Development of Latex Agglutination Technique for Diagnosis of Leptospirosis

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ชื่อวิทยานิพนธ์ การพัฒนาลาเทกแอกกลูทิเนชั่นเทคนิคเพื่อวินิจฉัยโรคฉี่หนู
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บทคัดย่อ

เพื่อพัฒนาการวินิจฉัยโรคเลปโตสไปโรซิส โดยใช้วิธีลาเทกแอกกลูทิเนชั่น ได้ทำการสกัด whole cell extract, total membrane protein และ outer membrane protein antigen จากเชื้อ *Leptospira interrogans* serovar Autumnalis Pomona และ Sejroe ทำการทดสอบหาปริมาณของ antigen และสภาพที่เหมาะสมในการจับกับ latex particle โดยการทดสอบกับตัวอย่าง serum ของผู้ป่วย leptospirosis ทำการประเมิน latex ที่เตรียมได้ โดยทดสอบกับ ตัวอย่างserum MAT บวก จำนวน 40 ตัวอย่าง ตัวอย่างserum MAT ลบจำนวน 50 ตัวอย่าง ตัวอย่างควบคุมลบคือ ผู้บริจาคโลหิต 30 ตัวอย่าง และคนไข้ไทฟอยด์ 30 ตัวอย่าง พบว่า latex ที่เตรียมมาจาก antigen แต่ละชนิดของเชื้อ leptospira ทั้ง 3 serovars ให้ผลที่ใกล้เคียงกัน ยกตัวอย่างเช่น latex ที่เตรียมมาจากเชื้อ serovar Autumnalis ชนิด WC-LAT ให้ผล ความไว ความจำเพาะ ความถูกต้อง positive predict value(PPV) negative predict value (NPV) และค่า kappa 95.0%, 82.0%, 87.7%, 80.8%, 95.3%, 0.98 ตามลำดับ ชนิด TM-LAT ให้ผล 92.5%, 92.0%, 92.2%, 90.2%, 93.8%, 0.97 ตามลำดับ และชนิด OMP-LAT ให้ผล 87.5%, 96.0%, 93.3%, 92.1%, 92.3%, 0.98 ตามลำดับ latex ที่เตรียมจาก antigen ทั้ง 3 ชนิดของเชื้อแต่ละ serovar ให้ผลที่ไปด้วยดีกับวิธี MAT ผลความไว ความถูกต้อง PPV และ NPV ของ OMP-LAT, TM-LAT และ WC-LAT ไม่มีนัยสำคัญทางสถิติ อย่างไรก็ตาม latex ชนิด OMP-LAT ให้ผลค่าความจำเพาะที่สูงกว่า WC-LAT อย่างมีนัยสำคัญทางสถิติ

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ABSTRACT

To develop serodiagnosis of leptospirosis using latex agglutination technique, whole cell extract, total membrane protein and outer membrane protein antigen were isolated from three serovars of *Leptospira interrogans*, Autumnalis, Pomona and Sejroe. The optimal concentration of antigen and condition for coupling with latex particle of each antigen were performed with positive human leptospirosis sera. The prepared latex was tested with 40 MAT positive sera, 50 MAT negative sera, negative control sera, 30 blood donors sera and 30 typhoid fever sera. Each antigen based latex from 3 serovars revealed similar results. For example, serovar Autumnalis latex type: WC- LAT resulted in sensitivity, specificity, accuracy, positive predict value(PPV), negative predict value (NPV) and Kappa as 95.0%, 82.0%, 87.7%, 80.8%, 95.3%, and 0.98 respectively; TM- LAT as 92.5%, 92.0%, 92.2%, 90.2%, 93.8%, and 0.97 respectively; OMP-LAT as 87.5%, 96.0%, 93.3%, 92.1%, 92.3%, and 0.98 respectively. The results of latex coated coupling with three antigens correlated with MAT test thus demonstrated perfect agreement method. The sensitivity, accuracy, PPV and NPV of OMP-LAT, TM-LAT and WC-LAT of three serovars were not statistically significant different. However, the specificity of OMP-LAT was statistically significantly different with WC-LAT.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ab	=	antibody
Ag	=	antigen
APS	=	Ammonium persulfate
BSA	=	bovine serum albumin
ºC	=	degree Celsius
EDTA	=	ethylenediaminetetraacetic acid
et al.	=	et ali (Latin) and others
g	=	gram
h	=	hours
i.e.	=	id. Est, for example
IgG	=	Immunoglobulin
kDa	=	kilo Dalton(s)
LAT	=	Latex Agglutination Test
M	=	Molar
mA	=	milliampare
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	milimolar
mm	=	millimeter
mol	=	mole
MW	=	molecular weight
nM	=	nanomolar
nm	=	nanometer
nmole	=	nanomole
O.D.	=	Optimum density
PBS	=	phosphate buffer saline

