* sv. Bataviae; (15) *L. interrogans* sv. Canicola; (16) *L. interrogans* sv. Celledoni; (17) *L. interrogans* sv. Grippotyphosa; (18) *L. interrogans* sv. Javanica; (19) *L. interrogans* sv. Louisiana; (20) *L. interrogans* sv. Panama; (21) *L. interrogans* sv. Pomona; (22) *L. interrogans* sv. Pyrogenes; (23) *L. interrogans* sv. Ranarum; (24) *L. interrogans* sv. Sejroe; (25) *L. interrogans* sv. Shermani; (26) LipL32

Group 4 consisted of one monoclone was F7-15/1H/2. This monoclone secreted MAbs which strong reacted with *L. interrogans* serovar Pomona and shown weak reactivity to serovars Sejroe and Canicola.

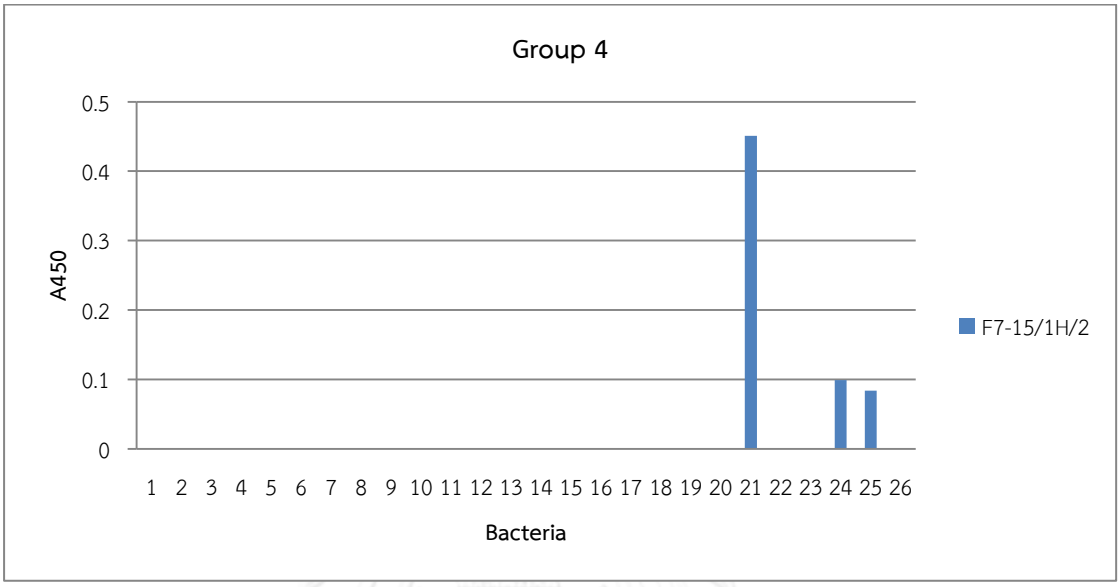

Figure 4.5 The specificity of MAbs (non-dilute) against various sonicated bacteria were determined by indirect ELISA, in group 4

Gram-negative bacteria: (1) *Enterobacter aerogenes*; (2) *Enterrococcus faecalis*; (3) *Escherichia coli*; (4) *Klebsiella pneumoniae*; (5) *Proteus mirabilis*; (6) *Pseudomonas aeruginosa*; (7) *Serratia marcescens*; Gram-positive bacteria: (8) *Staphylococcus epidermidis*; Pathogenic *Leptospira* spp. (9) *L. interrogans* sv. Manilae; (10) *L. interrogans* sv. M1352; (11) *L. interrogans* sv. Bratislava; (12) *L. interrogans* sv. Autumnalis; (13) *L. interrogans* sv. Ballum; (14) *L. interrogans* sv. Bataviae; (15) *L. interrogans* sv. Canicola; (16) *L. interrogans* sv. Celledoni; (17) *L. interrogans* sv. Grippotyphosa; (18) *L. interrogans* sv. Javanica; (19) *L. interrogans* sv. Louisiana; (20) *L. interrogans* sv. Panama; (21) *L. interrogans* sv. Pomona; (22) *L. interrogans* sv. Pyrogenes; (23) *L. interrogans* sv. Ranarum; (24) *L. interrogans* sv. Sejroe; (25) *L. interrogans* sv. Shermani; (26) LipL32

Group 5 consisted of two monoclones were F8-8/2C/3 and F8/2C/4. The MAbs that were secreted from both monoclones most high reacted with *L. interrogans* serovar Pomona and shown cross-reactivity to serovars Manilae, Bratislava, Canicola, Grippotyphosa, Pomona, Sejroe and Shermani. But they also weak reacted with *E. aerogenes*.

