DEVELOPMENT OF MONOCLONAL ANTIBODY-BASED LATEX AGGLUTINATION TEST FOR THE RAPID SEROGROUP IDENTIFICATION OF LEPTOSPIRES

UTANE RUNGPANICH

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (IMMUNOLOGY)

FACULTY OF GRADUATE STUDIES

MAHIDOL UNIVERSITY

2007

Thesis

entitled

DEVELOPMENT OF MONOCLONAL ANTIBODY-BASED LATEX AGGLUTINATION TEST FOR THE RAPID SEROGROUP IDENTIFICATION OF LEPTOSPIRES

……..
Mr. Utane Rungpanich
Candidate

……..
Assoc. Prof. Pattama Ekpo, Ph.D.
Major Advisor

……...
Prof. Yupin Suputtamongkol, M.D.,M.Sc.
Co-advisor

……....
Assist. Prof. Amornrut Leelaporn, Ph.D.
Co-advisor

……
Prof.M.R.Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies

……
Prof.Kovit Pattanapanyasat, Ph.D.
Chair
Master of Science Programmee in Immunology
Faculty of Medicine Siriraj Hospital

Thesis

entitled

DEVELOPMENT OF MONOCLONAL ANTIBODY-BASED LATEX AGGLUTINATION TEST FOR THE RAPID SEROGROUP IDENTIFICATION OF LEPTOSPIRES

was submitted to the Faculty of Graduate Student Studies, Mahidol University
for the degree of Master of Science (Immunology)
On
May 16, 2007

……..
Mr. Utane Rungpanich
Candidate

……..
Assoc.Prof. Chintana Chirathaworn, ph.D.
Chair

……..
Assoc. Prof. Pattama Ekpo, Ph.D.
Member

……
Dr. Stuart Blacksell, Ph.D.
Member

……..
Assist.Prof. Amornrut Leelaporn, Ph.D.
Member

……
Prof.M.R.Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

……..
Clin.Prof.Piyasakol Sakolsatayadorn
M.D., FRCST
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

ACKNOWLEDGEMENTS

I would like to express to my deep appreciation to my kind advisor, Assoc. Prof. Pattama Ekpo, for her expression, valuable advices, guidance, supervision, encouragement throughout the study and preparation of this thesis.

I also express my appreciation to my co-advisor, Prof. Yupin Suputtamongkol from Department of Medicine and Assist. Prof. Amornrut Leelaporn from Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, for their supervision, valuable advice, suggestion and encouragement in this thesis. My appreciation is also extended to both examiners, Assoc. Prof. Chintana Chirathaworn from Faculty of Medicine, Chulalongkorn University, Thailand and Dr. Stuart Blacksell from Mahidol-Oxford Tropical Medicine Research Unit, Thailand.

I would like to thank Dr. Duangjai Suwancharoen from the National Institute of Animal Health (NIAH), Department of Livestock Development, Ministry of Agricultural and Cooperatives, Thailand, Mrs. Pimjai Naigovit from The National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand, for providing of leptospires., Dr. Duangporn Phulsuksombati from Royal Thai Army-Armed Forces Research Institute of Medical Sciences (RTA-AFRIMS), Thailand, for culture isolation of leptospires from rats' kidneys, Mrs. Vanaporn Wuthiekanun from Mahidol-Oxford Tropical Medicine Research Unit, Thailand, for culture isolation of leptospires from patients' blood samples, and Dr. Lee Smythe from WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Center for Public Health Science, Queensland, Australia for identifying leptospires by CAAT.

I would like to express my sincere thank to Prof. Suttipant Sarasombath and Prof. Napatawn Banchuin for their kindness and permission for this study, and all laboratory staffs at the Department of Immunology, Faculty of Medicine Siriraj Hospital for their friendship and also special thank to Ms. Nujohn Junsong and Ms. Narintorn Gaudart for their kindly help. I would like to thanks Mrs. Surapee Tiengrim and the staffs at the Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University for their helpfulness.

Finally, great appreciation is especially to my family for their loves, morale and encouragement throughout my study.

Utane Rungpanich

DEVELOPMENT OF MONOCLONAL ANTIBODY-BASED LATEX AGGLUTINATION TEST FOR THE RAPID SEROGROUP IDENTIFICATION OF LEPTOSPIRES

UTANE RUNGPANICH 4536753 SIIM/M

M.Sc. (IMMUNOLOGY)

THESIS ADVISORS: PATTAMA EKPO, Ph.D., AMORNRUT LEERAPORN, Ph.D., YUPIN SUPUTTAMONGKOL, M.D., M.Sc.

ABSTRACT

Leptospirosis is caused by spirochete bacteria of the genus *Leptospira* that includes a large number of species. The disease has caused a major public health problem in Thailand. The annual reported cases have been increasing in Thailand since 1996. The source of infection in humans and animals has been studied by identification of Leptospires isolated from patient and animal specimens by the reference serological method, cross agglutination absorption test (CAAT). However, this method is laborious. Therefore, this study attempted to develop a rapid serological test for identification of leptospires into serogroups.

A monoclonal antibody-based latex agglutination test (LAT) was previously developed. Sixteen clones of in-house monoclonal antibodies specific to 15 serogroups of pathogenic *Leptospira interrogans* which were prevalent in Thailand during the past ten years and a non-pathogenic *Leptospira biflexa* were sensitized onto latex particles. The latex agglutination was performed for only 5 minutes. Evaluation of the panel latex reagents was evaluated with 125 leptospires isolated from rats' kidneys and 113 leptospires isolated from patients' blood samples. The identity of serogroups/serovars of these isolates was compared with the CAAT method. The results showed that latex agglutination gave 100% correlation with CAAT method (Kappa value = 1.00) when leptospires isolated from rats' kidneys were identified. However, the established latex reagents gave 99.11% of specificity and 100% of sensitivity compared with CAAT method (Kappa value = 0.99) when leptospires isolated from patients' blood samples were identified. These established panel latex reagents are very useful for rapid identification of leptospiral serogroups.

KEY WORDS: LEPTOSPIROSIS/*LEPTOSPIRA*/ IDENTIFICATION/ MONOCLONAL ANTIBODY/LATEX AGGLUTINATION

78 pp.

การพัฒนาชุดการตรวจวินิจฉัยจุลชีพ *Leptospira* อย่างรวดเร็วโดยการจับกลุ่มของเม็ดลาเท็กซ์ที่เคลือบด้วยโมโนโคลนัลแอนติบอดี (DEVELOPMENT OF MONOCLONAL ANTIBODY-BASED LATEX AGGLUTINATION TEST FOR THE RAPID SEROGROUP IDENTIFICATION OF LEPTOSPIRES)

อุเทน รุ่งพาณิชย์ 4536753 SIIM/M

วท.ม. (วิทยาภูมิคุ้มกัน)

คณะกรรมการควบคุมวิทยานิพนธ์ : ปัทมา เอกโพธิ์, Ph.D., อมรรัตน์ ลีลาภรณ์, Ph.D., ยุพิน ศุภพุทธมงคล, M.D., B.Sc.

บทคัดย่อ

โรคเล็ปโตสไปโรซิสเป็นโรคติดเชื้อที่เกิดจากเชื้อเล็ปโตสไปร่าซึ่งเป็นสไปโรคีตแบคทีเรีย แบคทีเรียในจีนัสนี้ประกอบด้วยสมาชิกจำนวนหลายสปีชีส์ ในประเทศไทยโรคเล็ปโตสไปโรซิสจัดเป็นโรคที่เป็นปัญหาสำคัญทางด้านสาธารณสุข โดยมีรายงานผู้ติดเชื้อเพิ่มสูงขึ้นอย่างต่อเนื่องตั้งแต่ปี พ.ศ. 2539 เป็นต้นมา ในการศึกษาเชื้อเล็ปโตสไปร่าชนิดที่เป็นสาเหตุที่ทำให้เกิดโรคในคนและสัตว์นั้น จำเป็นต้องทำการบ่งชี้ชนิดของเชื้อเล็ปโตสไปร่าที่แยกได้จากสิ่งส่งตรวจชนิดต่างๆ ของคนไข้และจากสัตว์รังโรค วิธีการบ่งชี้ชนิดของเชื้อเล็ปโตสไปร่าที่ใช้อยู่ในปัจจุบันและเป็นวิธีมาตรฐานคือวิธี cross agglutination absorption test (CAAT) แต่อย่างไรก็ตามวิธีนี้มีขั้นตอนการทดสอบหลายขั้นตอนและต้องใช้ระยะเวลานานประมาณ 2 เดือน จึงจะทราบผลการทดสอบ ในการศึกษานี้จึงได้พัฒนาชุดการตรวจวินิจฉัย serogroup ของเชื้อเล็ปโตสไปร่าอย่างรวดเร็วโดยวิธีการจับกลุ่มของเชื้อกับเม็ดลาเท็กซ์ที่เคลือบด้วยโมโนโคลนัลแอนติบอดีจำเพาะ นอกจากนั้นยังได้ศึกษาความไวและความจำเพาะของชุดการตรวจการเกาะกลุ่มโดยอาศัยโมโนโคลนัลแอนติบอดีจำนวน 16 ชนิด โดยที่ 15 ชนิด เป็นโมโนโคลนัลที่มีความจำเพาะกับ serogroup ของ *L. interrogans* ซึ่งเป็นเชื้อเล็ปโตสไปร่าชนิดก่อโรคที่พบบ่อยในประเทศไทย และโมโนโคลนัลอีก 1 ชนิด มีความจำเพาะกับ *L. biflexa* ซึ่งเป็นเชื้อไม่ก่อโรค ชุดการตรวจนี้สามารถให้ผลการทดสอบได้ภายในเวลา 5 นาที จากการประเมินชุดตรวจนี้เปรียบเทียบกับวิธี CAAT ซึ่งเป็นวิธีมาตรฐาน โดยทดสอบกับเชื้อเล็ปโตสไปร่าที่แยกได้จากไตหนูจำนวน 125 ตัวอย่าง พบว่าชุดทดสอบที่พัฒนาขึ้นมามีความไวและความจำเพาะ 100 % และมีค่า Kappa value เท่ากับ 1.00 และเมื่อทดสอบกับเชื้อเล็ปโตสไปร่าที่แยกได้จากเลือดผู้ป่วยจำนวน 113 ตัวอย่าง มีความไว 100 % ความจำเพาะ 99.11 % และมีค่า Kappa value เท่ากับ 0.99

การศึกษานี้แสดงให้เห็นว่าชุดน้ำยาลาเท็กซ์ที่ผลิตได้เป็นประโยชน์อย่างมากในการบ่งชี้ serogroup ของเชื้อเล็ปโตสไปร่าที่แยกได้จากผู้ป่วยหรือสัตว์รังโรค ซึ่งข้อมูลเหล่านี้จะเป็นประโยชน์อย่างมากต่อการศึกษาระบาดวิทยา การเฝ้าระวังป้องกันโรค การพัฒนาชุดตรวจวินิจฉัยทางห้องปฏิบัติการ และการพัฒนาวัคซีนป้องกันโรคเล็ปโตสไปโรซิส

78 หน้า

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF ABBREVIATION

Abbreviation	Term
BHI	Brain heart infusion
BSA	Bovine serum albumin
°C	Degree Celsius
CAAT	Cross agglutination absorption test
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EMJH	Ellinghausen-McCullought-Johnson-Harris
gm	Gram
hr	Hour
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect heamagglutination assay
κ	Kappa light chain immunoglobulin
λ	Lambda light chain immunoglobulin
LPS	Lipopolysaccharide
LAT	Latex agglutination test
Mab	Monoclonal antibody
MAT	Microscopic agglutination test
M	Molar
mM	Millimolar
mg	Milligram
µg	Microgram
µl	Microliter
ml	Milliliter
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PFGE	Pulsed field gel electrophoresis

LIST OF ABBREVIATION (Continued)

Abbreviation	Term
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
REA	Restriction endonuclease analysis
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
rpm	Round per minute
v/v	Volume by volume
w/v	Weight by volume
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Leptospirosis is a zoonotic disease caused by spirochete *Leptospira interrogans* which is an important cause of morbidity and mortality for both animals and human living in tropical and subtropical regions. The clinical manifestations may be mild and self limiting or severe syndrome of multiorgan infection with high mortality and fatality rates. This disease was a major public health problem in Thailand during the last ten years. The highest annual reported cases, 14,285, in Thailand was in 2000. The causative serovars were identified by microscopic agglutination which indicated the prevalence antibodies specific to leptospires in a panel antigens. However, cross-reaction between serogroups or serovars are common in which the initial immune response is directed to a heterologous serogroup or serovar (1-3). This non specific reaction is paradoxical reaction that may occur in up to 50% of cases (3). A narrow range of serogroup is represented in the panel of antigens used in the MAT, which may further reduce the ability of the serologic analysis to accurately predict the infecting serogroup (4, 5). Moreover, the potential of over interpretation of serologic data is much greater if acute or early convalescent phase serum samples are available for testing (2).

Leptospiral identification is based on serological typing or molecular typing. The former one is the reference method. Many groups of researchers have been tried to develop the molecular typing methods. However, up to now over 200 serovars which are identified by serotyping can not be classified properly by the molecular methods.

Leptospires are classified by serotyping into 2 species, *L. interrogans* and *L. biflexa* which are correlate to pathogenic and non pathogenic leptospires, respectively. No reference laboratory in Thailand has been developed the CAAT. Because the test is laborious to identify leptospires, the isolated bacteria have to deliver to the WHO

reference laboratory centre such as WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Center for Public Health Science, Queensland, Australia and some isolates die or contaminate during transfer to the lab and the CAAT can not identify these kinds of samples.

In this study, a simple identification method was developed. Sixteen monoclonal antibodies specific to leptospires that were commonly reported in Thailand during the past ten years were produced, partially purified and sensitized onto latex particles. The monoclonal antibody-based latex agglutination test (LAT) is simple, inexpensive, rapid and no requirement of complicate instrument. In adding, LAT can identify frozen samples or slightly contaminated samples. The test was evaluate for its sensitivity and specificity comparing with the reference CAAT using leptospires isolated from rats' kidneys and patients' blood samples. The stability kept at 4 °C was also evaluated for at least a year. Inaddition, the minimum bacteria that required for agglutination were also analyzed.

The developed identification test is the first test developed for rapid serological typing. The identification can be performed in any laboratories even in rural area. The knowledge of the prevalent serovars/serogroups and their maintenance hosts is essential to understanding the epidemiology of the disease, vaccine and diagnostic test development in Thailand.

CHAPTER II

OBJECTIVES

The objectives of this study are:

1. To develop a panel latex reagents by using in-house monoclonal antibodies for rapid serological identification of the isolated leptospires.

2. To evaluate the efficacy of a panel latex reagents for the identification of the isolated leptospires comparing with cross agglutination absorption test (CAAT).

CHAPTER III
LITERATURE REVIEWS

Genus *Leptospira*

Leptospira is spirochete of the family Leptospiraceae, order Spirochaetales (6). Observation under darkfield microscopic, leptospires are motile, thin, flexible helicoidal organisms with a length of 6-20 μm and diameter of 0.1-0.15 μm, and often hooked at one or both end. Leptospires are obligate aerobic bacteria with an optimum growth temperature of 28 to 30 °C. Leptospiral structures compose of axial filaments called endoflagella which wound around in the insertion pore of the protoplasmic cylinder that cause them highly motile. The protoplasmic cylinder consists of cell wall, cytoplasmic membrane, and cytoplasmic contents within an outer envelope. The outer envelope is a triple-layered structure composed of proteins, lipopolysaccharide (LPS), and lipids (7). Leptospiral LPS has a composition similar to other gram-negative bacteria (8). The genus *Leptospira* is divided into two species by serotypic classification as *L. interrogans* and *L. biflexa. L. interrogans* comprise all of pathogenic strains over 200 serovars that are grouped into 24 serogroups (Table 1) and *L. biflexa* composed of nonpathogenic strains over 60 serovars that are grouped into 3 serogroups (Table 2) (1). By using genotypic classification, the genus *Leptospira* can be classified into 18 genomospecies (Table 3)(9, 10).