LIST OF ABBREVIATIONS AND SYMBOLS

PAGE	=	Polyacrylamide gel electrophoresis
rpm	=	revolutions per minute
sec	=	second
SDS	=	sodium dodecyl sulfate
TBS	=	Tris buffered saline
TBS T	=	Tris buffered saline Tween 20
TEMED	=	tetramethylethylenediamine
ug	=	microgram
ul	=	microliter
uM	=	micromolar
U	=	unit (s)
V	=	volt
WB	=	Western Blotting
v/v	=	volume per volume
w/v	=	weight per volume

CHAPTER 1

INTRODUCTION

Background

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic *Leptospira interrogans.* Leptospirosis is an important health problem in Thailand. 392 cases of leptospirosis were reported to the Bureau of Epidemiology in Maha Sarakham Province in 2000 (James et al., 2005). Physicians can make an early leptospirosis diagnosis by using features of a clinical presentation such as febrile illness, chills, headache, malaise, myalgia, abdominal pain, conjunctival suffusion, anorexia, skin rash, nausea and vomiting (Levett, 2001) and occasionally severe cases will show jaundice (Shiemeld, 1999) and hepatomegaly. However, during early stages of the disease, its clinical presentation alone cannot be differentiated from other febrile illnesses such as dengue fever, malaria, typhoid, or viral hepatitis (Levett et al., 1998). Thus appropriate laboratory investigations should be included in the diagnosis protocol.

Laboratory diagnosis of leptospirosis mostly depends on checking for specific antibodies because of the difficulty in isolating any particular organism. Visualizing live leptospira organisms directly from tissue or body fluids by using dark-field microscopy requires much technical expertise. Culturing leptospira in Ellinghausen and McCullough modified Johnson and Harris (EMJH) medium is time-consuming (2-3 months) and lacks sensitivity (Faine et al., 1999). The gold standard method for serological diagnosis of leptospirosis is the microscopic agglutination test (MAT) that requires live strains of leptospira. The MAT has high sensitivity and can be used for leptospira classification (Faine, 1982; Terpstra et al., 1980). However, the MAT is unsuitable for rapid case identification since it can only be performed in a few reference laboratories and requires paired sera to achieve sufficient sensitivity (Cumberland et al., 1999; Faine et al., 1999). The MAT is also laborious and requires expertise and specialized equipment. Several alternative serological methods for the early diagnosis of leptospirosis have been reported such as the indirect hemagglutination assay (IHA) (Levett et al., 1998), the

enzyme-linked immunosorbent assay (ELISA) method for immunoglobulin M (IgM) antibodies (Terpstra et al., 1980; 1985), the dot-ELISA (Pappas et al., 1985), which give results in 2 – 3 hours. Ideally, a suitable routine test should give results within minutes, such as the LEPTO-Dipstick test (Gussenhoven et al., 1997) that requires no special equipment, is easy to use and less expensive - however, this Leptodipstick method uses crude antigen prepared from the non-pathogenic strain *L. biflexa,* and has low specificity. There is a PCR method for diagnosing Leptospirosis (Merien et al., 1992; 1995), with high specificity and sensitivity, but it is a complex procedure requiring special equipment and is quite expensive, so is not really suitable for day-to-day use in a clinical setting.

An ideal routine assay for leptospirosis diagnosis should be easy, have high sensitivityand specificity, and provide rapid results within minutes, such as the simple latex agglutination (LA) test. However, a major problem with this test is that is uses crude antigens, thus has a low specificity. Lip32, an outer membrane protein (OMPs) of leptospira, has recently been reported as a recombinant LipL32-based latex agglutination test for detection of specific leptospira antibodies (Dey et al., 2007). OMPs have been reported to be an integral part of the bacterium that reacts with the host cell. Therefore, the component of this part should play an important role in the host immunity. The application of OMPs to couple with latex particle may be increase the sensitivity and specificity of the simplified latex test.