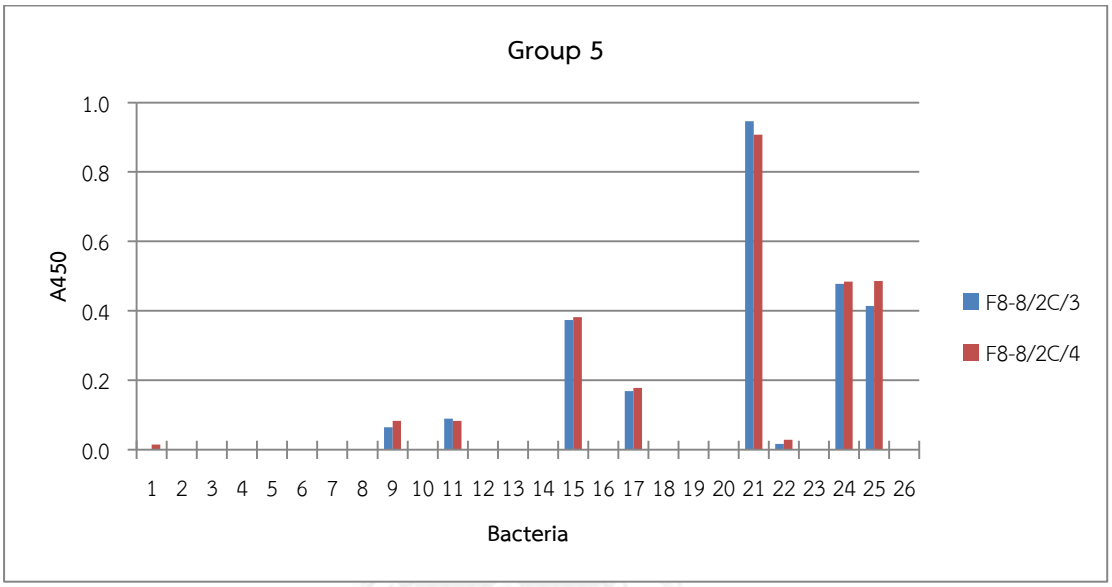

Figure 4.6 The specificity of MAbs (non-dilute) against various sonicated bacteria were determined by indirect ELISA, in group 5.

Gram-negative bacteria: (1) *Enterobacter aerogenes*; (2) *Enterrococcus faecalis*; (3) *Escherichia coli*; (4) *Klebsiella pneumoniae*; (5) *Proteus mirabilis*; (6) *Pseudomonas aeruginosa*; (7) *Serratia marcescens*; Gram-positive bacteria: (8) *Staphylococcus epidermidis*; Pathogenic *Leptospira* spp. (9) *L. interrogans* sv. Manilae; (10) *L. interrogans* sv. M1352; (11) *L. interrogans* sv. Bratislava; (12) *L. interrogans* sv. Autumnalis; (13) *L. interrogans* sv. Ballum; (14) *L. interrogans* sv. Bataviae; (15) *L. interrogans* sv. Canicola; (16) *L. interrogans* sv. Celledoni; (17) *L. interrogans* sv. Grippotyphosa; (18) *L. interrogans* sv. Javanica; (19) *L. interrogans* sv. Louisiana; (20) *L. interrogans* sv. Panama; (21) *L. interrogans* sv. Pomona; (22) *L. interrogans* sv. Pyrogenes; (23) *L. interrogans* sv. Ranarum; (24) *L. interrogans* sv. Sejroe; (25) *L. interrogans* sv. Shermani; (26) LipL32

Group 6 consisted of one monoclone was F8-6/10H/2. Cross-reactivity of this group was similar to group 5, but it not reacted with serovars Grippotyphosa and Pyrogenes.

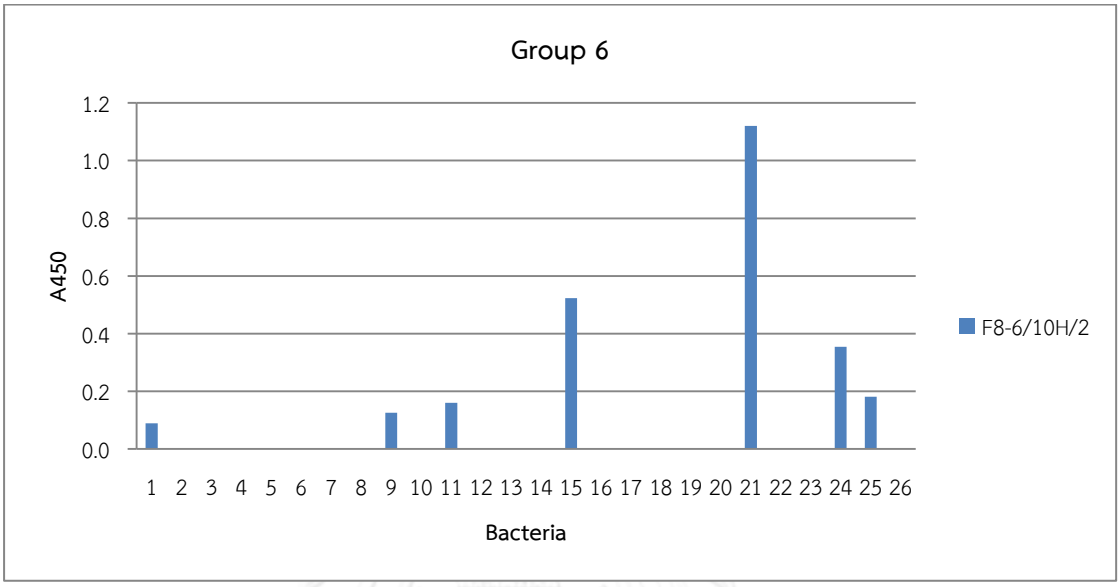

Figure 4.7 The specificity of MAbs (non-dilute) against various sonicated bacteria were determined by indirect ELISA, in group 6

Gram-negative bacteria: (1) *Enterobacter aerogenes*; (2) *Enterrococcus faecalis*; (3) *Escherichia coli*; (4) *Klebsiella pneumoniae*; (5) *Proteus mirabilis*; (6) *Pseudomonas aeruginosa*; (7) *Serratia marcescens*; Gram-positive bacteria: (8) *Staphylococcus epidermidis*; Pathogenic *Leptospira* spp. (9) *L. interrogans* sv. Manilae; (10) *L. interrogans* sv. M1352; (11) *L. interrogans* sv. Bratislava; (12) *L. interrogans* sv. Autumnalis; (13) *L. interrogans* sv. Ballum; (14) *L. interrogans* sv. Bataviae; (15) *L. interrogans* sv. Canicola; (16) *L. interrogans* sv. Celledoni; (17) *L. interrogans* sv. Grippotyphosa; (18) *L. interrogans* sv. Javanica; (19) *L. interrogans* sv. Louisiana; (20) *L. interrogans* sv. Panama; (21) *L. interrogans* sv. Pomona; (22) *L. interrogans* sv. Pyrogenes; (23) *L. interrogans* sv. Ranarum; (24) *L. interrogans* sv. Sejroe; (25) *L. interrogans* sv. Shermani; (26) LipL32