Table 1 **List of *Leptospira interrogans* serogroups and number of their strains**

No.	Serogroup	Number of strains
1	Australis	15
2	Autumnalis	16
3	Ballum	7
4	Bataviae	15
5	Canicola	16
6	Celledoni	5
7	Cynopteri	3
8	Djasiman	4
9	Grippotyphosa	11
10	Hebdomadis	23
11	Icterohaemorrhagiae	26
12	Javanica	18
13	Louisiana	3
14	Lyme	1
15	Manhao	5
16	Mini	10
17	Panama	3
18	Pomona	15
19	Pyrogenes	20
20	Ranarum	3

Table 1 **List of *Leptospira interrogans* serogroups and number of their strains (continued)**

No.	Serogroup	Number of strains
21	Sarmin	5
22	Sejroe	27
23	Shermani	5
24	Tarassovi	24

The data from http:// dfp.univ.trieste/spirolab/leptostrains.html

Table 2 **List of *Leptospira biflexa* serogroups and number of their strains**

No.	Serogroup	Number of strains
1	Andaman	1
2	Codice	1
3	Semaranga	3

The data from http:// dfp.univ.trieste/spirolab/leptosapro.html

Table 3 List of genomospecies, number of their strains and their major serogroups

No.	Species	Number of strains	Major serogroups
1	*L. alexanderi* (*genomospecies* 2)	5	Hebdomadis, Manhao
2	*L. biflexa*[b]	3	Andamana
3	*L. Borgpetersenii*	16	Ballum, Javanica, Sejroe, Tarassovi
4	*L. fainei*[a]	5	Hurstbridge
5	*L. inadai*[a]	6	Lyme, Manhao
6	*L. interrogans*	28	Australis, Autumnalis, Canicola, Sejroe, Icterohaemorragiae, Pomona, Pyrogenes,
7	*L. kirschneri*	6	Autumsnalis,Grippotyphosa, Icterohaemorragiae
8	*L. meyeri*[a]	2	Javanica, Mini
9	*L. noguchii*	8	Australis, Icterohaemorragiae
10	*L. santarosai*	28	Hebdomadis, Mini, Pyrogenes, Sejroe, Tarassovi
11	*L. weilii*	6	Cellidoni, Javanica, Tarassovi
12	*L. wolbachii*[b]	2	Codiac, Semaranga
13	*Leptonema illini*[b]	2	Leptonema

Table 3 List of genomospecies, number of their strains and their major serogroups (continued)

No.	Species	Number of strains	Major serogroups
14	*Turneria pava*[b]	1	Tuneri
15	*genomospecies* 1[a]	2	Saprophytic serogroup Ranarum
16	*genomospecies* 3[b]	1	Saprophytic tentative serogroup Holland
17	*genomospecies* 4	1	Icterohaemorrhagiae
18	*genomospecies* 5[b]	1	Saprophytic serogroup Ranarum

Reported by Faine *et al*.(6) and Brenner *et al*.(9)

[a] Pathogenic status not clear

[b] Saprophytes/other genera

Leptospirosis

Leptospirosis is an acute febrile illness occurring in humans and animals worldwide. The disease is caused by pathogenic *Leptospira interrogans*. High prevalence of leptospirosis is found in tropical countries which support the growth and transmission of leptospires. Other associated factors are such as occupational risk, poor sanitation system in both urban and rural areas, high seasonal rainfall, flooding and population density of reservoir host. Infection of animal or human occurs from contact with urine or other products of the leptospiral carrier, as well as contaminated water, soil, and mud in environment (11). The entry site of leptospires is through abrasions or cuts in the skin or via the conjunctiva and mucous membranes of the respiratory tract (12). The carriers may be wild or domestic animals, especially rodents, cattle, pigs, and dogs. Often infected animal has asymptomatic or mild symptoms and sometimes cause loss in productivity through abortion, stillbirth or loss of milk production. Leptospirosis in human varies in severity according to the infection serovar of *Leptospira,* age, health, and nutrition of patient. The symptom ranges from a mild influenza-like illness to severe renal, hepatic failure, myocarditis, hemorrhages, and death (Weil′s disease). A larger proportion of infection is mild type, and few cases present severe symptoms. However, serological studies in Nicaragua epidemic area and during an outbreak in Thailand in 1995 founded that asymptomatic infections occurred in 60-70% of all serologically identified infection (13, 14). Clinical diagnosis of Leptospirosis in human requires the confidence of current supported by laboratory confirmation because the symptoms alone may be confuse with other infection diseases such as influenza, dengue hemorrhagic fever, malaria, rickettsiosis, viral hepatitis, and etc (1). Treatment of leptospirosis differs depending on the severity and duration of symptoms at time of presentation. Patients with mild flu-like symptoms require only symptomatic treatment but should be cautioned to seek further medical help if they develop jaundice. Patients who present with more severe an icteric leptospirosis will require hospital admission. The antibiotic treatment by using Penicillin, Oxytetracycline, and Doxycycline were reported (1).

Epidemiology

Leptospirosis is particularly prevalent in warm humid countries. Based on global data collected by the International Leptospirosis Society (ILS) surveys, there are currently 350,000 to 500,000 severe cases of leptospirosis annually (15). Case-fatality rates in different parts of the world have been reported to range from < 5% to 30% (12). However, this is probably an underestimation of leptospirosis because worldwide surveillance is incomplete. Surveillance and notification is notably absent in some countries that have environmental condition are favorable for a high incidence of leptospirosis. Currently leptospirosis is recognized as a globally emerging disease with marked increased number of cases and frequent outbreaks in South East Asia and Latin America.

Leptospirosis is zoonotic disease, which worldwide distribution. The disease is maintained in nature by persistent colonization of renal tubules of carrier animals. An infected animal can remain symptom-free and shed infectious organisms in the urine for its entire life time (6, 16). The important maintenance hosts are rodents, which transfer several serovars of pathogenic leptospires to other animals and humans in both of rural and urban area in tropical or temperate region (17-19). Distinct variation in maintenance hosts and the serovars they carry occur throughout the world, so the knowledge of the prevalent serovars and their maintenance hosts is essential to understanding the epidemiology of the disease in any regions. The prevalence serogroups/serovars in patients and animals were studied in many countries by microscopic agglutination test (MAT) and other methods (Table 4, 5) (15).

Recently, the disease has become an urban problem of the developing countries because the poor people in rural areas have moved to cities and live in urban slum where rack of basic sanitation which produced the ecological conditions for rodent-borne transmission (18, 19). For example in Brazil where they found more than 10,000 cases of severe leptospirosis annually due to cyclic rainfall-associated urban epidemic area (18, 20-22), and in Peru found 12% of 195 urban slum residents had serologic evidence for a new infection during flood season (19). Investigation in Asia (13, 23-26) and Latin America (18-22, 27) provide evidences that leptospirosis has become an important public health problem.

Epidemiology of Leptospirosis in Thailand has been reported two periods, the first period started from 1942 to 1995 and the second period from 1996 to present. The disease was first reported in 1942 by Yunibandhu *et al*. (28). In 1972, leptospirosis was included as one of 58 reportable infection diseases under The National Passive Surveillance System. From 1972 to 1981, 10 to 20 leptospirosis cases were reported per year. The number of reported cases during 1982 to 1995 were increase to 55 - 272 cases (approximately 0.3/100,000 population)(www.moph.go.th). The second period outbreak was reported the increasing of the incidence cases (Figure 1A), morbidity, and mortality rates (Figure 1B). The disease shows a seasonal fluctuation with most of the cases occurred during June to December and the peak is between September and October each year. Over 50% of the cases were reported from Northeastern region. Common symptoms including fever, myalgia, headache, conjunctival suffusion, and meningism are reported (29). The most common serious complications jaundice and renal impairment were reported and case-fatality rate was around 10%. The epidemiological or serosurveillence studies based on MAT showed high titer of specific antibodies in patients' sera. However, this method needs the pair serum specimens for accurate interpretation of current infection. Acute sera from patients living in an endemic area have background of antibody titre specific to leptospires and these specific antibody can cause nonspecific reaction with another leptospiral serogroup. The strains of leptospires that are included in panel antigens are important for serosurveillance study. The causative serogroup that were not included in the panel antigens may result in false negative, and using the different causative serogroup in each panel antigens of each researcher group may give incorresponding results. In 1960s, the serosurveillance of Thai population were serovars Bataviae, Icterohaemorrahgiae, and Grippotyphosa (29). Epidemic in the late 1990s were found serovars Bratislava, Sejroe, and Pyrogenes as the major cause of leptospirosis (30-33). During 1996-1997, they demonstrated that *L.interrogans* serovars Icterohaemorrhagiae, Ballico, and Bratislava were the most frequently prevalence in human (32, 34, 35). The other serosurveillence in human are shown in Table 6. Farm animals' sera were studied by MAT for finding causative serovars of leptospirosis. The results showed that Wolffi, Pomona, Javanica, Hyos, and Pyrogenes were the causative serovars in bovine and buffalo and Ballico, Icterohaemorrhagiae, Canicola,

and Bataviae were causative serovars in pig (36). The prevalence study in rat from epidemic provinces and non epidemic provinces in the Northeast region during 1999 to 2000 by isolation of leptospires from kidney specimens and the isolated bacteria were identified by CAAT. The results showed that *L.interrogans* serovar Autumnalis, Bataviae, Pyrogenes, Javanica, and Australis are the predominant serovars persistent in rats' kidneys (37). These prevalence serovars isolated from rats' kidneys were identical to the result of prevalence serovars isolated from patients' blood samples in Udon Thani regional hospital (15). Then, it is reasonable to assume that the high seroprevalence of reservoir animal may be indicated the high endemic for leptospirosis. However, changing in serovars prevalence and the reservoir host are affected the epidemiology of the disease. Thus, the knowledge of the prevalence serovars and their maintenance hosts are essential to understand the epidemiology of the disease in any regions.

Table 4 **The prevalence leptospiral serogroups/serovars in human identified by MAT (15)**

Year	Countries	Numbers of patient	Serogroup/serovar
2004	Queenland, Australia	177	Zanoni 24%, Arborea 22%, Hardjo13%, Australis 12%
2002	India	58	Icterohaemorragiae (major serogroup), Grippothyphosa (minor serogroup)
2003	Guilan, Iran	304	Icterohaemorragiae (major serovar), Grippothyphosa, Hardjo, Pomona, Canicola, Bullum (minor serovar)
2003-2004	American Samoa	58	Bratislava 77.6%, Icterohaemorragiae 15.5%
2004	Nakhonratchasima, Thailand	280	Bratislava 97.1%
1981-2000	Croatia	1311	Sejroe, Saxkoebing, Grippotyphosa, Australis, Icterohaemorhagiae

Table 5 The prevalence leptospiral serogroups/serovars in animals identified by MAT, CAAT, or PCR (15)

Countries	Method	Animal	Serogroup/serovar
Trinidad and Tobago	MAT	40 dog	Autumnalis 42.5%, Mankarso 40%, Icterohaemorhagiae 12.5%
Papua New Guinea	MAT	79 cattle	Hardjo 49.6%, Szwajizak 24.9%, Tarassovi 15.8%, Medanensis9.8%, Kremastos 4.8%, Pomona 2.9%
France	MAT	feral rodent	Icterohaemorrhagiae, Grippotyphosa
Iran	MAT	cattle	Canicola, Grippotyphosa, Sejroe , Icterohaemorrhagiae, Pomona
Mexico	MAT	850 pig	Bratislava 27.7%, Icterohaemorrhagiae 14.8%, Palo Alto strain 14.2%, Panama 12%, Sinaloa ACR strain 11.4%

Table 5 The prevalence leptospiral serogroups/serovars in animals identified by MAT, CAAT, or PCR (15) (continued)

Countries	Method	Animal	Serogroup/serovar
Northeastern part, Thailand	CAAT	138 rodent	Pyrogenes 48.55%, Autumnalis, Bataviae, Javanica, Australis
Chiang Mai, Thailand	MAT	23 dog	Batavia 47.8%, Canicola 21.7%, Batislava 13%, Icterohaemorrhagiae 13%, (Ballum, Djasiman, Javanica, Mini, Sejroe 21.7%)
Peru	PCR	20 bat	*interrogans** 25%, *kirschneri** 5%, *borgpetersenii** 20%, *fainei** 5%, Unidentified species 45%

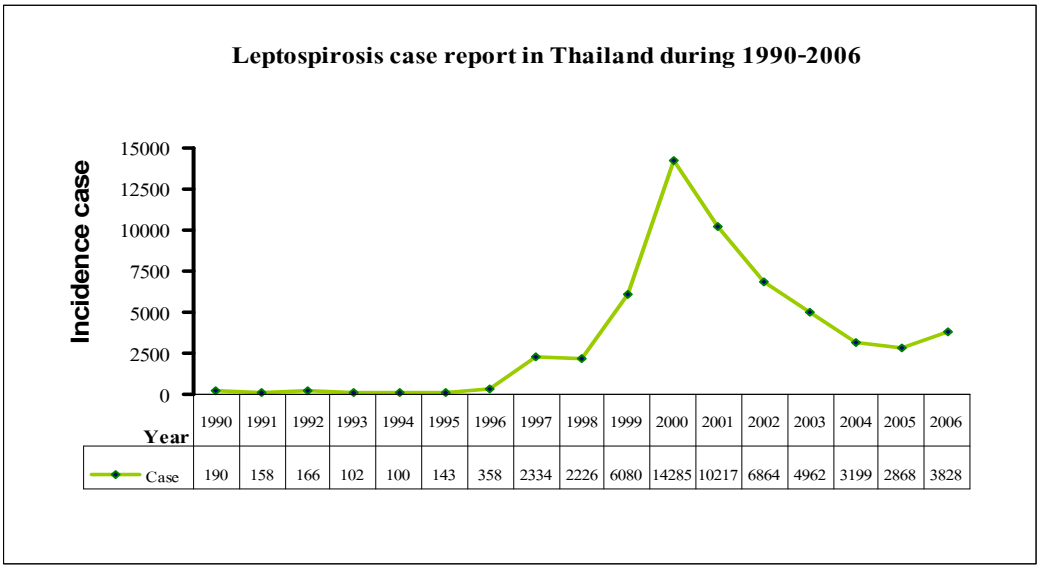
* Species based on molecular identification

Table 6 The prevalence leptospiral serogroups/serovars in Thai patients identified by MAT or CAAT (11)

Year	Province	Case	serogroup/serovar
1998-2003	Loei	43	Bratislava 13.9%, Sejroe 11.6%, Mini 7%, Australis and Rachmati 7%
1998-2003	Kamphangphet	90	Australis, Bratislava, Autumnalis, Bataviae, Canicola (Top 5 serovars)
2000	Songkhla	130	Bataviae 80.8%, Bratislava 19.2%
2004	Nahhon Ratchasrima	280	Bratislava 97%
2000-2002	Udon Thani*	204	Autumnalis 85%, Pyrogenes 5%, Javanica 4%

*Prevalence serovar from Udon Thani province characterized by CAAT

(A)



(B)

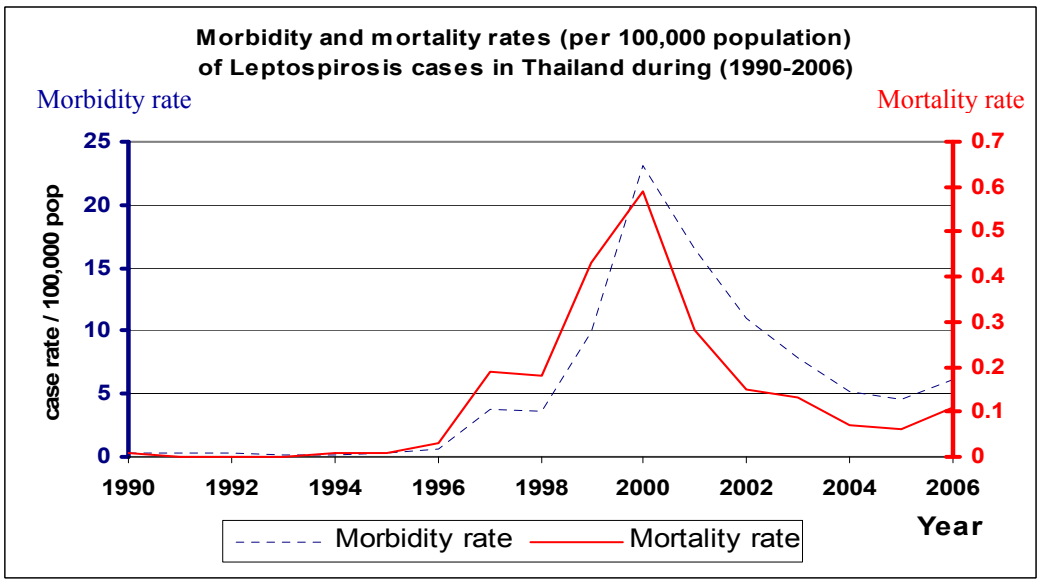


Figure 1 Case report (A), Morbidity rate and mortality rate (B) of leptospirosis in Thailand during 1990 – 2006

The data were kindly provided by the Epidemiology Division, Ministry of Public Health, Thailand

Laboratory diagnosis

The leptospirsis diagnostic methods can be divided into three categories. First category is direct diagnostic methods which compose of microscopic, antigen detection, and isolation methods. Second category is serological methods and the last category is molecular methods.

1. Direct diagnostic methods

1.1 Microscopic methods

Leptospires in clinical materials or natural sources can be visualized by dark-field microscope. The clinical specimens are collected for this direct diagnosis such as blood, urine, cerebrospinal fluid (CSF), and dialysate fluid. This method needs density approximately 10^4 leptospires/ml in sample for one cell/field detection (38). By light microscopic detection, leptospires can be stained by Gram's staining, Giemsa staining, and silver staining for higher sensitivity and specificity.

However, these methods also have disadvantages since false positive result can be occurred by misinterpretation of cellular fibrin or protein threads as leptospires and can not identify degenerated leptospire and antigen (1).

1.2 Antigen detection

Detection of *Leptospira* antigens in clinical specimens would offer greater specificity and sensitivity than direct detection by dark-field microscopy. This method can detect and identify intact form of leptospires in tissues, phagocytes, and in fluids, eventhough the specimen has been refrigerated or partially autolysated during transport to the laboratory. Antigen detection methods are including radioimmunoassay (RIA) (39), enzyme linked immunosorbent assay (ELISA)(40), chemiluminescent immunoassay (41), and dot blot ELISA (42).

1.3 Isolation method

Leptospires from clinical materials including blood, CSF, urine, tissue biopsy, and postmortem tissue fragments can be isolated by inoculation of an appropriate clinical materials into leptospira culture medium such as Ellinghausen-McCullought-Johnson-Harris (EMJH) medium. Samples destined for culturing should be removed before antibiotic therapy. Fluid media containing 5-fluorouracil are used for primary culture. Leptospires growth is relatively slow, with doubling time about 6-8 hours at optimal temperature 28-30 $^{\circ}$C. Growth may occasionally be detectable by

dark-field microscope after culture for about a week but often takes longer period to 4 months. The knowledge of pathogenesis and the stages of disease are necessary for choosing target site of sample collection. Hemoculture should be done within 10 days following the fever, which is symptomatic of the beginning of the disease. CSF should be collected between the end of the first week to second week of the illness. Urine samples can be used for culture from the third week or about one month of illness (43). This method has the advantage that the infecting agent can be further investigated. However, this method is very laborious and time consuming. Therefore it is used as a confirmatory test.