Literature review

1. *Leptospira* species

1.1 Characteristics

Leptospira are spirochetal bacteria, normally about 0.1 um wide and 6 – 20 um long. The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Faine et al., 1999). Leptospira has three distinct forms of movement: rotation (spinning rapidly around the long axis of the cell), progressive movement in the direction of the straight end, and

circular motion. Leptospira have 3-5 outer membranes; the inner cell is a protoplasmic cylinder consisting of a cytoplasmic membrane and a peptidoglycan cell wall. Leptospir*a* have two periplasmic flagella, one attached sub-terminally at each end (Figure 1).

Leptospira are obligate aerobes with an optimal growth temperature of 28-30°C and which die within 2-3 min at 57°C. They are grown for study purposes in media enriched with vitamins (vitamins B2 and B12 are growth factors), long-chain fatty acids and ammonium salts. Leptospira are catalase and oxidase positive. Motility and chemotaxis encoding genes of *L. interrrogans* are well conserved among 42 genes Genomic analysis indicates that the chemotaxis system of *L. interrogans* is more complex than that of either *Treponema pallidum* or *Borrelia burgdorferi.*The reason for the greater number of motility- associated genes in *L. interrogans* than in other spirochetes is not clear.

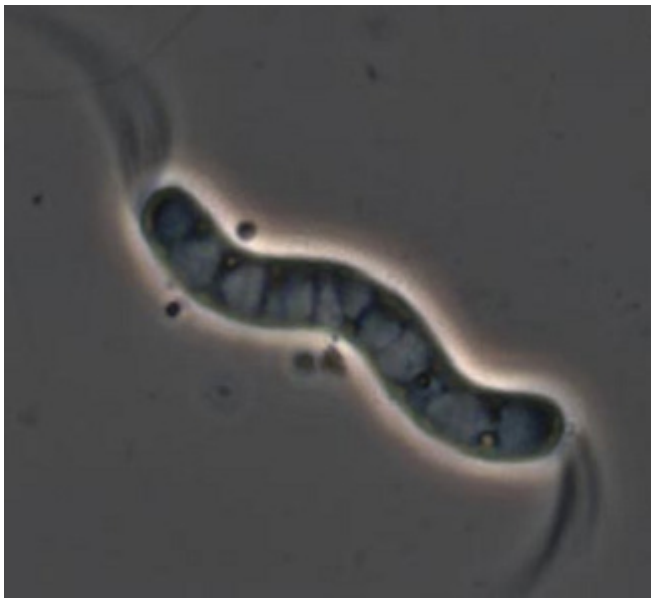


Figure 1. *Leptospira spp.*, taken using phase contrast optics (from Preisfeld and Patterson, 2003)

1.2 Taxonomy and classification

1.2.1 Serological classification

Class: Schizomycetes

Order: Spirochaetales

Family: Spirochaetaceae

Genus: Leptospira

Leptospira are divided into two species, *L. interrogans* and *L. biflexa*. *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing saprophytic strains isolated from the environment (Faine and Stallman, 1982). Both *L. interrogans* sensu lato and *L. biflexa* sensu lato are divided into serovars defined by agglutination after cross-absorption with homologous antigens (Kmety and Dikken, 1993). Serovars are considered distinct if more than 10% of the homologous titer remains in at least one of the 2 antisera on repeat testing. In *L. biflexa,* over 60 serovars have been recorded, while in *L. interogans* over 200 serovars are recognized (Johnson and Faine, 1984). The serogroup of *L. interrogans* and some common serovars of clinical importance are shown in Table 1 (from Levett, 2001). Serogroups have no taxonomic standing, but the concept has proved useful for epidemiological understanding.

Leptospira are obligate aerobes with an optimum growth temperature of 28 to 30°C., although some species of saprophytic leptospira are able to grow at temperatures as low as 13°C. The optimal growth temperature of the pathogenic leptospira species is 25°C. A comparison of both leptospira species is shown in Table 2 (from Cinco, 1986).