Table 4.3 Groups of monoclonal antibodies

Sonicated bacteria	Groups					
	1	2	3	4	5	6
Enterobacter aerogenes					+	+
Enterrococcus faecalis						
Escherichia coli						
Klebsiella pneumoniae						
Proteus mirabilis						
Pseudomonas aeruginosa						
Serratia marcescens						
Staphylococcus epidermidis						
L. interrogans sv. Manilae		+	+		+	+
L. interrogans sv. M1352	+	+	+			
L. interrogans sv. Bratislava					+	+
L. interrogans sv. Autumnalis						
L. interrogans sv. Ballum						
L. interrogans sv. Bataviae						
L. interrogans sv. Canicola					+	+
L. interrogans sv. Celledoni						
L. interrogans sv. Grippotyphosa					+	
L. interrogans sv. Javanica						
L. interrogans sv. Louisiana						
L. interrogans sv. Panama						
L. interrogans sv. Pomona		+		+	+	+
L. interrogans sv. Pyrogenes					+	+
L. interrogans sv. Ranarum						
L. interrogans sv. Sejroe				+	+	+
L. interrogans sv. Shermani				+	+	+
LipL32						

+ = MAb reacted against sonicated bacteria

4.3.2 Sensitivity

The sensitivity of fifteen monoclones with non-diluted antibodies was determined by the indirect ELISA in terms of EC50 and LOD values, the results were shown in Table 4.4. The EC50 values were quantified in the range of 2.08×10^{6} to 9.97×10^{6} cells/ml and the LODs were between 4.73×10^{5} and 3.51×10^{6} cells/ml.

Table 4.4 The EC50 and LOD values of fifteen MAbs

No.	Monoclone	EC50	LOD	R-square
1	F2-5/2E/1	5.83×10^{6}	2.42×10^{6}	0.96
2	F3-10/1G/1	7.74×10^{6}	1.86×10^{6}	0.96
3	F3-10/1G/2	5.86×10^{6}	1.52×10^{6}	0.95
4	F4-7/1B/1	5.21×10^{6}	2.18×10^{6}	0.96
5	F4-8/12D/1	6.94×10^{6}	3.22×10^{6}	0.98
6	F4-5/10A/1	6.87×10^{6}	3.51×10^{6}	0.96
7	F6-4/9E/1	7.53×10^{6}	1.64×10^{6}	0.96
8	F6-4/9E/2	7.71×10^{6}	2.01×10^{6}	0.95
9	F6-8/6C/1	9.97×10^{6}	3.27×10^{6}	0.97
10	F6-5/11H/1	2.25×10^{6}	4.73×10^{5}	0.73
11	F7-15/1H/2	3.71×10^{6}	6.95×10^{5}	0.92
12	F8-13/3B/3	5.07×10^{6}	2.19×10^{6}	0.96
13	F8-8/2C/3	2.25×10^{6}	7.02×10^{5}	0.93
14	F8-8/2C/4	2.08×10^{6}	6.19×10^{5}	0.93
15	F8-6/10H/2	3.06×10^{6}	7.73×10^{5}	0.97

4.3.3 Isotyping determination of MAbs

Isotyping of fifteen MAbs was identified by Sigma-Aldrich's mouse monoclonal antibody isotyping kit which base on principle of sandwich ELISA. The

absorbance values from the ELISA were measured at 450 nm and shown isotype class of each MAbs in Table 4.5, as bold number. The results indicated that the isotype of all MAbs was IgM, excepted for MAbs that were secreted from monoclone no. F6-5/11H/1 was indicated as class of IgG3 and IgM.

Table 4.5 The isotype of fifteen MAbs specific for IgG1, IgG2a, IgG2b, IgG3, IgM and IgA

No.	Monoclone	A450					
		IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
1	F2-5/2E/1	0.069	0.135	0.075	0.079	**0.713**	0.078
2	F3-10/1G/1	0.080	0.155	0.086	0.604	**1.919**	0.114
3	F3-10/1G/2	0.077	0.133	0.072	0.277	**0.737**	0.083
4	F4-7/1B/1	0.067	0.129	0.074	0.076	**1.214**	0.078
5	F4-8/12D/1	0.071	0.145	0.069	0.090	**1.346**	0.090
6	F4-5/10A/1	0.075	0.151	0.072	0.077	**0.985**	0.081
7	F6-4/9E/1	0.076	0.154	0.07	0.072	**1.687**	0.081
8	F6-4/9E/2	0.164	0.156	0.084	0.077	**2.075**	0.090
9	F6-8/6C/1	0.076	0.153	0.076	0.090	**2.566**	0.109
10	F6-5/11H/1	0.128	0.197	0.085	**1.806**	**1.949**	0.222
11	F7-15/1H/2	0.091	0.156	0.08	0.755	**1.676**	0.137
12	F8-13/3B/3	0.086	0.191	0.089	0.102	**2.630**	0.103
13	F8-8/2C/3	0.068	0.139	0.077	0.184	**2.208**	0.103
14	F8-8/2C/4	0.067	0.134	0.073	0.179	**2.088**	0.104
15	F8-6/10H/2	0.073	0.134	0.067	0.068	**0.998**	0.074

4.3.4 Antigen recognition by Western blotting

Antigen of *L. interrogans* serovar Manilae recognized by MAbs were determined by Western blotting. After antigens separation by one-well SDS-PAGE using Mini-PROTEAN® II Multiscreen Apparatus, the antigens were probed by MAbs obtained from eight fusions. In Figure 4.8, the nitrocellulose membrane was probed with fifteen non-diluted MAbs and 1:8000 dilution of Goat Anti-Mouse IgM-HRP

secondary antibody. The result showed that four MAbs from fifteen MAbs were able to detect antigens on the membrane. The MAbs secreted from clone F6-4/9E/1 (lane 9) and F6-4/9E/2 (lane 10) reacted to protein band of 42.9 kDa when calculated molecular weight from stand curve of marker (Appendix F, Figure F1). And from clone F6-5/11H/1 (lane 12) and F7-15/1H/2 (lane 13) reacted with a smear ladder-like pattern bands.