2. Serological methods

Most cases of leptospirosis are diagnosed by serology, because the direct detection of leptospires is either slow or limited reliability. Moreover, serology has been used to provide evidence of current or recent leptospirosis by determined class and quantity of antibodies. Antibodies are detectable in blood approximately 5-7 days after the onset. In general, blood for serology should be taken as soon as possible in the illness. A second specimen should be taken 5-7 days later, and repeated at similar intervals if necessary. The test may be negative in early stage, but the second specimen may be positive or show arising in titer compared with the first sample specimen. Serological method can be divided into genus specific and serogroup specific methods.

2.1 Genus specific method

Most genus specific methods are screening test for early diagnosis of leptospirosis. The methods are included complement fixation test (44), macroscopic slide agglutination(45, 46), indirect hemagglutination assay (IHA) (47), ELISA (48), Dot-ELISA (42), microcapsule agglutination (49, 50), IgM dipstick (51, 52), latex agglutination (53-55), and lateral flow (56). They use numbers of antigen prepared from *L.biflexa* serovar Patoc such as heat stable antigen, sonicated antigen, and lipopolysaccharide (LPS). Several tests for genus specific antibody detection are now commercial available either IgM or both IgM and IgG (36). However, the final result is confirmed by microscopic agglutination test (MAT).

2.2 Serogroup specific method

Serogroup specific method is based on MAT, which patients' sera are reacted with live antigen suspension of leptospiral serovars. After incubation the

serum-antigen mixtures are determined for the agglutination and antibody titers under dark-field microscope. The disadvantages of MAT are complex method that difficult to perform and interpret. Interpretation of MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute phase serum. Moreover, it presents risks of infection for laboratory workers. Because this test has to perform with live antigen which need to maintain culture in large amount. However, the MAT is also the suitable test for serodiagnosis and seroepidemiology, and it is considered the gold standard or reference method for antibody detection.

3. Molecular methods

Molecular methods are being developed for both leptospiral diagnosis and identification. These methods are more sensitive and specific than serological methods. Serological test often gives cross-reactivity between leptospires and difficult to distinguish the antibody titers of chronic infection and vaccination, particularly in animal (57). Previously, leptospiral DNA has been detected in clinical materials by dot blotting and in situ hybridization techniques (58, 59). Specific DNA probes were developed by DNA recombination, DNA cloning, and polymerase chain reaction (PCR) methods (57). DNA probes can be labeled with ^{32}P, biotin (59), or digoxigenin (60). However, the sensitivity of ^{32}P-labeled probe is approximate 10^3 leptospires (59) which much lower than PCR. Then, this probe has not been used extensively for diagnosis since PCR became available. PCR method is use to amplify specific segments of *Leptospira* DNA in clinical samples such as blood, urine, and tissue. Several primer pairs for PCR detection for leptospires have been developed. They are designed based on the specific genes of either 16S rRNA or 23S rRNA, repetitive elements (61-63), and genomic libraries (64, 65). PCR can rapidly confirm the diagnosis in the early phase of the disease, when the bacteria present and before the antibody titer detectable. However, PCR may also give false-negative results because inhibitors are present in the clinical samples or the number of leptospires in samples were too small to be detected. However, a limitation of PCR based diagnosis in most assays is the inability to identify the infecting serovar (66).

Identification

In clinical management and curly of leptospirosis, it is better to know whether the patient has leptospirosis or not. The public health perspective typing is a useful epidemiologic tool to establish the causative serogroup or serovar at the first step toward identifying the source of infection and reservoirs, thus determines the choice of methods for prevention and control. The serosurveillance results from MAT testing of patients' sera are used as a surrogate to infer the infecting serogroup or serovar of leptospires but its result is not absolutely conclusion. Cross-reaction between serogroups or serovars are common in which the initial immune response is directed to a heterologous serogroup or serovar (1-3). This non specific reaction is paradoxical reaction that may occur in up to 50% of cases (3). A narrow range of serogroup is represented in the panel of antigens used in the MAT, which may further reduce the ability of the serologic analysis to accurately predict the infecting serogroup (4, 5). Moreover, the potential of over interpretation of serologic data is much greater if acute or early convalescent phase serum samples are available for testing (2).

The genus *Leptospira* composes of two species, *L.interrogans* sensu lato and *L.biflexa* sensu lato which contained pathogenic and nonpathogenic strains, respectively. Two species can be recognized using phenotypic characteristic, including growth in the presence of 8-azaguanine and growth at 13°C (67). From two species are identified to serogroups and serovars level by serological method based on antigenic criteria. By molecular method based on genomic criteria, the genus *Leptospira* is classified into 18 species (9, 10, 67, 68), several of which contain both pathogenic and nonpathogenic serovars.

The identification method has been developed for identifying the leptospires which isolated from patient, reservoir animal, and environment. Basically, the MAT is the core principle of all serotyping methods which recognize antigenic of *Leptospira* by reference polyclonal or monoclonal antibody reagents such as the cross agglutinin absorption test (CAAT), the factor sera analysis, and monoclonal antibody testing. Recently, typing method based on different leptspiral DNA have been developed such as restriction endonuclease (REA), nucleic acid probe and hybridization, pulsed field gel electrophoresis (PFGE), ribotyping, and a number of PCR-based approaches for improve typing tool. Moreover, the most identification method is essential for

checking the reference strain in culture collection and checking on the identity of strains used for diagnostic test in the routine and reference laboratories.

1. Serological identification

1.1 Using polyclonal antibody

1.1.1 Cross agglutination absorption test (CAAT)

The cross agglutinin absorption test is conventional method for serotyping of unknown strains into serogroup and serovar (69).

1.1.1.1 Determination of the serogroup

During the early years, *Leptospira* strains could be distinguished by their antigens. As more difference strains were isolated, serological relationship were demonstrated between some of them. For practical reason, strains belonging to separate, but closely related serovars were grouped together into serogroups. Serogroup cannot be defined accurately and have no official taxonomic status but serve the practical purpose of grouping strains on the basis of their antigenic similarlity. However, serogrouping is necessary because more than 200 leptospiral reference strains cannot be used individually in serotyping experiment. First, leptospira is classified into serogroup belonging by the MAT using selected rabbit group sera. As representative group, serum is chosen a rabbit immune serum which agglutinates optimally all serovars of given serogroup and which gives the least cross-agglutination with strains of other serogroups. Therefore, the determination of serogroup is provide suggestion battery of group sera is to be used. Briefly, procedure for determining the serogroup is as following : well grown culture (approximately $2x10^8$ cell/ml) of the unknown strain is first submitted to the MAT with the standard group sera used at low dilutions (1:200 or 1:300 provided that the antisera have titres of about 1:10,000 with the homologous strain). Then, the positive reacting sera are further titrated with the unknown and homologous strains. Endpoint titers sera also compare with the titers of agglutination reactions of standard group sera with reference strains. This usually allows the serogroup to be determined.

1.1.1.2 Determination of the serovar

The original definition of serovar formulated by Wolff and Broom (1954), and a WHO Expert Group (World Health Organization, 1967) was intended not only for taxonomic purpose but also a practical way of

differentiating between leptospires on the basic of host-parasite relationship. Under the present definition, two strains are considered belong to different serovar if after cross-absorption with adequate amounts of heterologous antigen, more than 10% of the homologous titre regularly remains in at least one of the two antisera in repeated tests. If an unknown serovar is difference from all known reference serovars accoding to the criterion given in the definition above, it is seem to be a new serovar. If no homologous titre or less than 10% persist, the unknown strain belongs to the serovar concerned. Thus an unknown strain may either belong to a known serovar represented by a reference strain, or be a new serovar and become the reference strain for this new serovar. The 10% limit criterion is critical, this rule allows a 0-10% margin of difference for strains belonging to the same serovar. Briefly, procedure for determining the serovar of strain is as following: well grown culture (approximately $2x10^8$ cell/ml) of the unknown strain is first submitted to MAT with antisera of all reference strains of the serogroup. An immune serum is prepared against the unknown strain and this antiserum is similarly investigated with all reference strains of the determined serogroup to which the strain belongs. The next step is to carry out cross-agglutinin absorption test (CAAT) with all the reference strains which have some degree of serological relatedness to the unknown. Relatedness is considered to exist when the unknown strain reacts with the immune sera of a reference strains to at least a titer of 10% of that obtained with the homologous strains. If the unknown strain gives a reaction with a reference serum less than 10% of the homologous titer, an absorption test with the homologous strain is redundant. During the comparative absorption tests, serological identity is proved with one of the selected reference strains. Two strains are considered serologically identical if absorption with the heterologous antigen less than 10% residual homologous antibodies remain in both sera (69).

Even though, the CAAT is the conventional and reference method for classifying leptospires, but it is limiting in a few reference laboratories because the test is complicate, and time-consuming. Moreover, it requires the maintenance of all leptospiral reference strains and corresponding rabbit immune serum. Therefore, this method is not available for service laboratories in Thailand.

1.1.2 Factor analysis

The factor analysis involves more detail studied of the antigenic structure of each serovar which characterized by its own particular combination or mosaic of major and minor antigenic factors. Factor sera are prepared by absorbing rabbit antiserum with one or more difference leptospires until they react only with one serovar, subgroup or serogroup. Panels of factor sera can be used in similar way as monoclonal antibodies to identify strain to serovar quickly. However, factor sera preparation is time-consuming, and preparing batches does not always lead to reproducible results (12).

1.2 Using monoclonal antibody

Identification by using monoclonal antibodies (Mabs) is related to conventional typing based on the recognition of characteristic antigen patterns of serovars by panels of Mabs. In contrast with the cross-agglutination absorption test, large numbers of strains can be typed in a short time with Mabs.

Mabs react in the microscopic agglutination test (MAT) with a single antigenic epitope which specific for certain serovar or shared by various serovars. On the basis of combinations or mosaics of epitopes characteristic of certain serovar, panels of Mabs have been used for identification of leptospires to serovar level and sometimes to subserovar level. Differences in agglutination profiles obtained from a panel of Mabs can be used as indicative of new serovars and differences between strains belonging to the same serovar may be observed (12). Sehgal *et al.* confirmed AF61 isolated leptospiral strain from patient in Andaman Islands with monoclonal antibodies. The result showed that AF61 is closely related to the reference strain Lai serovar Lai, which similar result to CAAT (70). The isolation of leptospire 149 of 228 samples from mainland Portugal and Azorean wild mammals were rapid characterized at the serovar level by monoclonal antibodies (71).

These methods also allow a rapid check on the identity of leptospiral stains that used as antigens in the MAT for serodiagnosis. Mislabeled strains can be retyped and identified accurately and more easily than using conventional rabbit antisera. However, preparing monoclonal antibodies is difficult and time consuming.

2. Molecular identification

From serological identification system, the genus *Leptospira* consists of two species. The first one is *Leptospira interrogans sensu lato*, a group of pathogenic leptospires. The second one is *Leptospira biflexa sensu lato*, a group of non-pathogenic leptospires. In contrast, the molecular identification based on DNA homology separates *Leptospira* into 18 species, which some species members contain both pathogenic and non-pathogenic leptospires. Furthermore, some species may compose of more than one serogroup (Table 3). The molecular methods based on genomic analysis are used to identify and classify leptospires by comparing DNA fragments, which currently researches for leptospiral taxonomy and to develop simple and reliable methods that limit in serological identification and typing. Moreover, these identification system traits should ideally allow subspecies characterization. Methods employed include digestion of chromosomal DNA by restriction endonuclease (REA), Pulsed field gel electrophoresis (PFGE), ribotyping, and a number of PCR-based approaches.

2.1 Restriction endonuclease analysis (REA)

REA principle is based on digestion of purified leptospiral DNA with restriction enzymes and electrophoresis on an agarose gel. Restriction enzymes recognize and cleave dsDNA at specific sequences that generate a set of DNA fragments which differently migrate on agarose gel according to their molecular weight. This method generating characteristic DNA fingerprints for each type of leptospires. Relationship between leptospiral strains can then be established by comparing the patterns of unknown strains with leptospiral reference strains.

Use of REA for identification of leptospires was first proposed by Marshall *et a*l.. They reported that serovar Hardjo and Balcanica were clearly distinguished from each other by REA using *Eco*RI enzyme (72). Theirman *et al*. adopt REA for classification leptospiral isolates belonging to serogroup Pomona (73). They showed that REA could distinguish the differences between the organisms which were not detectable by conventional serological typing methods. Ellis *et al*. performed REA using 20 restriction enzymes in the study of 162 pig isolates and compared the electrophoretic pattern of serogroup Australis strains (74). Venkatesha analysed genomic DNA of 19 pathogenic serovars and 2 nonpathogenic serovars by REA using

*Eco*RI and *Hae*III enzymes and showed that REA was reproducible and created stable fragment patterns, which could be used for serovar differentiation (75). Moreover, these method can be used for typing by comparing restriction patterns with reference serovars. From these results, REA is a good method for identification and classification of leptospires. The disadvantage of REA method is the requirement of pure leptospira cultures and extraction of their DNA. Either contaminated or degraded leptospiral DNA can result in abnormal patterns. Furthermore, only few restriction enzymes give clear-cut differences between different serovars and the restriction patterns that are very complex and difficult to compare.

2.2 Nucleic acid probes and hybridization

Molecular cloning techniques make possibility to prepare gene-specific probes that can be used further in hybridization techniques for detection or identification of leptospires. Furthermore, nucleic acid hybridization provides the confirmation of diagnosis before the results of culture and biochemical tests are available. Using total genomic DNA probes in examining 66 serovars of pathogenic leptospires by slot blot hybridization proposed a new species *L. kirscherneri* that comprising nine serovars, which seven of them had not been studied (68). The recombinant probes derived from genomic library by serovar Lai were used to characterize DNA of 20 strains in different genus, species, serogroups and serovars by Southern hybridization (76). The advantage of this technique is very easy to distinguish the banding pattern since the probe hybridized with limited number of DNA fragments. Nucleic acid hybridization using a cloned probe is suitable for serovar differentiation and characterization of isolates as it produced limited number of bands and unique pattern for each serovar tested that easy for comparison and interpretation (75).

2.3 Pulsed field gel electrophoresis (PFGE)

PFGE is a variation of agarose gel eletrophoresis that permits analysis of bacterial DNA fragments over an order of magnitude longer than a conventional REA. This process produces highly reproducible restriction profiles that typically show distinct well resolved fragments, representing the entire bacterial genome in a single gel. PFGE analysis has become the standard method for molecular characterization of leptospiral isolates, and other molecular typing methods will be

validated against this method. However, it is technically more demanding, requires more expensive and specialized equipments.

Hermann *et al*. developed fingerprints for 72 reference strains by PFGE following *Not*I chromosomal DNA digestion (77). They found that 13 of 18 isolates identified by MAT and cross absorption procedures were correctly typed by PFGE. Then, PFGE was applied to identify 200 serovars of pathogenic leptospires of clinical isolates and the results showed specific fingerprints (78). However, using only *Not*I enzyme could not produce unique PFGE pattern of all serovars. For example, *L.interrogans* serovars Bratislava, Lora, Jalna, and Muenchen gave identical patterns when digested with *Not*I but were differentiated when digested with *Sgr*AI enzyme (77). Moreover, the patterns of DNA fringerprint produced by the *Not*I and *Sgr*AI restrictions of chromosomal DNA analysed by PFGE allowed 166 of 170 serovars reference strains to be characterized (77, 78). Thus, PFGE was more rapid than serology and was useful for identification in epidemiology.

2.4 Ribotyping

Ribosomal RNA gene restriction patterns have been demonstrated for the identification of species or epidemiological typing. The conserved nature of rRNA gene allows the use of single probe for typing bacteria for any phylogenic positions. Ribotyping has demonstrated reasonably good correlation with the phylogenetic classification that classified *Leptospira* into 11 genomospecies using *Eco*RI digestion, and 16S and 23S rRNA from *Escherichia coli* as the probe. This technique provided a large database construct (79, 80). Ribotypes of serovar within genomospecies could be grouped together by the possession of common fragments. This method has been shown to discriminate accurately between the serovar Hardjo genotypes Hardjobovis and Hardjoprajitno but not by conventional serological typing methods (81). However, particular serovars closely related such as Icterohaemorrhagiae and Copenhaegini could not be distinguished by ribotyping (82, 83) .

2.5 Polymerase chain reaction (PCR)

PCR method involves *in vitro* amplification of target DNA sequence, brought out by thermostable DNA polymerase. A pair of short DNA fragments known as primers is used for specific amplification of DNA fragments. This technique is modifying to several methods such as arbitrarily primed PCR (AP-PCR), and random

amplified polymeric DNA (RAPD) fingerprinting for diagnostic, identification, and taxonomy of bacteria including leptospires. However, several methods based on PCR requires isolation of leptospires in pure culture and their DNA isolation for carrying out PCR.

Arbitrarily primed PCR (AP-PCR) is based on the use of a single arbitrary primer in a PCR of low stringency to amplify segments of the genome. Highly diverse DNA banding patterns can be generated, which may allow distribution between and within species. This technique has been used to rapidly identify leptospires (84), genetic variability among strains of serovar Hardjo (85), and determine the taxonomic relationship between leptospiral serovars. However, few serovars like Copenhageni, Icterohaemorrhagiae, Pyrogenes, and Bataviae were indistinguishable by this method (86).