TABLE 1. Serogroups and some serovars of *L. interrogans* sensu lato (from Levett, 2001)

Serogroups	**Serovars**
Icterohaemorrhagiae	icterohaemorrhagiae, copenhageni, lai, zimbabwe
Hebdomadis	hebdomadis, jules, kremastos
Autumnalis	autumnalis, fortbragg, bim, weerasinghe
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynopteri
Djasiman	djasiman
Sarmin	sarmin
Mini	mini, georgia
Tarassovi	tarassovi
Ballum	ballum, aroborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

TABLE 2. Comparison of the pathogenic and saprophytic leptospira (Cinco, 1986)

Characteristic	**Pathogenic**	**Saprophytic**
Pathogenicity	+	-
Growth at 13°C	-	+
Inhibition of growth with 8-azaguanine	+	-
Conversion to spherical forms by 1M NaCl	+	-
Lipase	S	+
% G+C of DNA	35.3 – 39.9	38 – 41

+ Present in more than 90% of strains, - present in less than 10% of strains, S = present in 11 – 89% of strains

1.2.2 Genotypic classification

A number of genomospecies include all serovars of both *L. interrogans* and *L. biflexa.* DNA hybridization studies were used to elucidate the genomospecies of leptospira. The leptospira are taxonomically classified into twelve species and four unnamed species (Genomospecies 1, 3, 4 and 5) (Table 3) (Brenner et al., 1999)**.** Some leptospiral serovars are commonly associated with particular animal reservoirs (Table 4). The genotypic classification of leptospira is taxonomically correct and provides a foundation for future classifications. The molecular classification, however, still causes problems for clinical microbiologists due to incompatible system of serogrouping clinically and epidemiologically. Consequently, when new leptospira species are isolated they should be characterized both molecularly and serologically.

TABLE 3. Genomospecies of leptospira and distribution of serovars (Brenner et al., 1999)

Species	**Serovars**	**Reference strain**
Pathogens		
L. interrogans	icterohaemorrhagiae	RGA
	canicola	Hond Utrecht IV
	pomona	Pomona
	australis	Ballico
	pyrogenes	Salinem
	hebdomadis	Hebdomadis
	bataviae	Van Tienen
	batislava	Jez Bratislava
	copenhageni	M 20
	lai	Lai
	hardjo	Hardjoprajitno
L. noguchii	fortbragg	Fort Bragg
L. santarosai	Brasiliensis	An 776
	georgia	LT 117
L. meyeri	semaranga	Veldrat, Semaranga 173
L. fainei	Hurstbridge	BUT 6
L. alexanderi	Manhao	L60
L. borgpetersenii	Javanica	Veldrat, Bataviae 46
	Ballum	Mus 127
	Sejroe	M 84
	Tarassovi	Perepilitsin
	castellonis	Castellon 3
L. kirschneri	grippotyphosa	Moskva V
	cynopteri	3522C
	bim	1051
	mozdok	5621
	panama	CZ 214K

Species	**Serovars**	**Reference strain**
L. weilii	Celledoni	Celledoni
L. indadai	lyme	10
Genomospecies 1	Pingchang	80-412
Genomospecies 4	hualin	LT 11-33
Genomospecies 5	saopaulo	Sao Paulo
Saprophytes		
Genomospecies 3	holland	Waz Holland (P438)
L. wolbachiic	codice	CDC
L. biflexac	patoc	Patoc I

Table 4. Typical reservoir hosts of common *Leptospira* spp.

Reservoir host	**Serovars**
Swine	pomona, tarassovi
Cattle	hardjo, pomona
Horses	bratislava
Dogs	canicola
Sheep	hardjo
Raccoons	grippotyphosa
Rats	icterohaemorrhagiae, copenhageni
Mice	ballum, arborea, bim
Marsupials	grippotyphosa
Bats	cynopteri, wolffi

1.3 Leptospira antigens

1.3.1 Lipopolysaccharides (LPSs)

LPSs are the major surface component of leptospira and contribute to the pathology associated with disease. LPSs have high immunogenicity and are responsible for serovar specificity. LPSs are the target of agglutination studies so serological classification of leptospira is important. Leptospira lipopolysaccharides have a composition similar to the lipopolysaccharides of other gram negative bacteria, but they have lower endotoxic activity (Shimizu et al., 1987). The LPSs of *L. interrogans* is a structurally unique molecule of relatively low toxicity that activates macrophages in a distinct manner (Nally et al., 2005). The lack of efficient recognition of leptospira lipopolysaccharides by human TLR4 may lead to the human susceptibility to leptospira infection (Nahori et al., 2005).