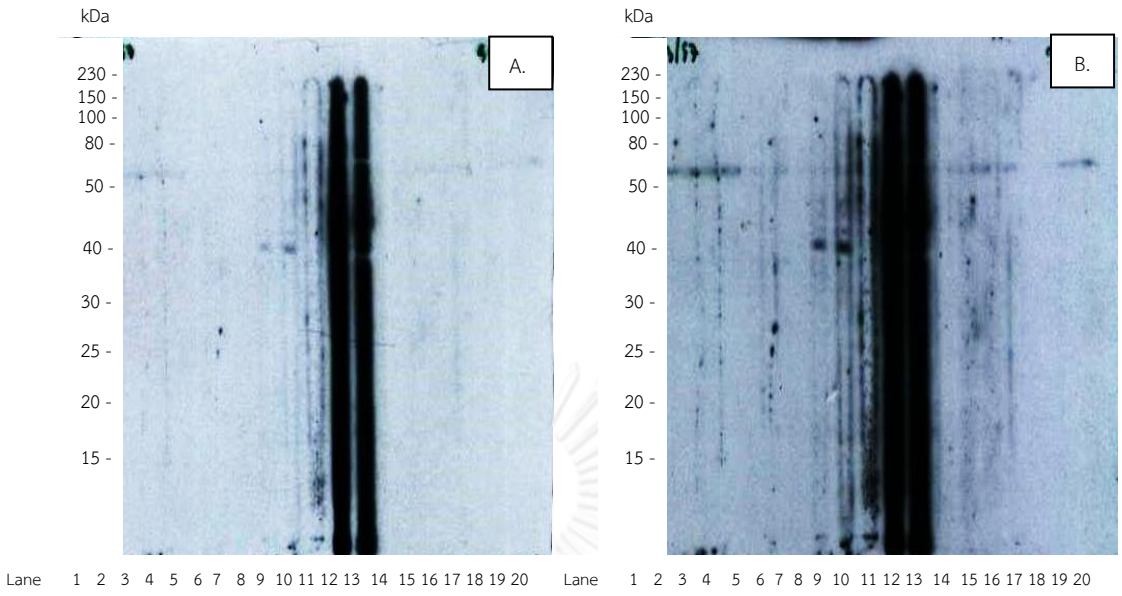

Figure 4.8 Westernblot analysis for the specific of MAbs against separating protein of *L. interrogans* serovar Manilae with mutiscreen apparatus.

A. exposed to X-ray film for 5 min, B. exposed to X-ray film for 30 min

Lane 1: Marker, Lane 2: Marker,

Lane 3: F2-5/2E/1, Lane 4: F3-10/1G/1,

Lane 5: F3-10/1G/2, Lane 6: F4-7/1B/1,

Lane 7: F4-8/12D/1, Lane 8: F4-5/10A/1,

Lane 9: F6-4/9E/1, Lane 10: F6-4/9E/2,

Lane 11: F6-8/6C/1, Lane 12: F6-5/11H/1,

Lane 13: F7-15/1H/2, Lane 14: F8-13/3B/3,

Lane 15: F8-8/2C/3, Lane 16: F8-8/2C/4,

Lane 17: F8-6/10H/2,

Lane 18: Polyclonal antibody against *L. interrogans* serovar Manilae from F6, dilution of 1:500,

Lane 19: Polyclonal antibody against LipL32, dilution of 10,000,

Lane 20: Culture medium of myeloma cell, non-dilute

And then, the results of these four MAbs were confirmed by conventional ten-well SDS-PAGE followed by Western blotting. Two MAbs that reacted to single-band protein in the previous multiscreen test, clone F6-4/9E/1 and F6-4/9E/2, were undiluted whereas another two MAbs which gave smear bands, clone F6-5/11H/1 and F7-15/1H/2, were used at the dilution of 1:100 and 1:200 to reduce the background. The results were shown at a larger scale of detection than those of multiscreen test. In Figure 4.9, F6-4/9E/1 weakly reacted to 42.8 kDa band protein and smear band protein above. F6-4/9E/2 was reacted strongly to the band protein of 42.8 kDa and reacted weakly to the band protein of 12.7 kDa. The molecular weights were calculated from standard curve of marker in Appendix F, Figure F2. However, when F6-5/11H/1 and F7-15/1H/2 were diluted, they could not detect any band. The positive result of bands was subtracted from the background of negative-control in lane 10.