Random amplified polymorphic DNA (RAPD) fingerprinting is based on PCR amplification and fingerprinting, using arbitrary oligonucleotide primers which produces reliable and easily obtainable typing schemes. Comparing RAPD fingerprinting with CAAT and REA for typing bovine leptospires 26 isolates, found that 23 isolates were identified as Hardjo genotype Bovis, 2 isolates were identified as Zanoni and one was identified as Pomona (84). Gerritsen *et al*. used RAPD for identification of leptospiral serovars within the serogroup Sejroe. They found the unique DNA banding patterns for each of the 17 reference strains by using *L. hardjo* type Hardjobovis-derived primers and all clinical isolates were identified as *L. hardjo* type Hardjobovis. Therefore, RAPD fingerprinting was a single and rapid method suitable for serovars identification (87). The other study using RAPD for characterization of the laboratory strains of leptospiral serovars demonstrated that each serovars produced a unique and distinct fingerprint pattern of DNA that difference from other bacterial species such as *Escherichia coli*, *Putida multocida*, *Salmonella* spp., and *Pseudomonas* spp. (75).

Principle of ammonium sulfate precipitation

Ammonium sulfate precipitation is one of the most commonly used methods for removing proteins from solution. Proteins in solution form hydrogen bonds with water through their exposed polar and ionic groups. When high concentrations of

small, highly charged ions such as ammonium or sulfate are added, these groups compete with the proteins for binding to water. This process removes the water molecules from the protein and decreases its solubility, resulting in protein precipitation. For this reason, ammonium sulfate precipitation can also be used in antibody concentration and partial purification. During purification one takes advantage of the fact that different proteins will be precipitated at different concentrations of ammonium sulfate. The factors that will affect the concentration at which a particular protein will precipitate include the number and position of the polar groups, the molecular weight of the protein, pH of the solution, and temperature at which the precipitation is performed. Although other salts such as sodium sulfate are sometimes used, precipitation of antibodies is commonly done with ammonium sulfate. The concentration at which antibodies will precipitate varies somewhat from species to species. Most rabbit antibodies can be precipitated with a 40% saturated solution, while mouse antibodies need 40-50% saturation. Because most of the protein components of hybridoma cell culture supernatant do not precipitate in this range, it is seldom worthwhile to distinguish between these concentrations, and 50% saturation is convenient level to use for most applications. One disadvantage of ammonium sulfate precipitation of antibodies is that the resulting antibodies will not be pured. They will be contaminated with other high molecular weight proteins such as albumin. Therefore, ammonium sulfate precipitation is not suitable for single-step purification but must be combined with other methods if pure antibody preparations are need. However, this method is cheap, easy, and convenient for large volume preparation. The precaution is the solutions of saturated ammonium sulfate should not be stored in containers that were previously used for laboratory wash-up detergents because the reagent trace can inhibit precipitation of antibodies by ammonium sulfate (88-90).

Latex agglutination

Latex agglutination is the indirect (passive) agglutination reaction for antibody or antigen detection. For antibody detection, the specific antigens were coated on latex particles to react with antibodies target. In contrast, the specific antibodies were coated on latex particles to react with antigens target for antigen detection. The nature of latex bead is polystyrene material, which is developed for a new choice of a carrier particle

in indirect agglutination test preparation. It has more stable, long shelf life, and simplify to preparation and storage than other carrier particle such as red blood cell. Moreover, it has various type, size, and density of surface charge group for individual coating substance such as protein, polysaccharide, low molecular weight peptide, and oligonucleotide (www.idclatex.com). The rapid diagnosis or identification test is simple to set up with latex particle. Attachment of antibodies, antigens, and other substances to latex particles is achieved by either physical absorption or covalent coupling to the surface of the latex particles. Most proteins can be absorbed on the latex particles by hydrophobic interaction. Polyclonal and monoclonal antibodies are commonly used to coat onto the latex particles. However, samples that contain protein or peptides may bind less well and require covalent coupling for increase attraction. The hydrophobic interaction is largely independent on pH since charges in different pH influence the conformation of the protein molecules. In general, proteins bind most efficiently at pH that is close to their isoelectric points (91). For passive absorption onto latex particles with antibodies, the antibody molecules are bound in random alignment to the surface of particles. However, the potential number of antigen binding site exposed to the outside is large because large numbers of antibody molecules are bound to each latex particle.

During agglutination reaction between the antigens and latex particles coated with specific antibodies, the antigens will bind to the antigen binding sites of the antibodies that exposed on the surface of the latex particles, forming cross-linked aggregatation of latex bead. The pH, osmolarity, and ionic concentration of the solution will influence the degree of cross-linking. Furthermore, the degree on lattice formation can be divided into three zones, the antibody-excess zone (prozone), the equivalence zone, and the antigen-excess zone (postzone) (92). In the prozone, practically every antigenic molecule is complexed with antibodies. However, there are not enough antigens to bind to all antibody molecules that allow low probability of a single antibody molecule's binding to two antigenic molecules simultaneously and some antibodies remain free in reaction, if the amount of antigens increases more antibody molecules crosslink to individual antigen molecules and form as stable lattice. In the equivalence zone, the highest degree of lattice formation is formed, resulted in maximum agglutination. As the amount of antigen further increase in

antigen excess zone, all antibody molecules are bound to antigen whereas several antigen molecules are remain free in solution and each antibody is separated from each others thus preventing lattice formation.

CHAPTER IV

MATERIALS AND METHODS

1. Bacterial strains

1.1 *Leptospira*

Twenty-six reference *Leptospira* serovars belonging to 22 serogroups (Table 7) used in the experiments were kindly provided by Dr. Duangjai Suwanchareon from the National Institute of Animal Health (NIAH), Department of Livestock Development, Ministry of Agricultural and Cooperatives, Thailand and Mrs. Pimjai Naikowit from the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand.

1.2 Other bacterial genera

Ten other bacterial genera (Table 8) that used in the experiments were kindly provided by Dr. Amornrut Leelaporn from the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

2 Antigen preparation

2.1 Leptospiral antigens

2.1.1 Whole cell antigen

Leptospires were grown at 30 °C in Johnson and Harris modification of the Ellinghausen and McCullough (EMJH) medium containing 3% rabbit serum until they were at exponential phase which took about 7-10 days. The leptospires were collected by centrifugation at 10,000 rpm (Superspeed centrifuge, Sorval; Model RC28S, USA) for 15 minutes at room temperature and washed two times with 0.01M phosphate buffer saline (PBS) pH 7.4, and treated with 0.02% formalin in 0.01M PBS (see Appendix) for 18 hours at 4 °C. The formalinized whole cell antigens were separated by centrifugation at 10,000 rpm (Superspeed centrifuge, Sorval; Model RC28S) for 15 minutes at room temperature, washed two times and

resuspended in 0.01M PBS pH 7.4. The protein concentration of the whole cell antigen was measured and the antigen was stored at -20 °C in small aliquots.

2.1.2 Secreted antigen

Leptospires at exponential phase were collected by centrifugation at 10,000 rpm (Superspeed centrifuge, Sorval; Model RC28S) for 15 minutes at room temperature, washed two times with 0.01M PBS pH 7.4 and resuspended in the PBS. The secreted antigen was prepared by shaking leptospires in 0.01 M PBS for 18 hr at 30 ºC. After incubation, antigen was prepared by centrifugation at 10,000 rpm for 15 minutes at room temperature; the supernatant was collected, measured for protein concentration, and stored at –20 ºC in small aliquots.

2.1.3 Live antigen

Representative serovars from each *Leptospira* serogroups were used for preparation of live antigens. The bacteria were grown in EMJH media for 7-14 days at 30 ºC and subcultured every two weeks. The bacteria were triplicate counted under dark-field microscope at 200x magnification by using hemocytometer (Fast-Read 102®, Hycor Biomedical Inc., Italy). The live antigen was used for characterization the sensitivity of the latex reagents**.**

2.2 Other bacteria antigens

The other bacteria genera were grown in Brain heart infusion (BHI) broth for 18-24 hr at 37 ºC. The whole cell antigen was prepared by treated the bacteria with 0.02% formalin in 0.15M PBS pH 7.4 for 18 hr at 4 ºC. The formalinized cells were separated by centrifugation at 5,000 rpm (Superspeed centrifuge, Sorval; Model RC28S) for 15 minutes at room temperature, washed for two times with 0.15M PBS, resuspend in 0.15M PBS and stored at –20 ºC in small aliquots.

Table 7 List of 26 reference *Leptospira* serovars belonging to 22 serogroups used in the experiment

No.	Serogroup	Serovar
1	Australis	Australis, Bangkok, Bratislava
2	Autumnalis	Autumnalis
3	Ballum	Bullum
4	Bataviae	Bataviae
5	Canicola	Canicola
6	Celledoni	Celledoni
7	Cynopteri	Cynopteri
8	Djasiman	Djasiman
9	Grippotyphosa	Grippotyphosa
10	Hebdomadis	Hebdomadis
11	Icterohaemorrhagiae	Icterohaemorrhagiae
12	Javanica	Javanica
13	Louisisana	Louisiana
14	Mini	Mini
15	Pomona	Pomona
16	Pyrogenes	Pyrogene
17	Ranarum	Ranarum
18	Sarmin	Sarmin

Table 7 List of 26 reference *Leptospira* serovars belonging to 22 serogroups used in the experiment (continued)

No.	Serogroup	Serovar
19	Sejroe	Sejroe, Hardjo, Wolffi
20	Shermani	Shermani
21	Tarassovi	Tarassovi
22	Semaranga	Patoc

Table 8 List of 10 genera of other bacteria used in the experiment

No.	Genus	Species
1	*Aeromonas*	*A. hydrophila*
2	*Burhkordoria*	*B. pseudomallei*
3	*Escherichia*	*E. coli*
4	*Krebsiella*	*K. pneumoniae*
5	*Pseudomonas*	*P. aeruginosa*
6	*Proteus*	*Proteus* spp.
7	*Salmonella*	*Salmonella* spp.
8	*Shigella*	*Shigella* spp.
9	*Staphylococcus*	*S. aureus*
10	*Streptococcus*	*Streptococcus* spp.

3 Specific *Leptospira* serogroups/serovars Monoclonal antibodies

Sixteen monoclonal antibodies (Mabs) (provided by the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand) were produced using a standard hybridoma technique and tested for antigenic specificity by indirect ELISA using panel of formalinized whole cell antigens prepared from 26 leptospires and 10 genera of other bacteria. The specificity of the monoclonal antibodies were further tested by MAT, which kindly performed by the National Institute of Animal Health (NIAH) and the National Institute of Health (NIH), Thailand. All of Mabs used in this study recognize the lipopolysaccharide (LPS). The characteristics of Mabs are shown in Table 9.

4 Precipitation of monoclonal antibody from the culture supernatant fluid by 50% ammonium sulfate precipitation method

The hybridoma cell culture supernatant was partially purified and concentrated by ammonium sulfate precipitation. A saturated ammonium sulfate, $(NH_4)_2SO_4$, solution buffer at pH 7.4 was added dropwise to the culture supernatant fluid for a final concentration of 50% $(NH_4)_2SO_4$ with continuous stirring for overnight at 4 ºC. The precipitated proteins were removed by centrifugation at 10,000 x g (Superspeed centrifuge, Sorval; Model RC-5C Plus, USA) for 20 minutes. The pellet was washed with 50% saturated ammonium sulfate solution and centrifuged at 10,000 x g (Superspeed centrifuge, Sorval; Model RC-5C Plus) for 20 minutes and resuspended in 0.15M PBS. The excess salt was removed by exhaustive dialysis against 0.15M PBS. After dialysis, the solution was centrifuged at 1,000 x g (High speed micro refrigerated centrifuge, Tomy; Model MRX-150, Japan) for 5 minutes at 4 ºC. The supernatant fluid was filtrated through sterile 0.45 μm membrane (Acrodisc®, MI, USA). Globulin protein concentration was measured by the BioRad protein assay. The antibody preparation was aliquoted and kept at –20 ºC until use.

Table 9 Characteristics of the Mabs used for preparation of the latex reagents

No.	Code	Isotype	Serovar specific
1	8C6C4A12	IgMκ	Australis, Bangkok
2	2D2A12G3	IgMκ	Autumnalis
3	1B4D3F10	IgMκ	Bataviae
4	15B5C2G1	IgMκ	Biflexa
5	4A2D5D6	$IgG_3κ$	Bratislava
6	16C4E11E5	IgMκ	Canicola
7	20A5A6	IgMκ	Cellidoni
8	2B3A8H9	$IgG_3κ$	Cynopteri
9	4D4E8F10	$IgG_3κ$	Grippotyphosa
10	9A6E1E11	IgMκ	Hebdomadis
11	7B5E11D1	IgMκ	Pomona
12	16A3A4	IgMκ	Javanica
13	1A4B10E1	IgMκ	Pyrogenes
14	13C2E4F8	$IgG_3κ$	Sarmin
15	16B1C9H	IgMκ	Sejroe, Hardjo, Wolffi
16	9D4B12F5	IgMκ	Tarassovi

5 Protein concentration assay

Protein concentration of antigen and antibody were measured by Bradford protein assay (Bio-Rad, CA, USA). The known concentrations of globulin and albumin were used as the standard proteins for measuring protein concentration in antibody and antigen, respectively. Two fold serial dilutions of protein standard starting from 10 μg/ml were prepared according to microassay procedure. Then, 800 μl of each standard dilution and sample solution were added with 200 μl of dye reagent, mixed by vortex, incubated at room temperature for 30 minutes, and measured the absorbance at 595 nm.

6 Passive adsorption of the monoclonal antibody onto latex particles

Each of 16 ammonium sulfate precipitated Mab was passively adsorbed onto latex particles. A mixture of Mab at a final concentration of 200-700 μg/ml and latex particles (0.84 μm in diameter; Interfacial Dynamics Corp., Portland, USA) at a final concentration of 1% in glycine-buffered saline was incubated with gently rotated at room temperature for overnight. The unbound Mab in the supernatant was removed by centrifugation at 5,000 x g (High speed micro refrigerated centrifuge, Tomy; Model MRX-150) for 10 minutes at 4 ºC and kept for protein determination. The remaining protein binding sites on the surface of the latex particles were blocked with 1% (w/v) solution of BSA for 2 hr at room temperature. Then, the coated latex particles were washed 2 times with glycine-buffered saline containing 0.1% BSA. Finally, the coated latex particles were resuspended with storage buffer for a final concentration of 0.5% latex suspension. The latex suspension were stored at 4 ºC up to one year.

7 Latex agglutination test (LAT)

The latex agglutination was performed by mixing 20 μl of leptospiral broth culture or antigen suspension with 1 drop (20 μl) of latex reagent on a clean glass slide and mixed with wooden applicator. Thereafter, the slide was gently mixed by manual. Agglutination could be seen in the dark background within 5 min. The agglutination reaction was scored from 1+ to 3+. Typically, a 3+ reaction was observed as large

floccules formed instantly with clear background fluid. A negative result was characterized by uniform milky fluid with no visible floccules.

8 Performance of the latex reagents

The performance of latex reagent product was observed on specificity, sensitivity and stability.

The specificity of 16 latex reagents were performed using the panel antigen prepared from 26 serovars of *Leptospira,* and 10 genera of the other bacteria. The reference representative serovars from each *Leptospira* serogroup and other bacterial antigens at concentration of 1 mg/ml were also tested with latex reagent by mixing 20 μl of whole cell antigen suspension with 1 drop (20 μl) of latex reagent on a clean glass slide and mixed with wooden applicator. Thereafter, the slide was gently mixed by manual. Agglutination could be seen within 5 min.

The sensitivity or lower limit of detection of 16 latex reagents was performed using viable leptospires. The minimal concentration of the bacteria that required for agglutination is the sensitivity of the test. The reference *Leptospira* were triplicate counted under dark-field microscope at 200x magnification by using hemocytometer (Fast-Read 102®, Hycor Biomedical Inc., Italy). These panel latex reagents were tested with various concentration of the corresponding *Leptospira* and the sensitivity of the test was calculated.

The stability of these panel latex reagents was performed every month using aliquot of latex reagent kept at 4 ºC to evaluate the sensitivity of the agglutination test with the homologous secreted antigen. By mixing 20 μl of secreted antigen with 1 drop (20 μl) of latex reagent on a clean glass slide and mixed with wooden applicator. Thereafter, the slide was gently mixed by manual. Agglutination could be seen within 5 min. The agglutination reaction was scored from 1+ to 3+.

9 Evaluation of the efficacy of the panel latex reagent for the identification of the culture isolated leptospires

To evaluate the efficacy of the panel latex reagents, 125 leptospires isolated from rats' kidneys (provided by Armed Forces Research Institute of Medical Science, Thailand) and 113 leptospires isolated from patients' blood samples (provided by the

Department of Medicine, Faculty of Medicine Siriraj Hospital and the Wellcome-Mahidol Laboratory, Faculty of Tropical Medicine, Mahidol University, Thailand) were agglutinated with the panel latex reagents. The identification results were analyzed in comparison to the reference method, cross agglutination absorption test.

10 Statistical analysis

The relative sensitivity and specificity of latex agglutination test (LAT) for the identification of leptospires in culture isolated from rats' kidneys and patients' blood samples were determined in comparison to the CAAT as described below:

- Sensitivity = $[a / (a + c)] \times 100$

  a is the number of sample positive by LAT and CAAT

  c is the number of sample positive by CAAT but negative by LAT
- Specificity = $[d / (b + d)] \times 100$,

  d is the number of sample negative by LAT and CAAT,

  b is the number of sample negative by CAAT but positive by LAT

The results obtained from the tests were analyzed for the percentage agreement with CAAT by using the kappa statistics. The kappa statistics is a decimal measure of agreement between two tests, and is defined as Kappa (κ). Kappa value was interpreted by following this guideline (54).