1.3.2 Flagella antigens

Leptospira have two periplasmic flagella, one attached subterminally at each end. The 33-34 kDa doublets, presumed to represent flagella core proteins, react similarly in immunoblots with anti-leptospiral or anti-flagella antisera (Kelson et al., 1998)

1.3.3 Heat shock proteins

Heat shock proteins (Hsps) are synthesized when cells are exposed to elevated temperature or to a variety of other stresses (Lindquist and Craig., 1988). The major heat shock proteins, GroEL/GroES and DnaK, of *Leptospira* have been described (Ballard et al., 1998). GroEL is a major immunogen recognized during infection in humans and animal, but plays no role in immunity to infection.

1.3.4 Protein and lipoprotein antigens

The outer membrane of leptospires contains lipopolysaccharides (LPSs), phospholipids and several proteins (Haake et al., 2000). There are at least three kinds of proteins: lipoproteins, transmembrane proteins and peripheral outer membrane proteins (OMPs) (Cullen et al., 2004). The structures of the outer membrane lipoproteins of several spirochetes have been shown to play an important role in infection and immunity. Using of imunoprecipitation technique to identify surface exposed proteins of OmpL1 and transmembrane protein. Subsequently identified LipL21, LipL32, LipL36, LipL41 are highly conserved outer membrane proteins among pathogenic leptospira (Shang et al., 1996). The extraction of leptospiral membrane proteins using Triton X-114 revealed the presence of various lipoproteins (Zuerner et al., 1991). Leptospira can survive outside as well as inside the host and some of the outer membrane proteins have been found to be differentially regulated between in vivo and in vitro conditions. For example, leptospira immunoglobulin-like protein A (LigA) and LipL 32 have been found to be upregulated during infection (Haake and Matsunaga, 2002). Lipoprotein LipL36 is present in in vitro cultures but it is downregulated in vivo (Barnett et al., 1999).

2. Leptospirosis

2.1 Epidemiology

Leptospirosis is presumed to be the most widespread zoonosis in the world (World Health Organization, 2000).The source of infection in humans is usually direct or indirect contact with urine of an infected animal or from rat bite. Direct contact with infected animals accounts for most infections in farmers, veterinarians, abattoir workers, meat inspectors, and rodent control workers. Indirect contact is a more likely source for those working in such fields as sewer workers, miners, soldiers, septic tank cleaners, fish farmers, canal workers, rice field workers, taro farmers, banana farmers, and sugar cane cutters. The highest prevalence rates remain in tropical, and especially developing, countries (Plank and Dean, 2000). Outbreaks have been related to heavy rainfall in various parts of the world (during the June-October rainy season in Thailand). Several outbreaks of leptospirosis associated with water have been reported (Table 5).

TABLE 5. Documented outbreaks of leptospirosis associated with water

Place and yr.	No. of cases	Exposure	Source of infection	Presumptive serogroup
Wyoming, 1942	24	Swimming in a pool	Unknown	Canicola
Okinawa, 1949	16	Swimming in a pond	Unknown	Autumnalis
Alabama, 1950	50	Swimming in a creek	Suspected to be pigs	Pomona
Georgia, 1952	26	Swimming in a creek	Suspected to be dogs	Canicola
Japan, 1953	114	Swimming in a river	Suspected to be dogs	Canicola; serovar canicola isolated
Russia, 1954	62	Drinking and bathing in well water	Contamination by pigs	Serovar pomona isolated
Iowa, 1959	40	Swimming in a stream	Contamination by cattle	Serovar pomona isolated from two cases and from cattle
Washington, 1964	61	Swimming in a canal	Suspected to be cattle	Pomona; serovar pomona isolated from cattle
Italy, 1984	35	Drinking from water fountain	Dead hedgehog in header tank	Australis
Okinawa, Japan, 1987	22	Swimming in a pool or jungle training	Unknown	Shermani
Sa˜o Paulo, Brazil, 1987	23	Swimming in a pool with river water	Unknown	Pomona

The annual number of reported cases in England and Wales ranged between 29 and 48 cases during 1990-92 (Ferguson, 1993). Hawaii has historically had the highest annual incidence of leptospirosis in the United States, with a mean annual incidence rate of 1.29 per 100,000 (Zitek and Benes, 2005) from 1974 to 1998. Data from the Czech Republic over the period 1963-2003 indicate that the incidence rate of leptospirosis was 0.3% per 100,000 populations. However, during periods of intense flooding (1997-2002) this increased to 0.9% per 100,000 (Katz et al., 2002).