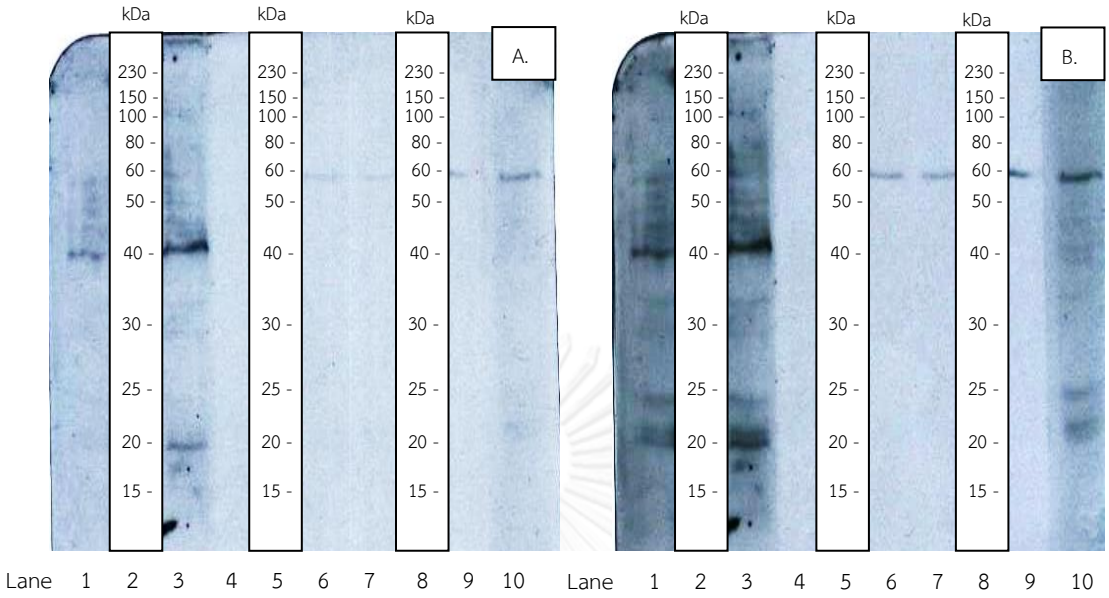

Figure 4.9 Western blot analysis for the specific of 4 MAbs against separating protein of *L. interrogans* serovar Manilae.

A. exposed to X-ray film for 5 min, B. exposed to X-ray film for 30 min

Lane 1: F6-4/9E/1,

Lane 2: Marker,

Lane 3: F6-4/9E/2,

Lane 4: F6-5/11H/1, dilution of 1:100,

Lane 5: Marker,

Lane 6: F6-5/11H/1, dilution of 1:200,

Lane 7: F7-15/1H/2, dilution of 1:100,

Lane 8: Marker,

Lane 9: F7-15/1H/2, dilution of 1:200,

Lane 10: Culture medium of myeloma cell, non-dilute

CHAPTER V
DISCUSSION

Productions of monoclonal antibodies against pathogenic *Leptospira* spp. were performed by conventional cell fusion between NS1 or P3X myeloma cells and spleen cells of the immunized mice. Five mice were immunized with either fixed form of *L. interrogans* serovar Manilae or *L. interrogans* serovar M1352. In general, immune system of the infected patient responses to the infection by secreting antibodies against antigens presented on the outer membrane of the pathogenic *Leptospira*. Serovar Manilae was used in this study because it is a new pathogenic serovar which has never been used in the MAbs production. While the mutant M1352 (LPS mutant) was selected in order to obtain antibodies that can recognize new antigens other than LPS since the LPS on the outer membrane was reduced (Bengoechea, Najdenski, & Skurnik, 2004; Morona & Bosch, 2003).

After immunization, the antibody titers of the immunized mice were found in the range of 1:4,000 to 16,000. Preliminary characterization of the obtained MAbs indicated that most of the MAbs were serovar-specific, especially those obtained by using the mutant M1352 in the immunization. Unlike the first five mice, another three mice were immunized with a mixed form of fixed and sonicated *L. interrogans* serovar Manilae. The mixed form of antigen was used to increase the possibility of obtaining broadly reactive MAbs. This was based on the reason that the mixed form consists of antigens from both outer membrane and inner cell components. Moreover, to increase the antibody titer, these three mice were immunized at longer period than the first group. The isotype of most obtained MAbs were identified as IgM but there was a MAb that gave positive result as both IgM and IgG3. This could be explained by the reason that it was not synthesized from a monoclone, or the other word it was not a MAb.

The obtained antibodies from eight fusions could be divided into 6 groups based on their specificities to the sonicated pathogenic *Leptospira* and other tested bacteria which could be found in samples or environments. The first group of MAbs

was specific only to the mutant M1352. These MAbs were mostly obtained from the fusion of the mouse immunized with the fixed form of serovar M1352. The second group of MAbs reacted to serovars Manilae, M1352 and Pomona. Most of them were obtained from the mouse immunized with the mixed form of serovar Manilae. The third group of MAbs was specific to both serovars Manilae and M1352. The MAbs in this group were obtained from mice (fusion 3 and 4) immunized with the fixed form of serovar Manilae and M1352, respectively. These indicated that antigens on the cell surface of both serovars are quite similar. It has been reported that the molecular mass of the Manilae wild-type and the LPS mutant (serovar M1352) are not significantly different (Murray et al., 2010). Therefore, MAbs in this group might be induced by common antigens which are not the LPS. MAbs in group 4, 5 and 6 (fusion 7 and 8) were obtained when the mixed form of serovar Manilae was ued. The forth group, MAbs reacted to serovars Pomona, Sejroe and Shermani while group 5 and 6 MAbs recognized various tested pathogenic leptospires and showed weakly cross-reactivity to *E. aerogenes*. These results indicated that the MAbs obtained when the mixed form of antigen was used are more generic to various pathogenic *Leptospira* than those obtained by using the fixed form. This could be due to the reason that preparation of the antigen by sonication results in a more common group of antigens found among the pathogenic *Leptospira.* It has been reported that MAbs obtained by using the sonicated form of *Leptospira* can react to either proteins of all pathogenic leptospires or proteins of all leptospires (Saengjaruk et al., 2002).

In addition, it has been known that LPS or lipopolysaccharide is the main component of the outer membrane of Gram-negative bacteria. It is composed of O polysaccharide side chain antigen, endotoxic lipid A anchor and a conserved core oligosaccharide (Raetz & Whitfield, 2002). LPS binds the CD14/TLR4/MD2 receptor complex in many cell types such as B-cells, monocytes and macrophage which promotes the secretion of pro-inflammatory cytokine (Abbas, Lichtman, & Pillai, 2012). Previous studies on LPS stimulation in mice reported that the spleen cells of LPS immunized mice preferentially secrete antibodies class of IgM and IgG3 (AK Singh & Jiang, 2003; Won & Kearney, 2002). In our study, the isotype of all obtained MAbs were identified as IgM and some MAbs were serovar-specific. These suggested that

leptospiral LPS may be one of major antigens that induce MAb production in our study. Furthermore, difference in specificity of the obtained MAbs might be due to the difference in the sugar composition and orientation of the LPS (Adler & de la Peña Moctezuma, 2010; Faine et al., 1999).