0.93-1.00	Excellent agreement
0.81-0.92	Very good agreement
0.61-0.80	Good agreement
0.41-0.60	Fair agreement
0.21-0.40	Slight agreement
0.01-0.20	Poor agreement
0.00	No agreement

$$\kappa = [(a + d) - P] / (1 - P)$$

P is the probability; $P = (a + b)(a + c) + (c + d)(b + d)$

a is the number of samples positive by both LAT and CAAT

b is the number of samples positive by CAAT but negative by LAT

c is the number of samples negative by CAAT but positive by LAT

d is the number of samples negative by both CAAT and LAT

CHAPTER V
RESULTS

1. Development of latex agglutination test for identification of leptospires

1.1 Precipitation of monoclonal antibody from the culture supernatant fluid by 50% ammonium sulfate

Each culture supernatant fluid from 16 clones of hybridoma cell culture containing monoclonal antibodies (Mabs) specific to leptospiral serogroup/serovar (Table 10) was precipitated with 50% ammonium sulfate and desalted by dialysis with 0.15M PBS pH 7.4. The protein concentrations of the ammonium sulfate precipitated monoclonal antibodies were measured by Bradford protein assay and the results are shown in Table 10.

1.2 Passive adsorption of the monoclonal antibodies onto the latex particles

The optimal conditions of Mabs for passive adsorption onto latex particles were investigated based on the percentage of Mab binding, the intensity of agglutination reaction, and the rapidity of agglutination time. The optimal concentration of Mabs both IgG and IgM to be adsorbed onto 0.84 μm polystyrene sulfate latex particles were between 400 – 500 μg/ml (Figure 2: A-D). The optimal Mab concentration for latex agglutination is the concentration that gives fastest and strongest agglutination reaction. The latex particles were coated with the antibody for 16 h at room temperature. The supernatants were kept for detection of unbound immunoglobulin by protein assay. The coated latex particles were blocked with 1.0% BSA in GBS for 2 hr. Then, the coated latex particles were resuspended in storage buffer and used for identification of the leptospires. The latex agglutination test has done by mixing equal volume of 20 μl leptospires culture and coated latex particle and gently rotated by manual. The agglutination was readed within 5 minutes by eyes. The

intensity of agglutination reaction was scored from 1+ to 3+ by strength of agglutination between latex reagent and leptospiral antigen as shown in Figure 3.

1.3 Specificity of the latex reagent

Latex reagent was determined for nonspecific of autoagglutination with 0.15M PBS pH 7.4 and leptospiral EMJH culture medium. The results showed no agglutination of all latex reagents with both PBS and EMJH medium.

The specificity of the latex reagents was further tested with whole cell antigens prepared from 26 serovars of leptospires and 10 genera of the other bacteria (see section 2 in materials and methods). The results in Table 11 and 12 showed that the whole cell antigen suspension of individual serovar of *Leptospira* agglutinated with the homologous latex reagent whereas the panel of heterologous leptospiral whole cell antigens and other bacterial antigens showed no agglutination with all latex reagents.

1.4 Sensitivity of the latex reagent

The sensitivity of the latex agglutination was tested by using a different concentration of homologous leptospira varied from 10^6 – 10^8 cells/ml. The latex reagent that can agglutinate the bacteria at a minimum concentration is the sensitivity of the test. The sensitivity of 16 latex reagents are shown in Table 13.

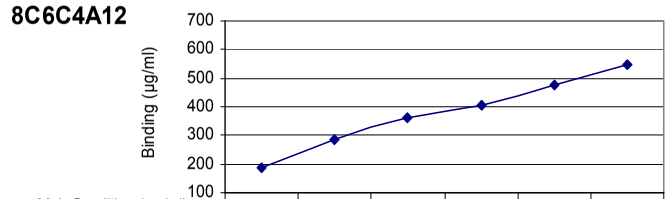
1.5 Stability of the latex reagent

An aliquot of latex reagent kept at 4 °C was taken at monthly interval to evaluate the sensitivity of agglutination test with the homologous secreted antigen. The sensitivity and stability of latex agglutination reaction with secreted antigen is shown in Table14. All latex reagents maintained their sensitivity and specificity properties for at least 12 months.

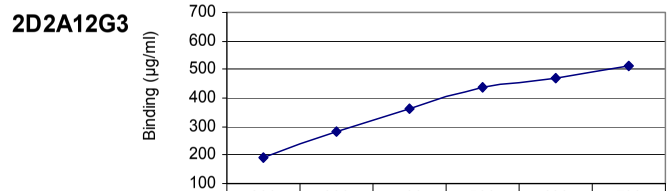
Table 10 Characteristics and concentrations of Mabs used for latex reagent preparations.

No.	Code	Isotype	Serovar specific	Protein concentration* (μg/ml)
1	8C6C4A12	IgMκ	Australis, Bangkok	145
2	2D2A12G3	IgMκ	Autumnalis	250
3	1B4D3F10	IgMκ	Bataviae	173
4	15B5C2G1	IgMκ	Biflexa	150
5	4A2D5D6	$IgG_3κ$	Bratislava	136
6	16C4E11E5	IgMκ	Canicola	119
7	20A5A6	IgMκ	Cellidoni	123
8	2B3A8H9	$IgG_3κ$	Cynopteri	131
9	4D4E8F10	$IgG_3κ$	Grippotyphosa	153
10	9A6E1E11	IgMκ	Hebdomadis	141
11	7B5E11D1	IgMκ	Pomona	109
12	16A3A4	IgMκ	Javanica	203
13	1A4B10E1	IgMκ	Pyrogenes	112
14	13C2E4F8	$IgG_3κ$	Sarmin	220
15	16B1C9H	IgMκ	Sejroe, Hardjo, Wolffi	122
16	9D4B12F5	IgMκ	Tarassovi	163

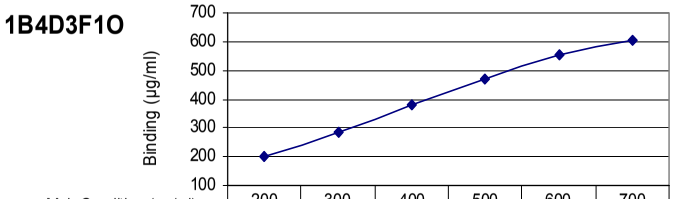
* yield of partially purified Mabs per milliliter of culture supernatant.



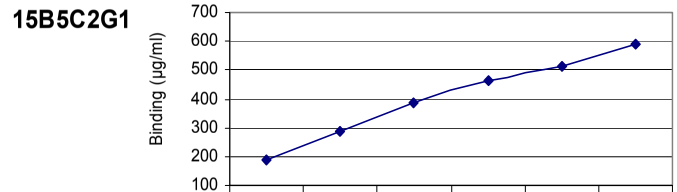
Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	190	285	360	405	474	548
Agglutination reaction	2+	2+	3+	3+	3+	2+
Agglutination time (Sec)	120	60	45	45	90	120



Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	190	283	364	435	467	512
Agglutination reaction	2+	3+	3+	3+	3+	2+
Agglutination time (Sec)	120	120	90	90	120	240

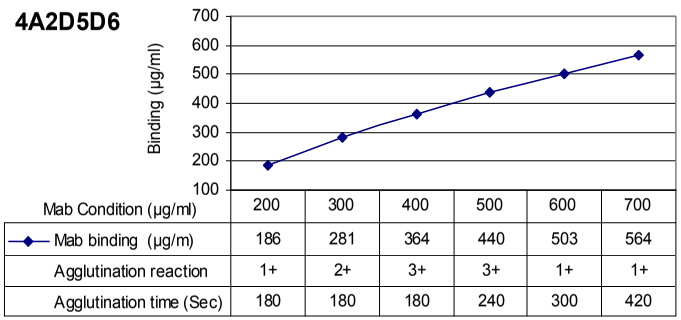


Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	200	287	380	472	555	606
Agglutination reaction	1+	2+	3+	3+	3+	2+
Agglutination time (Sec)	150	90	60	45	45	60

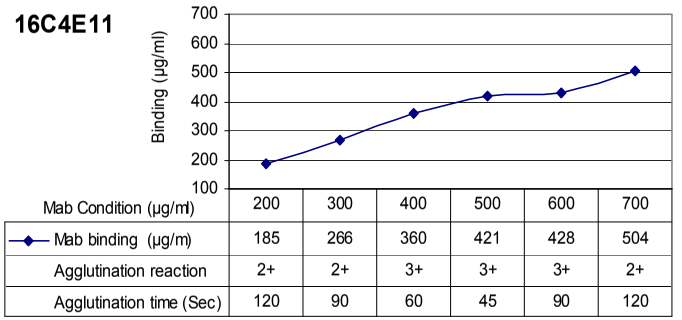


Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	189	287	387	461	512	588
Agglutination reaction	3+	3+	3+	3+	3+	2+
Agglutination time (Sec)	120	90	45	45	90	180

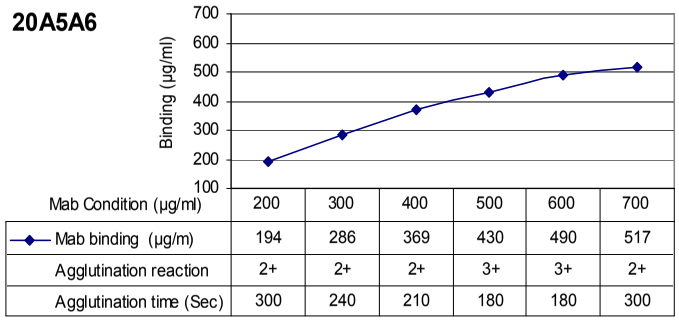
Figure 2 (A) Optimization of Mab, 8C6C4A12, 2D2A12G3, 1B4D3F10, or 15B5C2G1 bound onto fixed amount of 0.84 µm polystyrene sulfate latex particles. Various concentration of Mab, 200-700 µg/ml, were adsorbed onto the latex particles. The optimal concentration of Mabs to be adsorbed onto latex particles were between 400-500 µg/ml that showed fasten and strongest agglutination reaction.



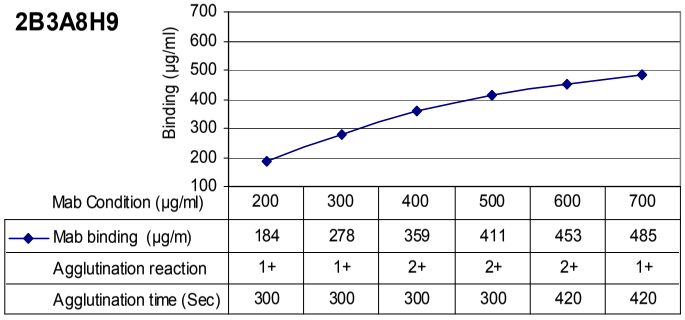
Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	186	281	364	440	503	564
Agglutination reaction	1+	2+	3+	3+	1+	1+
Agglutination time (Sec)	180	180	180	240	300	420



Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	185	266	360	421	428	504
Agglutination reaction	2+	2+	3+	3+	3+	2+
Agglutination time (Sec)	120	90	60	45	90	120

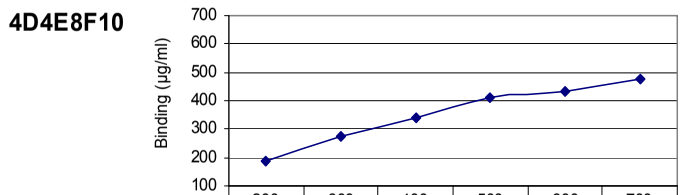


Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	194	286	369	430	490	517
Agglutination reaction	2+	2+	2+	3+	3+	2+
Agglutination time (Sec)	300	240	210	180	180	300

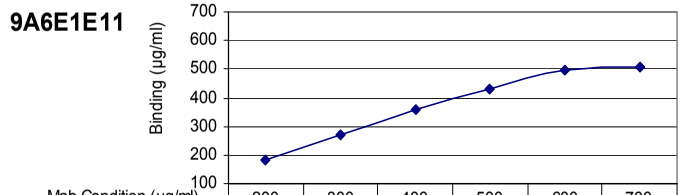


Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	184	278	359	411	453	485
Agglutination reaction	1+	1+	2+	2+	2+	1+
Agglutination time (Sec)	300	300	300	300	420	420

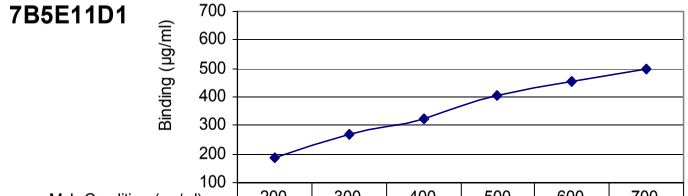
Figure 2 (B) Optimization of Mab, 4A2D5D6, 16C4E11, 20A5A6, or 2B3A8H9 bound onto fixed amount of 0.84 µm polystyrene sulfate latex particles. Various concentration of Mab, 200-700 µg/ml, were adsorbed onto the latex particles. The optimal concentration of Mabs to be adsorbed onto latex particles were between 400-500 µg/ml that showed fasten and strongest agglutination reaction.



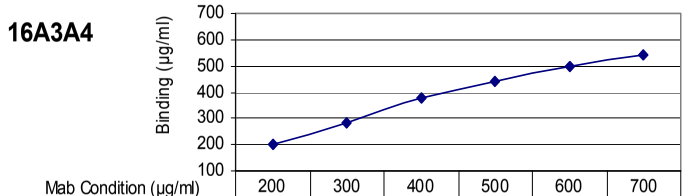
Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	187	273	338	409	433	479
Agglutination reaction	2+	3+	3+	2+	1+	1+
Agglutination time (Sec)	240	240	240	300	480	480



Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	181	272	360	432	496	508
Agglutination reaction	2+	2+	3+	3+	3+	2+
Agglutination time (Sec)	180	150	120	120	150	240

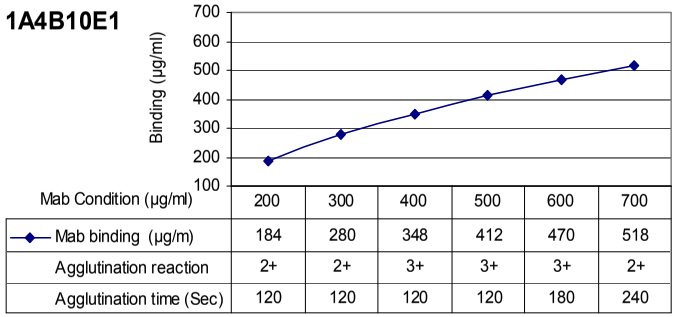


Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	188	269	322	408	452	500
Agglutination reaction	1+	2+	2+	2+	1+	1+
Agglutination time (Sec)	300	300	300	240	360	420

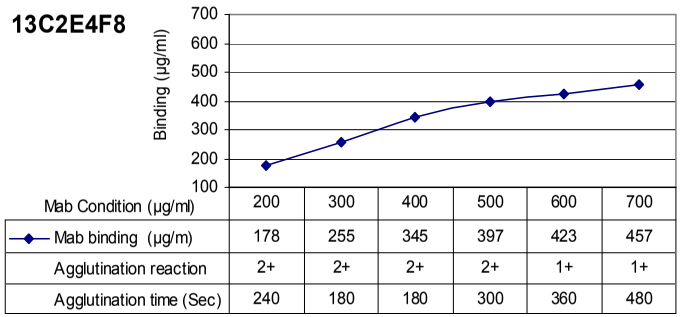


Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	200	282	375	443	498	545
Agglutination reaction	1+	2+	3+	3+	3+	1+
Agglutination time (Sec)	180	180	180	180	240	240

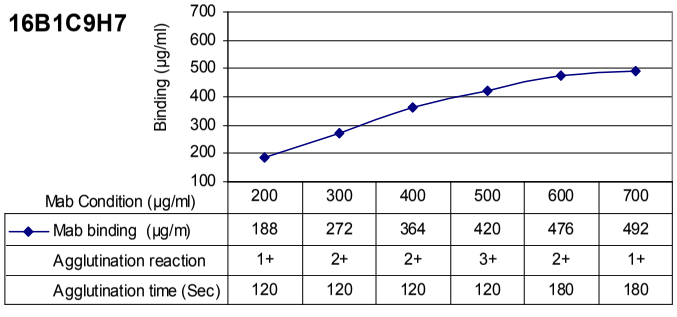
Figure 2 (C) Optimization of Mab, 4D4E8F10, 9A6E1E11, 7B5E11D1, or 16A3A4 bound onto fixed amount of 0.84 µm polystyrene sulfate latex particles. Various concentration of Mab, 200-700 µg/ml, were adsorbed onto the latex particles. The optimal concentration of Mabs to be adsorbed onto latex particles were between 400-500 µg/ml that showed fasten and strongest agglutination reaction.



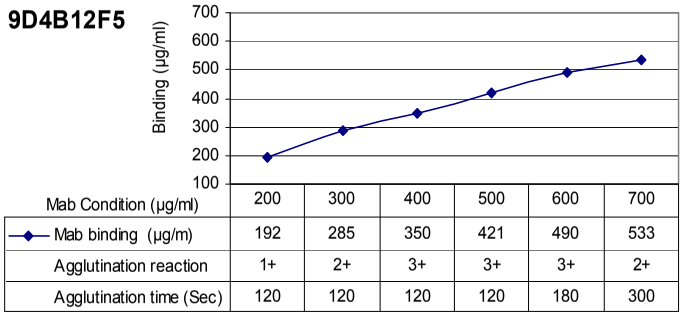
Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	184	280	348	412	470	518
Agglutination reaction	2+	2+	3+	3+	3+	2+
Agglutination time (Sec)	120	120	120	120	180	240



Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	178	255	345	397	423	457
Agglutination reaction	2+	2+	2+	2+	1+	1+
Agglutination time (Sec)	240	180	180	300	360	480



Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	188	272	364	420	476	492
Agglutination reaction	1+	2+	2+	3+	2+	1+
Agglutination time (Sec)	120	120	120	120	180	180



Mab Condition (µg/ml)	200	300	400	500	600	700
Mab binding (µg/m)	192	285	350	421	490	533
Agglutination reaction	1+	2+	3+	3+	3+	2+
Agglutination time (Sec)	120	120	120	120	180	300

Figure 2 (D) Optimization of Mab, 1A4B10E1, 13C2E4F8, 16B1C9H7, or 9D4B12F5 bound onto fixed amount of 0.84 µm polystyrene sulfate latex particles. Various concentration of Mab, 200-700 µg/ml, were adsorbed onto the latex particles. The optimal concentration of Mabs to be adsorbed onto latex particles were between 400-500 µg/ml that showed fasten and strongest agglutination reaction.