Before 1996, the number of cases reported in Thailand by the Department of Disease Control was estimated at 200 cases/year, a figure which increased to 358 in 1996 (Tangkanankul, 2000). In 2000, the estimated annual incidence was 14,285 cases leptospirosis **(Figure 2)**. This was followed by a continual decline to 2,868 cases in 2005. A vast majority of the cases (90%) were reported from the Northeast region (Tangkanankul et al., 2005)**.** Recently report**,** number one to third cases were recorded in 3 provinces in the north or northeast (Wuthiekanun et al., 2007**)** (Table 6). The median age of patients was 35 years (range 10-68 years), with 85% of the cases reported in men. The *L.* interrogans serovars were Autumnalis, Bataviae, Pyrogenes, Hebdomadis, Grippotyphosa, and the *L. borgpetersenii* serovars Javanica.

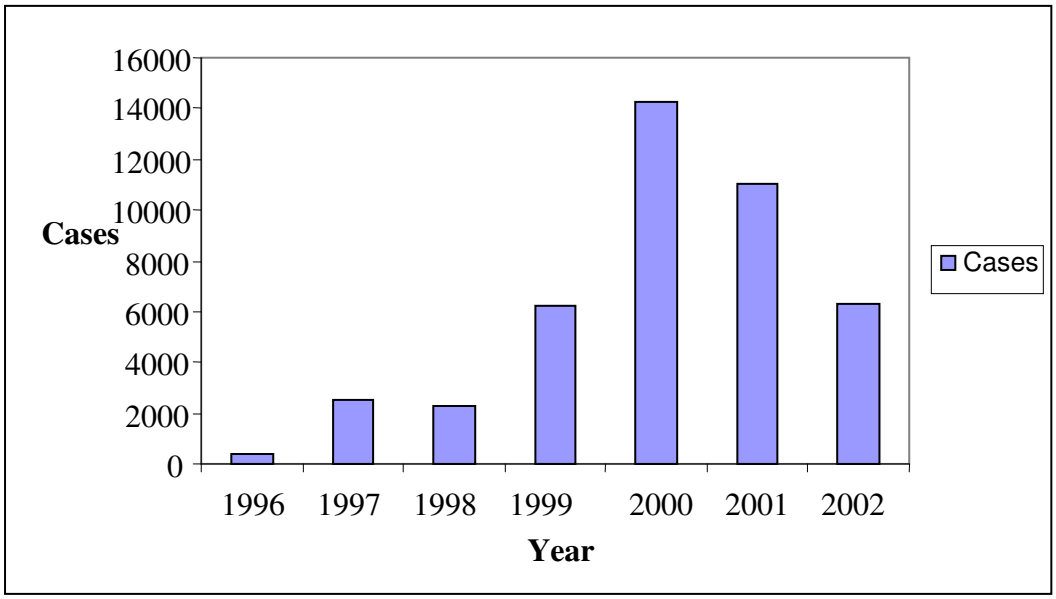


Figure 2. Number of reported cases of leptospirosis per year, 1996-2002, Thailand.

Table6. Distribution of suspected and confirmed cases of leptospirosis according to province, Thailand

Province	Geographic region	Clinically suspected cases (%)	Laboratory confirmed cases (%)	Culture positive cases
Lumpang	North	161(23)	28(20)	2
Udon Thani	Northeast	223(32)	64(45)	10
Maha Sarakham	Northeast	181(26)	26(18)	1
Ya sothon	Northeast	32(5)	6(4)	1
Chainut	Central	13(2)	3(2)	0
Rayong	Central	45(6)	13(9)	1
Chanthaburi	Central	4(0.6)	2(1)	0
Prachuap Khiri Khun	South	33(5)	1(0.7)	0
Phattalung	South	8(1)	0	0
Total		700	143	15

2.2 Clinical features of leptospirosis

Leptospirosis is an acute, febrile illness occurring in humans or animals all over the world. In humans the disease is contracted from infected animals. The carriers may be wild or domestic animals, especially rodents and small marsupials, cattle, pigs, and dogs. Infections of animals or humans occur from direct contact with urine or indirectly from contaminated water or soil. Leptospira can survive for as long as six months outdoors under favorable conditions. Leptospira bacteria can enter the body through cuts or other skin damage or the mucous membranes. The characteristic serovar associations are Hardjo with cattle, Pomona with pigs, cattle, and rodents, and Icterohaemorrhagiae, Copenhageni, Bataviae, Autumnalis, Australis, and Zanoni with rats and small rodents.