Two MAbs, F6-4/9E/1 and 4/9E/2, were shown to recognize a protein band of approximately 43 kDa which was calculated from the standard curve (Appendix F, Figure F1 and F2) or approximately 41 kDa as observed by the naked eyes. This finding is consistent with previous studies showing that IgM from leptospirosis patients reacted to p41/42 antigen (Doungchawee et al., 2008; Natarajaseenivasan, Vijayachari, Sugunan, Sharma, & Sehgal, 2004) which appears to be two proteins. One is the outer membrane lipoprotein, LipL41 (Shang, Summers, & Haake, 1996) and the other one is a 42-kDa inner membrane protein (Guerreiro et al., 2001). However, amino acid sequence analysis is required to determine if our MAbs recognize these two proteins. In addition, two MAbs obtained from mice (fusion 6 and 7), immunized with the mixed form, strongly reacted to the smear bands (Figure 4.8). The smear (ladder-like) pattern suggested that the MAbs may react to multiple isoforms or antigens with various MW such as LPS, or bind non-specifically to antigens on the blot. To test whether the nature of antigens recognized by these MAbs is protein, the whole-cell lysate should be treated with proteinase K before Western blotting analysis (Doungchawee et al., 2008). If the MAbs do not recognize the treated antigens, it suggests that they react to some protein antigens. On the contrary, if MAbs recognize to the treated antigen, it suggest that they possibly react to LPS, saccharide or some proteins which are not digested by proteinase K.

In this study, fixed and mixed form (fixed and sonicated) of *L. interrogans* serovar Manilae and the mutant M1352 were used to immunize mice. The MAbs that recognize specific epitope of carbohydrate on LPS may be useful for serotype-specific-detection of *Leptospira* (Gaudart et al., 2005). The MAbs that are broadly reactive but specific to leptospiral antigens may be used in screening tests for differential diagnosis of leptospirosis from other bacterial infections. Since, the obtained MAbs in group 5 and 6 cross-reacted to other bacteria besides pathogenic

leptospires, there were not suitable for leptospirosis detection. On the contrary, MAbs of group 2, 3 and 4 were specific to some of the tested pathogenic *Leptospira*, they were suitable for the detection of leptospires. In addition, they should be combined as a mix MAbs to increase their capability to detect various types of leptospires.

In case of sensitivity, preliminary study showed that the sensitivity of the obtained MAbs was in the range of 2×10^6 and 1×10^7 cells/ml. However, the leptospires that can be found in blood and urine are about 10^6 to 10^7 and 10^2 to 10^4 cells/ml, respectively. Therefore, use of purified MAb and other detection methods such as gold nanoparticle and fluorescence are recommended to improve the sensitivity in leptospirosis detection (Widiyanti et al., 2013).

CHAPTER VI
CONCLUSION

1. Fourteen monoclonal antibodies were obtained from eight fusions of NS1 or P3X myeloma cells and spleen cells of immunized mice. Eight mice were immunized by *L. interrogans* serovar Manilae and serovar M1352 with fixed form or mixed of fixed and sonicated form. These MAbs were divided by indirect ELISA into 6 groups according to their specificity to pathogenic *Leptospira* spp. and other bacteria that maybe contaminated in urine or environment.
2. MAbs in group 1, 2, 3 and 4 were specific to some pathogenic serovars of *Leptospira* spp.
3. MAbs in group 5 and 6 were able to detect more serovars of pathogenic *Leptospira* spp. than group 1, 2, 3 and 4, but they showed weakly cross-reactivity to other bacteria such as *E. aerogenes*.
4. The sensitivity by the half maximal effective concentration (EC50) and limit of detection (LOD) by individual MAb was in the range of 2.08×10^{6} to 9.97×10^{6} and 4.73×10^{5} to 3.51×10^{6}, respectively, which detected by indirect ELISA.
5. The classes of MAbs obtained from this study were IgM.
6. Four MAbs were shown to recognize leptospiral antigens by Western blotting. Two of four MAbs reacted to 41 kDa, and the other two MAbs reacted to smear-band antigens of undetermined nature of *L. interrogans* serovar Manilae.

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APPENDICES

APPENDIX A

Culture media for bacterial growth

1. EMJH media

1.1 Albumin fatty acid supplement stock solution

$CaCl_2$+$MgCl_2$·6H2O	0.076	g	Store at -20 °C
$ZnSO_4$·7H2O	0.04	g	Store at -20 °C
$CuSO_4$·5H2O	0.03	g	Store at -20 °C
Vitamin B12	0.002	g	Store at -20 °C
Tween 80	1	g	Store at -20 °C
Glycerol	1	g	Store at -20 °C

Each reagent was separately dissolved in 10 ml of distilled water.

1.2 Albumin fatty acid supplement solution, ready to use (50 ml)

BSA	5	g
$CaCl_2$+$MgCl_2$·6H2O	0.75	ml
$ZnSO_4$·7H2O	0.5	ml
$CuSO_4$·5H2O	0.05	ml
Vitamin B12	0.5	ml
Tween 80	6.25	ml
Glycerol	0.5	ml
FeSO4	0.025	g
Sodium pyruvate	0.02	g

The reagents were dissolved in distilled water, adjusted pH to 7.4-7.6 with HCl (conc.) and adjusted total volume to 50 ml using distilled water. The solution was sterilized by filtration and store at -20 °C.

1.3 Basal media

Bacto *Leptospira* Media Base EMJH dehydrated	0.23	g

The medium was dissolved and adjusted volume to 90 ml with distilled water. The solution was sterilized by autoclaving at 121 °C for 15 min.

1.4 EMJH media

Basal media	90	ml
Albumin fatty acid supplement solution	10	ml

The solution was mixed well and store at 4 °C.

2. LB broth

Tryptone	10	g
Yeast extracts	5	g
Nacl	5	g

The reagents were dissolved and adjusted volume to 1000 ml with distilled water. The solution was sterilized by autoclaving at 121 °C for 15 min.