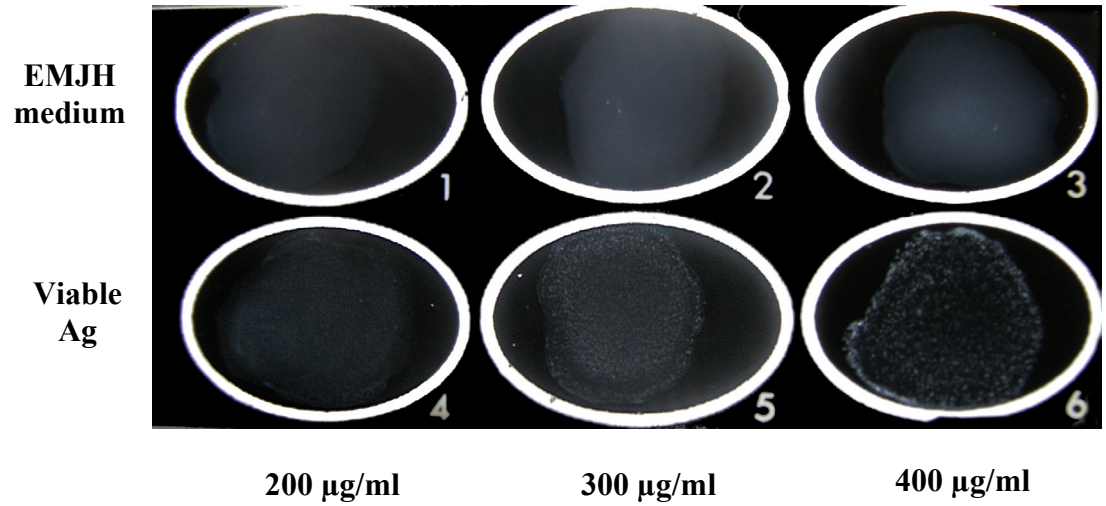


Figure 3 Latex agglutination reactions using latex reagents prepared by coating the latex particles with various concentrations of Mab (4A2D5D6), at 200 µg/ml (No. 1, 4); 300 µg/ml (No. 2, 5); and 400 µg/ml (No. 3, 6). The negative control was EMJH medium (No.1-3). *L. interrogans* serovar Bratislava in EMJH medium at concentration of 10^6 cell/ml was No.4-6. The results were read after mixing for 4 minutes.

No.1 Negative control, no agglutination

No.2 Negative control, no agglutination

No.3 Negative control, no agglutination

No.4 Positive 1+, mild agglutination

No.5 Positive 2+, moderate agglutination

No.6 Positive 3+, heavy agglutination

Table 11 The specificity of the latex reagents tested with whole cell antigens prepared from 26 serovars of leptospires

Latex code \ Leptospira whole cell Ag	Australis	Bangkok	Autumnalis	Ballum	Bataviae	Bratislava	Canicolaa	Celledoni	Cynopteri	Djasiman	Grippotyphosa	Hepdomadis	Icterohaemo-rrhaiae
8C6C4A12	+	+	-	-	-	-	-	-	-	-	-	-	-
2D2A12G3	-	-	+	-	-	-	-	-	-	-	-	-	-
1B4D3F10	-	-	-	-	+	-	-	-	-	-	-	-	-
4A2D5D6	-	-	-	-	-	+	-	-	-	-	-	-	-
16C4E11E5	-	-	-	-	-	-	+	-	-	-	-	-	-
20A5A6	-	-	-	-	-	-	-	+	-	-	-	-	-
2B3A8H9	-	-	-	-	-	-	-	-	+	-	-	-	-
4D4E8F10	-	-	-	-	-	-	-	-	-	-	+	-	-
9A6E1E11	-	-	-	-	-	-	-	-	-	-	-	+	-
7B5E11D1	-	-	-	-	-	-	-	-	-	-	-	-	-
16A3A4	-	-	-	-	-	-	-	-	-	-	-	-	-
1A4B10E1	-	-	-	-	-	-	-	-	-	-	-	-	-
13C2E4F8	-	-	-	-	-	-	-	-	-	-	-	-	-
16B1C9H	-	-	-	-	-	-	-	-	-	-	-	-	-
9D4B12F5	-	-	-	-	-	-	-	-	-	-	-	-	-
15B5C2G1	-	-	-	-	-	-	-	-	-	-	-	-	-

(+) agglutination; (-) no agglutination

Table 11 The specificity of the latex reagents tested with whole cell antigens prepared from 26 serovars of leptospires (continued)

Leptospira whole cell Ag / Latex code	Javanica	Louisiana	Mini	Pomona	Pyrogenes	Ranarum	Sarmin	Sejroe	Hardjo	Wolffi	Shermani	Tarassovi	Patoc
8C6C4A12	-	-	-	-	-	-	-	-	-	-	-	-	-
2D2A12G3	-	-	-	-	-	-	-	-	-	-	-	-	-
1B4D3F10	-	-	-	-	-	-	-	-	-	-	-	-	-
4A2D5D6	-	-	-	-	-	-	-	-	-	-	-	-	-
16C4E11E5	-	-	-	-	-	-	-	-	-	-	-	-	-
20A5A6	-	-	-	-	-	-	-	-	-	-	-	-	-
2B3A8H9	-	-	-	-	-	-	-	-	-	-	-	-	-
4D4E8F10	-	-	-	-	-	-	-	-	-	-	-	-	-
9A6E1E11	-	-	-	-	-	-	-	-	-	-	-	-	-
7B5E11D1	-	-	-	+	-	-	-	-	-	-	-	-	-
16A3A4	+	-	-	-	-	-	-	-	-	-	-	-	-
1A4B10E1	-	-	-	-	+	-	-	-	-	-	-	-	-
13C2E4F8	-	-	-	-	-	-	+	-	-	-	-	-	-
16B1C9H	-	-	-	-	-	-	-	+	+	+	-	-	-
9D4B12F5	-	-	-	-	-	-	-	-	-	-	-	+	-
15B5C2G1	-	-	-	-	-	-	-	-	-	-	-	-	+

(+) agglutination; (-) no agglutination

Table 12 The specificity of the latex reagents tested with whole cell antigens prepared from 10 genera of the other bacteria

Latex code \ Other bacterial whole cell Ag	*A. hydrophila*	*B. pseudomallei*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*Proteus* spp.	*Salmonella* spp.	*Shigella* spp.	*S. aureau*	*Streptococcus* spp.
8C6C4A12	-	-	-	-	-	-	-	-	-	-
2D2A12G3	-	-	-	-	-	-	-	-	-	-
1B4D3F10	-	-	-	-	-	-	-	-	-	-
4A2D5D6	-	-	-	-	-	-	-	-	-	-
16C4E11E5	-	-	-	-	-	-	-	-	-	-
20A5A6	-	-	-	-	-	-	-	-	-	-
2B3A8H9	-	-	-	-	-	-	-	-	-	-
4D4E8F10	-	-	-	-	-	-	-	-	-	-
9A6E1E11	-	-	-	-	-	-	-	-	-	-
7B5E11D1	-	-	-	-	-	-	-	-	-	-
16A3A4	-	-	-	-	-	-	-	-	-	-
1A4B10E1	-	-	-	-	-	-	-	-	-	-
13C2E4F8	-	-	-	-	-	-	-	-	-	-
16B1C9H	-	-	-	-	-	-	-	-	-	-
9D4B12F5	-	-	-	-	-	-	-	-	-	-
15B5C2G1	-	-	-	-	-	-	-	-	-	-

(+) agglutination; (-) no agglutination

Table 13 The sensitivity of the latex reagents tested with their homologous viable leptospires

No.	Latex code	*Leptospira* (serovar)	Sensitivity (cells/ml)
1	8C6C4A12	Australis	6.40×10^6
2	2D2A12G3	Autumnalis	5.92×10^6
3	1B4D3F10	Bataviae	8.90×10^6
4	15B5C2G1	Biflexa	8.66×10^6
5	4A2D5D6	Bratislava	3.38×10^6
6	16C4E11E5	Canicola	5.53×10^6
7	20A5A6	Cellidoni	2.58×10^6
8	2B3A8H9	Cynopteri	1.03×10^7
9	4D4E8F10	Grippotyphosa	6.66×10^6
10	9A6E1E11	Hebdomadis	7.50×10^6
11	7B5E11D1	Pomona	2.68×10^7
12	16A3A4	Javanica	8.56×10^6
13	1A4B10E1	Pyrogenes	9.66×10^6
14	13C2E4F8	Sarmin	7.75×10^6
15	16B1C9H	Sejroe	7.73×10^6
16	9D4B12F5	Tarassovi	9.10×10^6

Table 14 The sensitivity and stability of the latex reagents tested with their homologous secreted leptospiral antigens

No.	Latex code	Secreted Ag (serovar)	Sensitivity (CHO, ng/20 µl)	Stability (month)[@]			
				1	4	8	12
1	8C6C4A12	Australis	70#	3+	3+	3+	3+
2	2D2A12G3	Autumnalis	70#	3+	3+	3+	3+
3	1B4D3F10	Bataviae	20#	3+	3+	3+	3+
4	15B5C2G1	Biflexa	168#	3+	3+	3+	3+
5	4A2D5D6	Bratislava	420#	3+	3+	3+	3+
6	16C4E11E5	Canicola	270#	3+	3+	3+	3+
7	20A5A6	Cellidoni	1720*	3+	3+	3+	3+
8	2B3A8H9	Cynopteri	540*	2+	2+	2+	2+
9	4D4E8F10	Grippotyphosa	294*	3+	3+	3+	3+
10	9A6E1E11	Hebdomadis	920*	3+	3+	3+	3+
11	7B5E11D1	Pomona	750*	2+	2+	2+	2+
12	16A3A4	Javanica	870*	3+	3+	3+	3+
13	1A4B10E1	Pyrogenes	199#	3+	3+	3+	3+
14	13C2E4F8	Sarmin	420*	2+	2+	2+	2+
15	16B1C9H	Wolffi	80#	3+	3+	3+	3+
16	9D4B12F5	Tarassovi	680*	3+	3+	3+	3+

Measured with secreted antigen.

* Measured with supernatant of freezing whole cell antigen.

[@]All latex reagents maintained their stability for at least 12 months. The same score of agglutination reaction at sensitivity level of secreted Ag and homologous latex reagent showed between first to twelve month.

2. Evaluation of the latex agglutination test with the cultured leptospires isolated from rats' kidneys

One hundred and twenty five leptospires isolated from rats' kidneys identified by the latex agglutianation test were compared with the reference CAAT method. The results are shown in Table 15. Two of 125 isolates could not be identified by CAAT. However, one of them could identified by latex agglutination as Javanica.

Both latex agglutination and CAAT methods demonstrated 100% comparable in sensitivity and specificity. The Kappa value of this test is 1.00 which suggests that the 2 methods are excellent agreement.

3. Evaluation of the latex agglutination test with the cultured leptospires isolated from patients' blood specimens

One hundred and thirteen leptospires isolated from patients' blood samples were double blind identified by latex agglutianation compared with the reference CAAT method. The results (Table 16) showed that *Leptospira* serogroup/serovar identified by both techniques were almost identical. However, one isolate was identified as Pyrogenes by CAAT while latex agglutination identified as Autumnalis.

The sensitivity and specificity of latex agglutination for identification of leptospires isolated from patients' blood samples were 100%, and 99.11%, respectively. The Kappa value of this testing is 0.99 which suggests the excellent agreement of these 2 tests.

In additional, 84 leptospires isolated from patients' blood samples were also identified by latex agglutination. The results are shown in Table 17. Fifteen samples were sent to WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Center for Public Health Science, Queensland, Australia and the bacteria did not grow in the subculture medium therefore, the CAAT could not be performed. The other 69 samples were not sent to identify by CAAT. Fifty seven (67.86%) of 84 could be identified by latex agglutination. These leptospires were identified as serovars Australis (1 isolate), Autumnalis (23 isolates), Bataviae (10 isolates), Canicola (2 isolates), Grippotyphosa (3 isolates), Javnica (3 isolates), Pyrogenes (5 isolates), and Sejroe (10 isolates). However, with a limit of latex

reagents in the panel, 27 samples (32.14%) could not be identified by latex agglutination.

Table 15 One hundred and twenty five leptospires isolated from rats' kidneys identified by latex agglutination and cross absorption agglutination test (CAAT)

serogroup/serovar	LAT	CAAT
Australis	5	5
Autumnalis	13	13
Bataviae	42	42
Pyrogenes	63	63
Javanica	1*	-
Unidentify	1*	2*
Total	125	125

* Two samples could not be identified by CAAT and one of them was Javanica when identified by LAT, another one was unidentified which may be due to the limitation of the produced 16 latex reagents which are not covered the serogroup/serovar in the samples.

Table 16 One hundred thirteen leptospires isolated from patients' blood samples double blind identified by latex agglutination (LAT) compared with the reference cross agglutination absorption test (CAAT)

serogroup/serovar	LAT	CAAT
Autumnalis	100*	99
Bataviae	1	1
Hebdomadis	1	1
Javanica	3	3
Pyrogenes	8	9*
Total	113	113

* One isolate was identified as Pyrogenes by CAAT and as Autumnalis by LAT.

Table 17 The capability of 16 latex reagents that could identify the leptospires isolated from patients' blood samples

Method	CAAT			Total
	Result	Unidentified	Not done	
LAT	Identified	15	42	57
	Unidentified	0	27	27
Total		15	69	84

One hundred and twenty eight leptospires (113 from table 16 and 15 from table 17) were sent to WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Center for Public Health Science, Queensland, Australia for identifying the serogroup/serovar by CAAT and 113 (table 16) were able to identify. All of them (128 leptospires) could be identified by LAT. However, 42 of 69 leptospires which had no data of serogroup/serovar identified by CAAT could be identified by LAT. Put up together, 170 of 197 (86.30%) leptospires could be identified by LAT using 16 latex reagents.

CHAPTER VI

DISCUSSION

Leptospirosis is an important worldwide zoonotic disease caused by *Leptospira interogans*. During 1996-2002, there were outbreaks of the disease in the Northeastern part of Thailand. Then, in 2003-2006 the morbidity rates were decrease. In 2006, the number of leptospirosis patient maintained highly about 6.13 per 100,000 population (3,828 cases) (Figure 1A, B). This serosurveillance data were obtained from microscopic agglutination test (MAT) which detected the specific leptospiral antibodies in patients' sera using the panel of reference leptospiral serovars or serogroups as antigens (3). However, the false negative results reported may be occurred, if the existed serovars or serogroups were not included in panel antigens. Thus, the antigens in the assay should include a representative serovar from each serogroup of *Leptospira*. Moreover, cross-reaction between serogroups is common, as paradoxical reaction that the initial immune response is directed to heterologous serovar or serogroup. Paradoxical reactions may occur in up to 50% of cases (1). Overinterpretation of serological data is much greater if only acute-phase serum samples are available for testing. The usefullness of MAT for identification of the infecting leptospiral serogroup or serovar was evaluated by Levett *et al*. (3). The data of culture proven cases of leptospirosis that occurred in Barbados between 1980-1998 and the specificity and sensitivity of MAT for the prediction of the infecting serovars were determined. This study indicated that MAT using of the predominant serogroups to predict the infecting serovar is relatively insensitive. The prediction matches the serovar for only 46.4% and low specificity of samples isolated from recovered patients. Therefore, serological analysis appeared to be of little value for the identification of the infecting serovar in individual cases of leptospirosis in human. The presumptive serogroup reactivity data should be used for broad ideas of the common serogroups present in population but not for interprete reliably in individual patient. Therefore, the identification of major serogroup and serovar of leptospires that

caused leptospirosis is necessary for vaccine development and epidemiological study in Thailand. Several antigenic and genetic assays were developed for identification of leptospires. *Leptospira* classification is relied on two methods which are serological and molecular methods. The conventional serological method is microscopic agglutination test (MAT), using reference anti-leptospira sera and followed by cross agglutination adsorption test (CAAT). These assays are based on the reaction of unknown strains with different serogroup or serovar reference antisera which were prepared in rabbits (2). However, the disadvantages of this method are long time performing, laborious, and require the availability of all the reference strains and their antisera. Therefore, the test is restricted its usage for only at the reference laboratory centre. In addition, cross-agglutination reaction of reference sera with heterologous serogroup can be occurred at the low titer. Thus many laboratories have developed new identification techniques based on genetic heterogeneity among member of the genus *Leptospira*. The molecular techniques which were arbitrarily primed PCR (AP-PCR) (84-86), random amplified polymorphic DNA (RAPD) (84, 87), pulsed field gel electrophoresis (PFGE) (77, 78), and ribotyping (79-81) have been used for identification leptospires at serogroup and serovars level. However, these molecular techniques are not easy to apply for identification in the routine laboratories in Thailand because these techniques require the specialist and an expensive equipment to perform. The *Leptospira* classification based on serological method have been used for long time and more intensive used than molecular methods. Therefore, the rapid serological assays are required for leptospiral identification. Mouse monoclonal antibodies have been prepared for identification of leptospiral serogroups or serovars. The serogroups or serovars are identified based on the characteristic of agglutination pattern and panels of monoclonal antibodies allowed identification of leptospires at the serovar or sub-serovar level (93). Furthermore, the identification using monoclonal antibodies related well with conventional serological method. Thus, the monoclonal antibodies specific to serogroups or serovars of leptospires are very useful for rapid identification of isolated leptospires. Using of monoclonal antibodies provides several advantages including the high specificity of the monoclonal antibody with no crossreactivity as occur when polyclonal antibody against whole bacteria is used, minimal batch to batch variation, scale up production of the test reagents, and

reduction of the amount of laboratory animals. Mouse antibodies have been prepared and used for leptospiral identification (93-96). The difference in agglutination profile obtained from panel of monoclonal antibodies may indicate new leptospiral serogroup or serovar. However, the agglutination test need to read under dark-field microscopy. Therefore, the simple assay such as the latex agglutination test (LAT) is required for identification of leptospiral serogroups or serovars. The LAT is based on the immunological agglutination of monoclonal antibody-coated latex particles with the specific antigen on the surface of the bacteria. LAT is a simple, rapid, low cost and easy to perform. LAT has been successfully used for rapid identification for variety of clinically important microorganisms such as *Burkholderia pseudomallei*, *Candida dubliniensis*, and *Legionella pneumophila* (97-99).