The clinical significance (Shiemeld, 1999) of human leptospirosis ranges from sub-clinical to severe, depending on several factors including the portal of entry, the infecting

serovar, the infectious dose, and the host condition. Sub-clinical cases are often misdiagnosed as meningitis or fever of unknown origin. In more severe cases the three organ systems that are most often involved are the CNS, kidneys, and liver. After infection, the incubation period lasts from 2-20 days, and in clinical cases become to acute phase. Symptoms include fever 102-106 °F, chills, severe headache, back pain, and joint pain; neck stiffness, conjunctival suffusion, malaise, myalgia, and anorexia are also common. Nausea and vomiting were also common and jaundice was seen in 39% of cases. Less often are a skin rash and acute meningitis (Arzouni et al., 2002; Panicker et al., 2001) (Table 7). Occasionally severe cases experience jaundice, renal failure and toxic hepatitis, which can last from 4-8 days. Leptospirosis can be detected in blood and spinal fluid about 1 week after the onset of symptoms and in urine for several months after that time.

The immune phase begins during the first week. Patient symptoms are variable, with recurrent fever lasting from 1-2 days. In this time most of the leptospira have been eliminated from the body. Around 90% of human leptospirosis is self-limiting with recovery occurring in about 2-3 weeks, although severe cases may take several months to full recovery. Severe cases often present late in the course of the disease, and this contributes to the high mortality rate, which ranges between 5-15%. Renal failure is the cause of death in the 5-30% of untreated patients who experience jaundice. The true incidence of pulmonary involvement is unclear but may range from 20–70%. Serum bilirubin concentrations may be as high as 30-40 mg/dl, while transaminase levels (AST, ALT) are moderate with a minor increase of alkaline phosphatase concentrate (Vinetz et al., 1996). Thrombocytopenia is typical, developing in up to 50% of patients with leptospirosis, and correlates with the occurrence of renal failure (Edwards et al., 1982).

The clinical presentation of leptospirosis is biphasic (Figure 3) with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospira in the urine.

Table 7. Signs and symptoms of leptospirosis patients as recorded in large studies (adapted from Levett, 2001**)**

Symptom	% of Patients					
	China, 1965 N=168	**Puerto Rico,1963 N=208**	**Vietnam, 1973 N=150**	**Korea, 1987 N=93**	**Barbados, 1990 N=88**	**Brazil, 1999 N=193**
Jaundice	0	49	1.5	16	95	93
Anorexia	46	-	-	80	85	-
Headache	90	91	98	70	76	75
Conjunctival suffusion	57	99	42	58		28.5
Vomiting	18	69	33	32	50	-
Myalgia	64	97	79	40	49	94
Abdominal pain	26	-	28	40	43	-
Nausea	29	75	41	46	37	-
Cough	57	24	20	45	32	-
Hemoptysis	51	9	-	40	-	20
Hepatomegaly	28	69	15	17	27	-
Lymphadenopathy	49	24	21	-	21	-
Diarrhea	20	27	29	36	14	-