APPENDIX B

Reagents

1. 2X treatment buffer (10 ml)

1 M Tris-Cl, pH 6.8	1.25	ml
10% SDS	4	ml
Glyceral (87%)	2.29	ml
2-mercaptoethanol	1	ml

The reagents were dissolved in distilled water and adjusted total volume to 10 ml.

2. 1 N HCl

HCl (37%)	8.28	ml

The reagents were dissolved in distilled water and adjusted total volume to 100 ml.

3. 1 N NaOH

NaOH	4	g

The reagents were dissolved in distilled water and adjusted total volume to 100 ml.

4. 0.85% Nacl

Nacl	8.5	g

The reagent was dissolved in distilled water and adjusted total volume to 1000 ml.

APPENDIX C

Media and reagents for hybridoma production

1. Stock 100X HT

Hypoxanthine	0.1360 g	in 20 ml distilled water
Thymidine	0.0388 g	in 20 ml distilled water

The solutions were dissolved and adjusted total volume to 100 ml using distilled water. Then, the solution was divided into aliquots and stored at -20 °C before use.

2. Stock 100X HAT

Hypoxanthine	0.1360 g	in 20 ml distilled water
Aminopterin	0.0018 g	in 20 ml distilled water
Thymidine	0.0388 g	in 20 ml distilled water

The solutions were dissolved and adjusted total volume to 100 ml using distilled water. Then, the solution was divided into aliquots and stored at -20 °C before use.

3. RPMI 1640 medium

RPMI 1640 (Roswell Park Memorial Institute)	10.43	g
NaHCO3	2	g
L-glutamine	0.1	g
Glucose	2	g
Sodium pyruvate	0.11	g
Distilled water	1000	ml

The solution of penicillin G and streptomycin were added to a final concentration of 100,000 units and 100 mg per liter, respectively. The medium was sterilized by Millipore membrane (pore size 0.22 μm) filtration and stored at 4 °C.

4. HT medium

RPMI 1640 medium	1000	ml
100X HT	10	ml

The medium was mixed well and sterilized by Millipore membrane (pore size 0.22 μm) filtration and stored at 4 °C.

5. HAT medium

RPMI 1640 medium	1000	ml
100X HAT	10	ml

The medium was mixed well and sterilized by Millipore membrane (pore size 0.22 μm) filtration and stored at 4 °C.

6. 50% (w/v) polyethylene glycol (PEG)

10 g of PEG was thawed in a 60 °C water bath and dissolved in 10 ml of RPMI 1640 medium. The solution was divided into aliquots with the volume of 1 ml and stored at 4 °C. The aliquot of PEG solution was placed in a humidified 5% CO_2 incubator at 37 °C before use.

7. Freezing medium (10% DMSO)

Dimethyl sulfoxide (DMSO)	10	ml
RPMI 1640 medium	90	ml

The medium was stored at 4 °C before use.

APPENDIX D

Buffers and reagents for SDS-PAGE and Western blotting

1. 10% SDS

Sodium dodecyl sulfate (SDS)	10	g

The reagents were dissolved in deionized water and adjusted total volume to 100 ml.

2. 10% APS

Ammonium persulfate (APS)	1	g

The reagents were dissolved in deionized water and adjusted total volume to 10 ml.

3. 1 M Tris-HCl, pH 6.8

Trisma base	12.11	g

The small volume of deionized water was added and adjusted pH to 6.8 with 1 N HCl. Then deionized water was added to reach 100 ml final volume.

4. 1.5 M Tris-HCl, pH 8.8

Trisma base	18.17	g

The small volume of deionized water was added and adjusted pH to 8.8 with 1 N HCl. Then deionized water was added to reach 100 ml final volume.

5. 2X Laemmli buffer (SDS-dye) (10 ml)

1 M Tris-HCl, pH 6.8	1	ml
10% SDS	4	ml
Glycerol (87%)	2.29	ml

Bromphenol blue	0.001	g
HPLC water adjusted volume to	10	ml

The solution was stored at -20 °C.

6. SDS staining dye

2X Laemmli buffer (SDS-dye)	900	µl
2-mercaptoethanol	100	µl

The solution was stored at -20 °C.

7. SDS-polyacrylamide gel preparation

7.1 12% separating gel (8 ml)

Sterile water	3.436	ml
40% Acrylamide and Bis-acrylamide solution	2.4	ml
1.5 M Tris-HCl, pH 8.8	2	ml
10% SDS	0.08	ml
10% APS	0.08	ml
TEMED	0.004	ml

7.2 5% stacking gel (2 ml)

Sterile water	1.204	ml
40% Acrylamide and Bis-acrylamide solution	0.25	ml
1 M Tris-HCl, pH 6.8	0.504	ml
10% SDS	0.02	ml

10% APS	0.02	ml
TEMED	0.002	ml

8. 5X running buffer for SDS-PAGE

Trisma base	15.1	g
Glycine	94	g
SDS	5	g
Deionized water	1000	ml

For working solution, 1X running buffer was prepared by diluting 100 ml of 5X running buffer to total volume 500 ml using deionized water.

9. Transfer buffer for Western blotting

Trisma base	5.08	g
Glycine	2.9	g
SDS	0.37	g
Deionized water	800	ml
Absolute methanol	200	ml

The buffer was stored at 4 °C until use.

APPENDIX E

Buffers and reagents for immunoassay

1. 0.15 M Phosphate buffer saline (PBS), pH 7.4

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g

The reagents were dissolved in distilled water and adjusted pH to 7.4. Then distilled water was added to reach 100 ml final volume.