The present study aimed at developing the latex agglutination test based on specific monoclonal antibodies to 15 serogroups of pathogenic leptospires, *L. interrogans* and one serogroup of non pathogenic leptospire, *L. biflexa* for detect and identify the bacterial in isolated cultures. This panel latex reagents will be used for identification of leptospires isolated from patient and animal specimen.

The 16 clones of in-house monoclonal antibodies have been produced in our laboratory. Panel monoclonal antibody was tested for their specificity by indirect ELISA using panel antigen prepared from representative leptospiral serovars of each leptospiral serogroup (Table 7) and other bacterial genera (Table 8). Each monoclonal antibody was highly specific to representative leptospiral serovars of each homologous leptospiral serogroup (Table 10). It did not react with representative leptospiral serovars of heterologous leptospiral serogroups and other bacterial genera. However, there are many more serovars in each leptospiral serogroup which were not included in the panel antigens because the limitation of stock leptospiral culture in Thailand. To confirm the serovar specific property, the monoclonal antibodies should be evaluated by using all serovars which are member of each serogroup.

Latex particles provide a convenient carrier for antigen or antibody in agglutination test. Although this rapid identification assay is simple to setup without expensive equipments, the finding of appropriate condition that provides high degree of agglutination reaction should be carefully determined. This study, we used polystyrene hydrophobic latex, because it has an excellent substrate which show large

surface area, negligible solubility in water, invariant surface characteristic, and homogeneous size (100). Most proteins can be readily adsorbed onto polystyrene bead by hydrophobic interaction which is largely independent on pH with most efficiency at pH that closes to theirs isoelectric point (pI). Various concentrations of each monoclonal antibody from 200 to 700 μg/ml were titrated for optimal absorption onto latex particles. Monoclonal antibody at concentration of 400 and 500 μg/ml showed the rapid and high levels of agglutination reaction (Figure 2A-D). The sensitivity of each latex reagent was characterized by agglutination with its homologous viable leptospires. The minimum quantity of leptospires that could be agglutinated by the latex reagent was ranged from 2.58×10^6 to 2.68×10^7 leptospires/ml (Table 14). Even though, the sensitivity of latex reagents is quite low but their sensitivity is not the problem for identification. Because the concentration of the leptospires to be identified can be increased after longer incubation, or increased bacterial cell concentration by centrifugation and adjust into small volume. This panel latex reagent was highly specific to representative leptospiral serovars of each homologous leptospiral serogroup with no crossreaction with representative leptospiral serovars of heterologous leptospiral serogroups (Table 12) and other bacterial genera (Table 13). The specificity of each latex reagents is correlated with the specificity of its corresponding monoclonal antibody. Furthermore, the sensitivity and specificity of latex reagent is stable for at least 12 months when kept at 4 °C. Thus, this panel latex reagent could be a new tool for the rapid serogroup identification of leptospires in Thailand.

The efficacy of the panel latex reagents was evaluated and compared with the reference CAAT method (performed by Dr. Lee Smythe from WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Center for Public Health Science, Queensland, Australia). The samples were 125 leptospires isolated from rats' kidney. The results (Table 15) showed that the panel latex reagents could identify 124 leptospiral isolates while CAAT could identify 123 leptospiral isolates. Two leptospiral isolates that could not be identified by CAAT were died during transportation to the reference laboratory. The latex agglutination overcomes this problem because it can recognize with either live or death leptospires. However, the panel latex reagents could not identify one culture isolate. This result may be due to

the limitation of the 16 produced latex reagents which are not covered the serogroup/serovar in this sample. Another samples are 113 leptospires isolated from patients' blood samples identified by CAAT. The correspondence of the identification by using panel latex reagents and CAAT was found in 112 of 113 samples (Table 16). However, one isolate was identified as serovar Pyrogenes by CAAT while latex agglutination identified as serogroup/serovar Autumnalis. This isolate was related with new lot of this latex reagents that specific to serogroup/serovar Autumnalis and Pyrogenes. The result showed that this isolate agglutinated with anti-Autumnalis latex reagent but not anti-Pyrogenes latex reagent

In addition, 84 leptospires isolated from patients' blood samples with no results of identification by CAAT were also included for evaluation of latex agglutination identification (Table 17). Fifteen samples were sent to WHO reference laboratory (WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Center for Public Health Science, Queensland, Australia) but the bacteria did not grow in the subculture medium. Therefore, the CAAT could not be performed. These 15 leptospiral isolates could be identified by latex agglutination. So 113 of 128 leptospires isolated from patients' blood samples (88.28%) were able to identify by CAAT while all 128 isolates were readily identified by latex agglutination. These evidences support that latex agglutination can reduce the risk factors for unidentification such as sample contamination or died during transportation. It could identify death leptospires in older sample or other bacteria contaminated sample (data not show). Another 69 samples which were not sent to identify by CAAT, but it were further identified by latex agglutination. Forty two of 69 samples could be identified by this method. To clear up this limitation, more monoclonal antibody specific to other leptospiral serogroups should be produced to develop the full panel of latex reagents for identification of all serogroup of *L. interrogans*. This panel latex reagents could be used for identification of leptospiral serogroup of the isolated bacteria or to check the specificity of stock antigens in MAT testing. Furthermore, this test can be used for identification and tracing the relationship of prevalence serogroups/serovars in reservoirs and in patients. These data are beneficial for epidemiological study, proper protection and vaccine development.

CHAPTER VII

CONCLUSION

In the present study, 16 latex reagents were developed using 16 clones of in-house monoclonal antibodies that specific to 16 leptospriral serogroups/serovars commonly reported in Thailand during the past ten years which were Australis, Autumnalis, Bataviae, Bratislava, Canicola, Cellidoni, Cynopteri, Grippotyphosa, Hebdomadis, Pomona, Javanica, Pyrogenes, Sarmin, Sejroe, Tarassovi, and Semaranga. The latex reagents were evaluated for the identification of leptospires isolated from rats' kidneys and patients' blood samples comparing with cross agglutination absorption test (CAAT)

The monoclonal antibodies were partially purified and concentrated by 50% ammonium sulfate precipitation and passively adsorbed onto the latex particles. The optimal concentrations of the monoclonal antibody to be adsorbed onto 1% of polystyrene latex particles (0.84 µm in diameter; Interfacial Dynamics Corp., Portland, USA) were found to be 400 – 500 µg/ml. The latex particles coated with the monoclonal antibodies were used in the latex agglutination test. The latex agglutination test was performed by mixing an equal volume, 20 µl, of the bacterial culture and the latex reagent. Agglutination reaction can be visualized by eyes within 5 minutes. The intensity of agglutination reaction is graded from 1+ to 3+ that depend on the concentration of specific antigen.

The minimal bacterial quantity that can be agglutinated by each latex reagent was ranged from 5.16×10^4 - 5.36×10^5 cells. The latex particles coated with monoclonal antibodies were stable and maintained their original sensitivity and specificity for at least 12 months.

The panel latex agglutination was evaluated with 125 leptospires isolated from rats' kidneys comparing with the reference CAAT method. Both latex agglutination and CAAT methods demonstrated 100% correlation in sensitivity and specificity.

The Kappa value of this test is 1.00 which suggests that the 2 methods are excellent agreement.

One hundred and thirteen leptospires isolated from patients' blood samples were double blind identified by latex agglutination comparing with the reference CAAT method. The results showed that *Leptospira* serogroup/serovar identified by both techniques were almost identical. The sensitivity and specificity of latex agglutination for identification of leptospires isolated from patients' blood samples were 100%, and 99.11%, respectively. The Kappa value of this testing is 0.99 which suggests the excellent agreement of these 2 tests.

In additional, 84 leptospires isolated from patients' blood samples which were unknown serogroup/serovar were including for identification by latex agglutination. Fifteen samples were sent to WHO reference laboratories and the bacteria did not grow in the subculture medium therefore, the CAAT could not be performed. These 15 leptospiral isolates could be identified by latex agglutination. The other 69 samples were not sent to identify by CAAT, but it were further identified by latex agglutination.

In conclusion, 128 leptospires isolated from patients' blood samples were sent to WHO reference laboratories. Only 113 of 128 leptospires isolated from patients' blood samples (88.28%) were able to identify by CAAT while all 128 isolates (100%) were readily identified by latex agglutination. In other 69 samples were further identified by latex agglutination. Because the incomplete of latex reagents in the panel, only 42 of 69 leptospires isolated from patients' blood samples (60.87%) were able to identify by latex agglutination. However, all of 197 leptospires isolated from patients' blood samples, 170 samples (86.30%) could identify by latex agglutination.

The panel latex reagents are useful for rapid identification of leptospiral serovars/serogroups including Australis, Autumnalis, Bataviae, Bratislava, Canicola, Cellidoni, Cynopteri, Grippotyphosa, Hebdomadis, Pomona, Javanica, Pyrogenes, Sarmin, Sejroe, Tarassovi, and Semaranga.

REFERENCES

1. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001; 14(2): 296-326.
2. Turner LH. Leptospirosis.II Serology. Trans R Soc Trop Med Hyg. 1968; 62(6): 880-99.
3. Levett PN. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin Infect Dis. 2003; 36(4): 447-52.
4. Murhekar MV, Sugunan AP, Vijayachari P, Sharma S, Sehgal SC. Risk factors in the transmission of leptospiral infection. Indian J Med Res. 1998; 107 : 218-23.
5. Vanasco NB, Sequeira G, Dalla Fontana ML, Fusco S, Sequeira MD, Enria D. [Report on a leptospirosis outbreak in the city of Santa Fe, Argentina, March-April 1998]. Rev Panam Salud Publica. 2000; 7(1): 35-40.
6. Faine S AB, Bolin C, Perolat P. *Leptospira* and Leptospirosis. 2 nd ed. Melbourne, Australia: MediSci; 1999. p. 57-72.
7. Ruud PAMS. The Molecular Analysis of Sphingomyelinase Gene of Leptospiraceae. Netherland; 1991. p. 11-2.
8. Vinh T, Adler B, Faine S. Ultrastructure and chemical composition of lipopolysaccharide extracted from Leptospira interrogans serovar copenhageni. J Gen Microbiol. 1986; 132(1): 103-9.
9. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for Leptospira alexanderi sp. nov. and four new Leptospira genomospecies. Int J Syst Bacteriol. 1999; 49 (Pt 2): 839-58.
10. Perolat P, Chappel RJ, Adler B, Baranton G, Bulach DM, Billinghurst ML, et al. Leptospira fainei sp. nov., isolated from pigs in Australia. Int J Syst Bacteriol. 1998; 48 (Pt 3) : 851-8.

11. Tangkanakul W, Tharmaphornpil P, Plikaytis BD, Bragg S, Poonsuksombat D, Choomkasien P, et al. Risk factors associated with leptospirosis in northeastern Thailand, 1998. Am J Trop Med Hyg. 2000; 63(3-4): 204-8.
12. Organization WH. Human leptospirosis : guidance for diagnosis, surveillance and control. Malta: world Health Organization; 2003. p 1-108
13. Phraisuwan P, Whitney EA, Tharmaphornpilas P, Guharat S, Thongkamsamut S, Aresagig S, et al. Leptospirosis: skin wounds and control strategies, Thailand, 1999. Emerg Infect Dis. 2002; 8(12): 1455-9.
14. Ashford DA, Kaiser RM, Spiegel RA, Perkins BA, Weyant RS, Bragg SL, et al. Asymptomatic infection and risk factors for leptospirosis in Nicaragua. Am J Trop Med Hyg. 2000; 63(5-6): 249-54.
15. International Leptospirosis Society. Leptospirosis: Disarming the Threat, 4th Scientific Meeting of The International Leptospirosis Society 2005. Chiang Mai, Thailand: 2005. p. 42-332.
16. Leonard FC, Quinn PJ, Ellis WA, O'Farrell K. Duration of urinary excretion of leptospires by cattle naturally or experimentally infected with Leptospira interrogans serovar hardjo. Vet Rec. 1992; 131(19): 435-9.
17. Matthias MA, Levett PN. Leptospiral carriage by mice and mongooses on the island of Barbados. West Indian Med J. 2002; 51(1): 10-3.
18. Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr., Riley LW. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet. 1999; 354 (9181): 820-5.
19. Johnson MA, Smith H, Joeph P, Gilman RH, Bautista CT, Campos KJ, et al. Environmental exposure and leptospirosis, Peru. Emerg Infect Dis. 2004; (6): 1016-22.
20. Sarkar U, Nascimento SF, Barbosa R, Martins R, Nuevo H, Kalafanos I, et al. Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. Am J Trop Med Hyg. 2002; 66(5): 605-10.
21. Romero EC, Bernardo CC, Yasuda PH. Human leptospirosis: a twenty-nine-year serological study in Sao Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 2003; 45(5): 245-8.

22. Tassinari Wde S, Pellegrini Dda C, Sabroza PC, Carvalho MS. [Spatial distribution of leptospirosis in the city of Rio de Janeiro, Brazil, 1996-1999]. Cad Saude Publica. 2004; 20(6): 1721-9.

23. Karande S, Bhatt M, Kelkar A, Kulkarni M, De A, Varaiya A. An observational study to detect leptospirosis in Mumbai, India, 2000. Arch Dis Child. 2003; 88(12): 1070-5.

24. Karande S, Gandhi D, Kulkarni M, Bharadwaj R, Pol S, Thakare J, et al. Concurrent outbreak of leptospirosis and dengue in Mumbai, India, 2002. J Trop Pediatr. 2005; 51(3): 174-81.

25. LaRocque RC, Breiman RF, Ari MD, Morey RE, Janan FA, Hayes JM, et al. Leptospirosis during dengue outbreak, Bangladesh. Emerg Infect Dis. 2005; 11(5): 766-9.

26. Murdoch DR, Woods CW, Zimmerman MD, Dull PM, Belbase RH, Keenan AJ, et al. The etiology of febrile illness in adults presenting to Patan hospital in Kathmandu, Nepal. Am J Trop Med Hyg. 2004; 70(6): 670-5.

27. Segura ER, Ganoza CA, Campos K, Ricaldi JN, Torres S, Silva H, et al. Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity with quantification of leptospiral burden. Clin Infect Dis. 2005; 40(3): 343-51.

28. Yunibandhu J. First report of Weil's disease in Thailand. Journal of Medical Associated of Thailand. 1943; 26(4): 83-147.

29. Tangkanakul W, Smits HL, Jatanasen S, Ashford DA. Leptospirosis: an emerging health problem in Thailand. Southeast Asian J Trop Med Public Health. 2005; 36(2): 281-8.

30. Arjkien W. Leptospirosis in northern Thailand. Monthly Epidemiology Surveill Rep. 2000; 3 : 1224-9.

31. Boonyod D, Tanjatham S, Luppanakul P, Kiatvitchukul C. Leptospira in patient sera in the lower north. Journal of Health Science. 2001; 10 : 508-15.

32. Chaifoo W, Tharmapornpilas P, Limpaiboon R, Braggs A. Clinical study for finding leptospirosis definition at Udon Thani Hospital, Udon Thani, and Octobe, 1997. Reg 6/2 Med J. 1998; 6 : 168-83.

33. Tangkanakul W, Hinjoy S, Smithsuwan P, Phulsuksombati D. Leptospira serovar in humans and animals, Nakhon Ratchasima. Monthly Epidemiology Surveill Rep. 2002; 33 : 155-62.

34. Montian A, Kusum M, Naigowit P, Kamaswat S. Leptospirosis in Northeastern province of Thailand in 1996. Journal of Health Science. 1997;6(2):241-7.

35. Tankanaakul W, Kingnate D. Leptospirosis epidemic in Northeastern provinces of Thailand in 1997. Journal of Health Science. 1998; 7(3): 386-95.

36. Levett PN, Branch SL, Whittington CU, Edwards CN, Paxton H. Two methods for rapid serological diagnosis of acute leptospirosis. Clin Diagn Lab Immunol. 2001; 8(2): 349-51.

37. Phulsuksombati D SN, Khoprasert Y, Kingnate D, Tangkanakul W. Leptospires in rodent, northeastern region 1999-2000. Journal of Health Science. 2001; 10 : 516-25.

38. Turner LH. Leptospirosis 3 Maintenance, isolation and demonstration of leptospires. Trans R Soc Trop Med Hyg. 1970; 64(4): 623-46.

39. Bahaman AR, Marshall RB, Moriarty KM. Experimental trials on the use of radioimmunoassay for the detection of leptospiral antigens in urine. Vet Microbiol. 1986; 12(2): 161-7.

40. Nizamuddin M, Tuteja U, Shukla J, Nair L, Sudarsana J. Early diagnosis of human leptospirosis by antigen detection in blood. Indian J Med Microbiol. 2006; 24(4): 342-5.

41. Palmer M, Hookey J. The chemiluminescent detection of leptospiral antigen. Zentralbl Bakteriol. 1992; 277(3): 300-8.

42. Saengjaruk P, Chaicumpa W, Watt G, Bunyaraksyotin G, Wuthiekanun V, Tapchaisri P, et al. Diagnosis of human leptospirosis by monoclonal antibody-based antigen detection in urine. J Clin Microbiol. 2002; 40(2): 480-9.

43. Postic D, Merien F, Perolat P, Baranton G. Laboratory methods, biological diagnostic for leptospirosis and lyme borreliosis. 2 nd ed. France: Institute Pasteur; 2000. p. 161-172.

44. Koury MC, Cisalpino EO, Rangel Hde A. The use of methanol extract of Leptospira interrogans in complement fixation tests for leptospirosis. Can J Microbiol. 1991; 37(6): 455-8.

45. Brandao AP, Camargo ED, da Silva ED, Silva MV, Abrao RV. Macroscopic agglutination test for rapid diagnosis of human leptospirosis. J Clin Microbiol. 1998; 36(11): 3138-42.

46. Cermeno-Vivas JR, Sandoval-De Mora M, Bognanno JF, Caraballo A. [Clinical and epidemiological features of leptospirosis in Bolivar state, Venezuela. Comparison of diagnostic methods: LEPTO-Dipstick and plaque macroscopic agglutination test]. Invest Clin. 2005; 46(4): 317-28.

47. Effler PV, Domen HY, Bragg SL, Aye T, Sasaki DM. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis in Hawaii. J Clin Microbiol. 2000; 38(3): 1081-4.

48. Petchclai B, Hiranras S, Kunakorn M, Potha U, Liemsuwan C. Enzyme-linked immunosorbent assay for leptospirosis immunoglobulin M specific antibody using surface antigen from a pathogenic Leptospira: a comparison with indirect hemagglutination and microagglutination tests. J Med Assoc Thai. 1992; 75 (Suppl 1): 203-8.

49. Suputtamongkol Y, Sarawish S, Silpasakorn S, Potha U, Silpapojakul K, Naigowit P. Microcapsule agglutination test for the diagnosis of leptospirosis in Thailand. Ann Trop Med Parasitol. 1998; 92(7): 797-801.

50. Arimitsu Y, Kmety E, Ananyina Y, Baranton G, Ferguson IR, Smythe L, et al. Evaluation of the one-point microcapsule agglutination test for diagnosis of leptospirosis. Bull World Health Organ. 1994; 72(3): 395-9.

51. Gussenhoven GC, van der Hoorn MA, Goris MG, Terpstra WJ, Hartskeerl RA, Mol BW, et al. LEPTO dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human sera. J Clin Microbiol. 1997 ; 35(1): 92-7.

52. Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LEPTO Dipstick: a rapid and simple method for serodiagnosis of acute leptospirosis. Trans R Soc Trop Med Hyg. 1999; 93(2): 161-4.

53. Smits HL, Chee HD, Eapen CK, Kuriakose M, Sugathan S, Gasem MH, et al. Latex based rapid and easy assay for human leptospirosis in a single test format. Trop Med Int Health. 2001; 6(2): 114-8.

54. Beth D, Robert GT. Basic and Clinical Biostatistics. 4 nd ed. New York, USA: Mc Graw-Hill Company; 2004. p.118-9.

55. Pradutkanchana S, Nakarin J. The use of latex agglutination for the diagnosis of acute human leptospirosis. J Med Assoc Thai. 2005; 88(10): 1395-400.

56. Smits HL, Eapen CK, Sugathan S, Kuriakose M, Gasem MH, Yersin C, et al. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin Diagn Lab Immunol. 2001; 8(1): 166-9.

57. LeFebvre RB. DNA probe for detection of the Leptospira interrogans serovar hardjo genotype hardjo-bovis. J Clin Microbiol. 1987; 25(11): 2236-8.

58. Terpstra WJ, Schoone GJ, Ligthart GS, ter Schegget J. Detection of Leptospira interrogans in clinical specimens by in situ hybridization using biotin-labelled DNA probes. J Gen Microbiol. 1987; 133(4): 911-4.

59. Terpstra WJ, Schoone GJ, ter Schegget J. Detection of leptospiral DNA by nucleic acid hybridisation with 32P- and biotin-labelled probes. J Med Microbiol. 1986; 22(1): 23-8.

60. Letocart M, Baranton G, Perolat P. Rapid identification of pathogenic Leptospira species (Leptospira interrogans, L. borgpetersenii, and L. kirschneri) with species-specific DNA probes produced by arbitrarily primed PCR. J Clin Microbiol. 1997; 35(1): 248-53.

61. Woo TH, Smythe LD, Symonds ML, Norris MA, Dohnt MF, Patel BK. Rapid distinction between Leptonema and Leptospira by PCR amplification of 16S-23S ribosomal DNA spacer. FEMS Microbiol Lett. 1996; 142(1): 85-90.

62. Woo TH, Patel BK, Smythe LD, Symonds ML, Norris MA, Dohnt MF. Comparison of two PCR methods for rapid identification of Leptospira genospecies interrogans. FEMS Microbiol Lett. 1997; 155(2): 169-77.

63. Woo TH, Patel BK, Smythe LD, Symonds ML, Norris MA, Dohnt MF. Identification of pathogenic Leptospira genospecies by continuous monitoring of fluorogenic hybridization probes during rapid-cycle PCR. J Clin Microbiol. 1997; 35(12): 3140-6.

64. Kee SH, Kim IS, Choi MS, Chang WH. Detection of leptospiral DNA by PCR. J Clin Microbiol. 1994; 32(4): 1035-9.

65. Van Eys GJ, Gravekamp C, Gerritsen MJ, Quint W, Cornelissen MT, Schegget JT, et al. Detection of leptospires in urine by polymerase chain reaction. J Clin Microbiol. 1989; 27(10): 2258-62.

66. Bal AE, Gravekamp C, Hartskeerl RA, De Meza-Brewster J, Korver H, Terpstra WJ. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol. 1994; 32(8): 1894-8.

67. Morey RE, Galloway RL, Bragg SL, Steigerwalt AG, Mayer LW, Levett PN. Species-specific identification of Leptospiraceae by 16S rRNA gene sequencing. J Clin Microbiol. 2006; 44(10): 3510-6.

68. Ramadass P, Jarvis BD, Corner RJ, Penny D, Marshall RB. Genetic characterization of pathogenic Leptospira species by DNA hybridization. Int J Syst Bacteriol. 1992; 42(2): 215-9.

69. Dikken H, Kmety E. Methods in microbiology: Serological typing methods of leptospires. London, U.K.: Academic Press; 1978. p 259-307

70. Sehgal SC, Vijayachari P, Smythe LD, Norris M, Symonds M, Dohnt M, et al. Lai-like leptospira from the Andaman Islands. Indian J Med Res. 2000; 112: 135-9.

71. Collares-Pereira M, Korver H, Cao Thi BV, Santos-Reis M, Bellenger E, Baranton G, et al. Analysis of Leptospira isolates from mainland Portugal and the Azores islands. FEMS Microbiol Lett. 2000; 185(2): 181-7.

72. Marshall RB, Wilton BE, Robinson AJ. Identification of Leptospira serovars by restriction-endonuclease analysis. J Med Microbiol. 1981; 14(1): 163-6.

73. Thiermann AB, Handsaker AL, Moseley SL, Kingscote B. New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar kennewicki. J Clin Microbiol. 1985; 21(4): 585-7.

74. Ellis WA, Montgomery JM, Thiermann AB. Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the Australis serogroup of Leptospira interrogans. J Clin Microbiol. 1991; 29(5): 957-61.

75. Ramadass P, Meerarani S, Venkatesha MD, Senthilkumar A, Nachimuthu K. Characterization of leptospiral serovars by randomly amplified polymorphic DNA fingerprinting. Int J Syst Bacteriol. 1997; 47(2): 575-6.

76. Shen C, Baomin D. [Characterization of leptospiral DNA by southern hybridization serovar lai recombinant probe pCX7]. Hua Xi Yi Ke Da Xue Xue Bao. 1994; 25(3): 248-52.

77. Herrmann JL, Bellenger E, Perolat P, Baranton G, Saint Girons I. Pulsed-field gel electrophoresis of NotI digests of leptospiral DNA: a new rapid method of serovar identification. J Clin Microbiol. 1992; 30(7): 1696-702.

78. Herrmann JL. Genomic techniques for identification of Leptospira strains. Pathol Biol (Paris). 1993; 41(10): 943-50.

79. Perolat P, Grimont F, Regnault B, Grimont PA, Fournie E, Thevenet H, et al. rRNA gene restriction patterns of Leptospira: a molecular typing system. Res Microbiol. 1990; 141(2): 159-71.

80. Perolat P, Lecuyer I, Postic D, Baranton G. Diversity of ribosomal DNA fingerprints of Leptospira serovars provides a database for subtyping and species assignation. Res Microbiol. 1993; 144(1): 5-15.

81. Perolat P, Merien F, Ellis WA, Baranton G. Characterization of Leptospira isolates from serovar hardjo by ribotyping, arbitrarily primed PCR, and mapped restriction site polymorphisms. J Clin Microbiol. 1994; 32(8): 1949-57.

82. Hookey JV, Palmer MF. A comparative investigation and identification of Leptospira interrogans serogroup icterohaemorrhagiae strains by monoclonal antibody and DNA fingerprint analyses. Zentralbl Bakteriol. 1991; 275(2): 185-99.

83. Tamai T, Sada E, Kobayashi Y. Restriction endonuclease DNA analysis of Leptospira interrogans serovars Icterohaemorrhagiae and Copenhageni. Microbiol Immunol. 1988; 32(9): 887-94.

84. Corney BG, Colley J, Djordjevic SP, Whittington R, Graham GC. Rapid identification of some Leptospira isolates from cattle by random amplified polymorphic DNA fingerprinting. J Clin Microbiol. 1993; 31(11): 2927-32.

85. Corney BG, Colley J, Graham GC. Simplified analysis of pathogenic leptospiral serovars by random amplified polymorphic DNA fingerprinting. J Med Microbiol. 1997; 46(11): 927-32.

86. Brown PD, Levett PN. Differentiation of Leptospira species and serovars by PCR-restriction endonuclease analysis, arbitrarily primed PCR and low-stringency PCR. J Med Microbiol. 1997; 46(2): 173-81.

87. Gerritsen MA, Smits MA, Olyhoek T. Random amplified polymorphic DNA fingerprinting for rapid identification of leptospiras of serogroup Sejroe. J Med Microbiol. 1995; 42(5): 336-9.

88. Harlow E, David P. Anibodies a laboratory manual. USA: Cold Spring Harbor Laboratory; 1988. p. 298-9.

89. James WG. Monoclonal Anibodies: Principles and Practice. 3 rd ed. London, U.K.: Academic Press; 1996. p. 196-7.

90. Robert AC. Methods for protein analysis. New York, USA: Chapman and Hall Press; 1994. p. 33-4.

91. Raymond E. Immunodiagnostics. New York, USA: Oxford University Press; 1999. p. 51-60.

92. Kelly PJ. Basic Immunology. Maryland, USA: Williams and Wilkins; 1998. p 168-170.

93. Terpstra WJ, Korver H, van Leeuwen J, Klatser PR, Kolk AH. The classification of Sejroe group serovars of Leptospira interrogans with monoclonal antibodies. Zentralbl Bakteriol Mikrobiol Hyg [A]. 1985; 259(4): 498-506.

94. Adler B, Faine S. A pomona serogroup-specific, agglutinating antigen in Leptospira, identified by monoclonal antibodies. Pathology. 1983; 15(3): 247-50.

95. Masuzawa T, Kumagai M, Shimizu T, Yanagihara Y. Classification of Leptospira interrogans serovar lai strain 017 by using monoclonal antibodies. J Clin Microbiol. 1988; 26(11): 2332-7.

96. Masuzawa T, Sekiguchi T, Shimizu T, Iwamoto Y, Morita T, Mifuchi I, et al. Monoclonal antibody against Leptospira interrogans serovar canicola. Chem Pharm Bull (Tokyo). 1989; 37(2): 414-7.

97. Pongsunk S, Thirawattanasuk N, Piyasangthong N, Ekpo P. Rapid identification of Burkholderia pseudomallei in blood cultures by a monoclonal antibody assay. J Clin Microbiol. 1999; 37(11): 3662-7.

98. Marot-Leblond A, Beucher B, David S, Nail-Billaud S, Robert R. Development and evaluation of a rapid latex agglutination test using a monoclonal antibody to identify Candida dubliniensis colonies. J Clin Microbiol. 2006; 44(1): 138-42.

99. Reyrolle M, Ratat C, Leportier M, Jarraud S, Freney J, Etienne J. Rapid identification of Legionella pneumophila serogroups by latex agglutination. Eur J Clin Microbiol Infect Dis. 2004; 23(11): 864-6.

100. Adamczyk Z, Musial E, Jachimska B, Szyk-Warszynska L, Kowal A. Polystyrene latex adsorption at the gold/electrolyte interface. J Colloid Interface Sci. 2002; 254(2): 283-6.

APPENDIX

GENERAL REAGENTS

0.15 M phosphate buffer saline (PBS) pH 7.4

NaCl (Merk)	8.00 gm
KCl (Sigma)	0.20 gm
Na2HPO4 (Merk)	1.44 gm
KH2PO4 (Merk)	0.24 gm

Dissolve in distilled water, adjust to pH 7.4 with 1.0N HCl and adjust to 1,000 ml final volume with distilled water. Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches.

REAGENTS FOR LATEX REAGENT PREPARATION

0.1 M Phosphate buffer pH 7.4

Add 0.1 M $Na_2H_2PO_4$ solution (220 ml) into 0.1 M Na_2HPO_4 solution (500 ml) until these mixing solution is pH 7.4, filter sterilization (0.2 µm) and stored at 4 °C

Storage buffer

NaCl	0.88 gm
BSA (Sigma)	1.00 gm
NaN3 (Merk)	0.10 gm
99% Glycerol (Sigma)	5.00 ml
0.1 M Phosphate buffer pH 7.4	20.00 ml

Adjust to 100 ml final volume with distilled water, filter sterilization (0.2 µm) and stored at 4 °C

0.17 M Glycine buffer saline (GBS) pH 7.3

Glycine (Sigma)	0.28 gm
NaCl (Merk)	7.50 gm
NaN_3 (Merk**)**	0.04 gm

Dissolve in deionized water, adjust to pH 7.3 with 1.0 N HCl or 1.0 N NaOH and adjust to 1,000 ml final volume with deionized water, filter sterilization (0.2 µm) and stored at 4

REAGENTS FOR MONOCLONAL ANTIBODY PRECIPITATION

Saturated Ammonium sulfate solution

Ammonium sulfate (Carlo Erba) 761 gm

Dissolve in distilled water 1,000 ml, adjust to pH 7.4 with 1.0N HCl or 1.0 N NaOH , filtrate by Whatman filter paper number 1 and stored at 4 °C

50% Ammonium sulfate solution

Ammonium sulfate (Carlo Erba) 313 gm

Dissolve in distilled water, adjust to pH 7.4 with 1.0 N HCl or 1.0 N NaOH and adjust to 1,000 ml final volume with distilled water, filtrate by Whatman filter paper number 1 and stored at 4 °C

MEDIA FOR BACTERIAL CULTURE

Ellinghausen-McCullough-Johnson-Harris (EMJH medium)

Stock solution for EMJH medium preparation

Each reagent is prepared to 100 ml with distilled water and kept at 4 °C as stocks separately

NH_4Cl (Merk)	25	gm
$ZnSO_4$. $7H_2O$ (Merk)	0.4	gm
$MgCl_2$ (Merk)	0.695	gm
$CaCl_2$. H_2O (Merk)	1.5	gm
$FeSO_4$. $7H_2O$ (Merk)	0.5	gm
$CuSO_4$. $5H_2O$ (Merk)	0.3	gm
Sodium Puruvate (Merk)	10	gm
Thiamine HCl (Merk)	0.5	gm
Cyanocobalamine (Merk)	0.02	gm
Glycerol (Merk)	10	ml
Tween 80 (Merk)	10	ml

Basal Medium (1 liter)

Na_2HPO_4 (Merk)	1.0	gm

KH_2PO_4 (Merk)	0.3	gm
NaCl (Merk)	1.0	gm

Dissolve its in distilled water 996 ml before adding these stock solutions:

NH4Cl	1	ml
Thiamine	1	ml
Sodium Pyruvate	1	ml
Glycerol	1	ml

Adjust to pH 7.4. Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches. stored at 4 °C

Albumin Supplement (100 ml)

Bovine serum albumin (BSA) (fraction V, Sigma)	10	gm

Completely dissolve BSA by autoclave distilled water 60 ml before adding these stock solutions:

$MgCl_2$	1	ml
$CaCl_2$	1	ml
$ZnSO_4$	1	ml
$CuSO_4$	300	μl
$FeSO_4$	10	ml
Cyanocobalamine	1	ml
Tween 80	12.5	ml

Adjust to 100 ml final volume with distilled water. Filtration through a 0.2 μm filter and stored at 4 °C

Brain heart infusion broth (BHI broth)

Brain heart infusion (Gibco BRL)	38	gm
Distilled water	1000	ml

Sterilized by autoclaving for 15 minutes at 121 °C, 15lb/square inches.

BIOGRAPHY

NAME	Utane Rungpanich
DATE OF BIRTH	30 November 1976
PLACE OF BIRTH	Pathumtani, Thailand
INSTITUTION ATTENDED	Mahidol University, 1994: Bachelor of Science (Medical Technology) Mahidol University, 2002: Master of Science (Immunology)
SCHOLARSHIP	Royal Foundation of Siriraj Development
POSITION & OFFICE	Medical Technologist. Department of Immunology, Facculty of Siriraj Hospital Medicine, Mahidol University, Bankok, Thailand. E-mail : utane_r@yahoo.com