2. 0.05% Tween 20 in PBS (PBST)

Tween 20	500	µl
PBS	1000	ml

3. 5% skim milk

Skim milk	5	g
PBS	100	ml

4. 3% skim milk

Skim milk	3	g
PBS	100	ml

5. 3% skim milk in PBST

Skim milk	3	g
PBST	100	ml

6. 0.15 M Phosphate Citrate buffer, pH 5.0

Na_2HPO_4	9.5	g
Citric acid	7.3	g
Distilled water adjusted volume to	1000	ml

The buffer was adjusted pH to 5.0 and stored at 4 °C in a dark bottle before use.

7. TMB substrate solution

Tetramethylbenzidine (TMB)	3	mg
Dimethyl sulfoxide (DMSO)	0.3	ml
30% H_2O_2	0.0034	ml
0.15 M Phosphate citrate buffer	9.9	ml

The substrate solution was freshly prepared in a dark bottle before use.

8. 1 M H_2SO_4 (stopping solution)

H_2SO_4 (96%)	55.53	ml
Distilled water adjusted volume to	1000	ml

APPENDIX F

Molecular weight determination

The relative mobility (Rf) of the unknown was calculated according to the following formula:

$$\text{Relative mobility } (\text{Rf}) = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

Table F1 The molecular weight and relative mobility of protein markers (1)

Molecular weight (kDa)	Relative mobility (Rf)
80	0.20
60	0.25
50	0.32
40	0.41
30	0.52
25	0.63
20	0.72
15	0.81

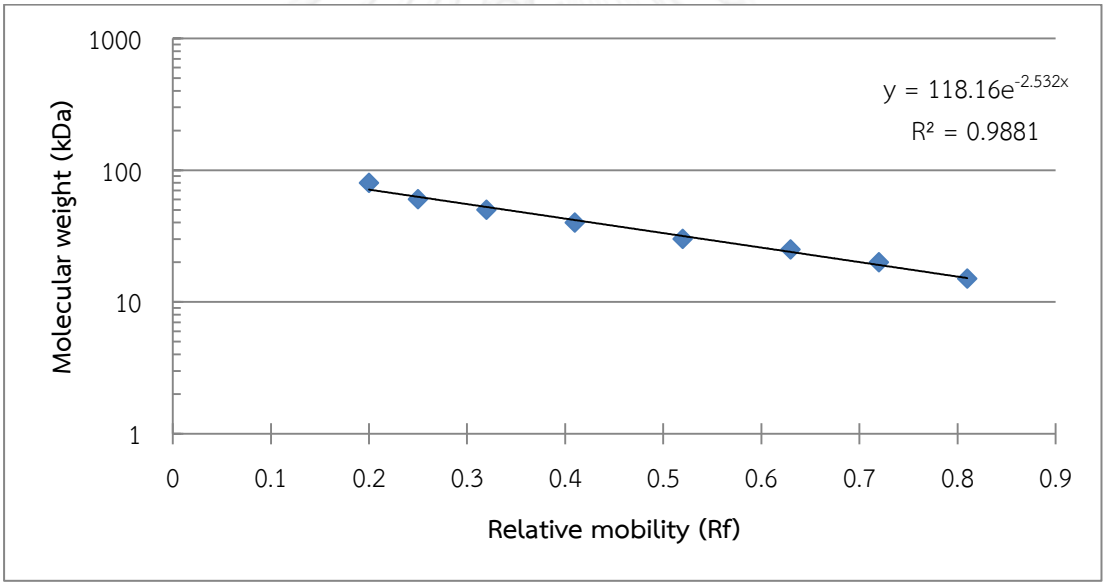

Figure F1 Standard curve of protein marker used for molecular weight determination (1).

Table F2 The molecular weight and relative mobility of protein markers (2)

Molecular weight (kDa)	Relative mobility (Rf)
80	0.18
60	0.24
50	0.30
40	0.41
30	0.53
25	0.66
20	0.75
15	0.85

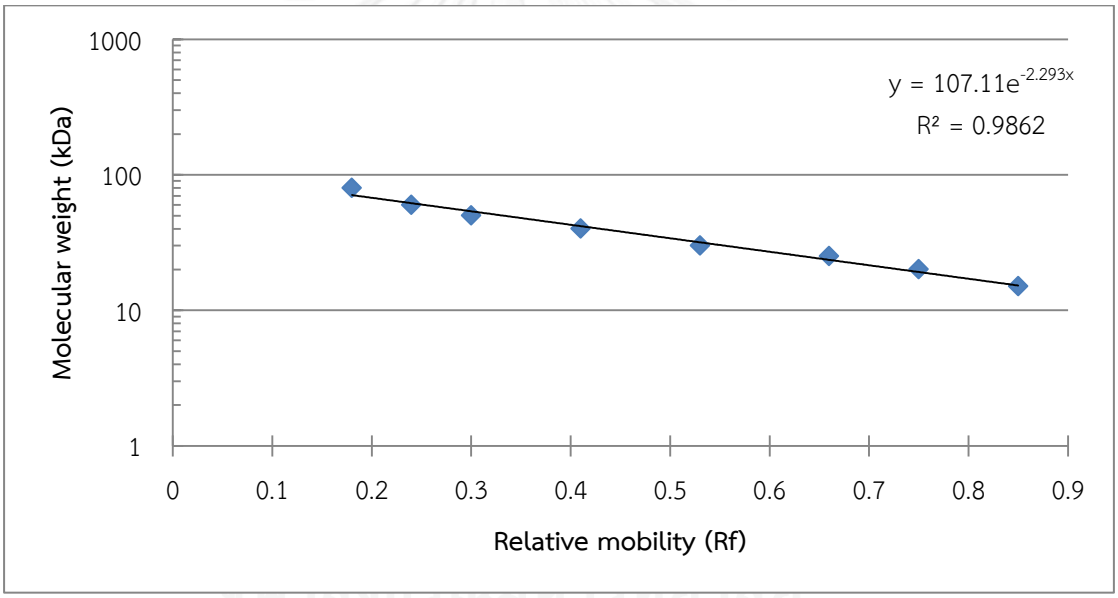

Figure F1 Standard curve of protein marker used for molecular weight determination (2).

VITA

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