(-) symptom not recorded

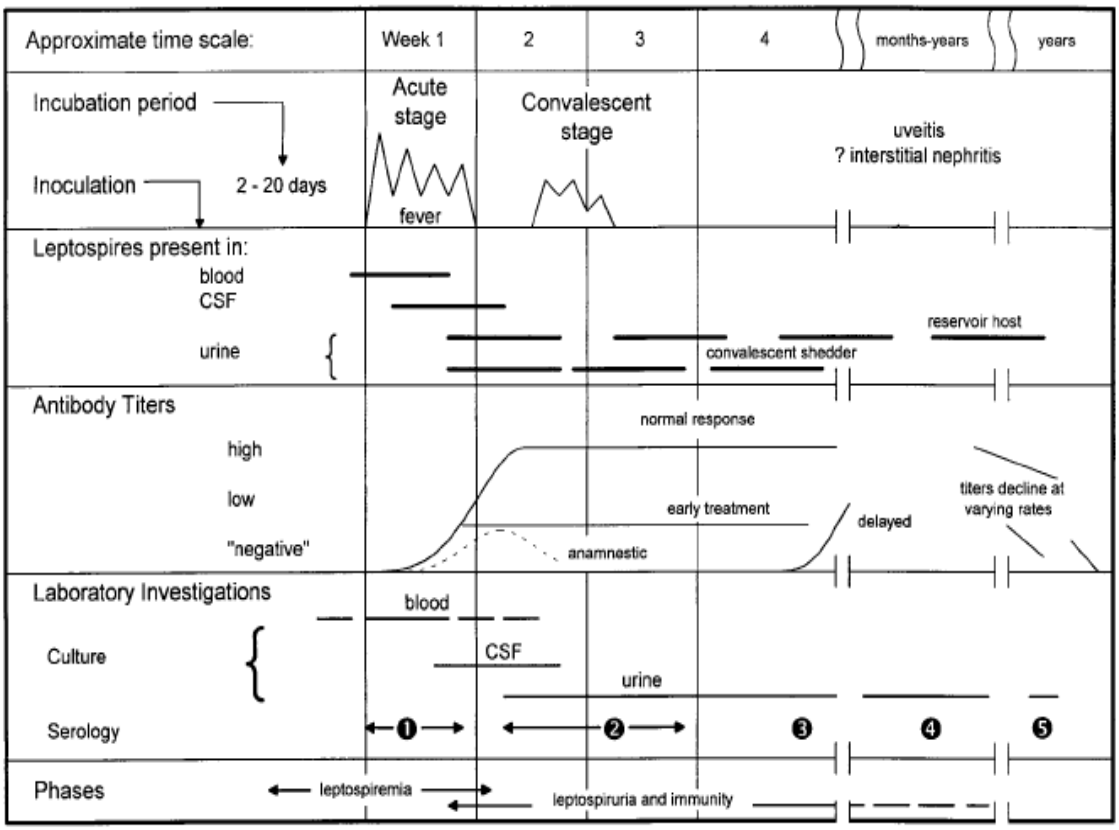


Figure 3. Biphasic nature of leptospirosis and relevant investigations at different stages of disease. Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumptive infecting serogroup.

2.3 Pathogenesis

Understanding of the mechanism(s) of leptospirosis pathogenesis is limited. Answers to such questions as whether the mild or severe manifestation is due to direct pathogen effects or determined by host immune responses remain elusive. The variable clinical of leptospirosis that diverse range of acute and chronic infection processes in people and reservoir hosts. Pathogenic mechanisms of virulence are motility is probably important in initial infection and in dissemination of organisms from the site of entry to sites of end-organ damage in, for

example, the lung, liver, kidney, eye or brain. That the commonest manifestation and estimated to complicate between 5 – 40 % of cases. Haemolytic, sphingomyelinase, and phospholipase activities have been described in vitro (Bernheimer et al., 1986). Specific genes have been characterised including haemolysins, sphingomyelinase C, sphingomyelinase H, and haemolysis-associated protein-1 (Hap1, also known as LipL32) (Lee et al., 2002). The leptospiral immunoglobulin-like protein A (LigA) contains domains homologous to proteins with attachment and invasion functions, and is expressed in vivo but not in vitro (Palaniappan et al., 2002). Histological examination of kidneys of infected carriers may show interstitial nephritis, believed to be a direct result of the presence of *Leptospira* in tissue, but chronic carriers typically have no renal pathology. Lipopolysaccharides and the outer membrane protein OmpL1 are also implicated in interstitial nephritis (Barnett et al., 1999).

Acute infection indicates the more serious icteric Weil's disease reported in human patients and, in particular, the more serious haemorrhagic syndromes. In acute infected animal models, liver and kidney pathology are related to large numbers of leptospira and associated cytotoxic factors in tissues**.** Lung pathology, lower numbers of leptospira per g of lung tissue relative to liver and blood counts have been reported, and may be due to exposure of circulating toxins produced at distant sites such as the liver (Miller et al., 1974). However, the lower numbers of leptospires in haemorrhagic lung tissue also supports an indirect pathogenic mechanism mediated by the host immune response to infection. The virulence factors may directly activate haemostasis pathways or, alternatively, induce an autoimmune response. The knowledge of mechanisms of host immunity to *Leptospira* or the role of host immunity in leptospirosis pathogenesis does not have detailed. Naturally acquired immunity that protects against reinfection by *Leptospira* does occur and has been assumed to be humourally mediated (Sonrier et al.., 2000). Protective immunity may be engendered by antibodies directed against serovar-specific leptospira lipopolysaccharide. Some evidence suggests that antibodies against *Leptospira* membrane-associated proteins may have a role in host defence, but such evidence is not definitive**.** Leptospira are highly effective pathogens as shown by their distribution and the diversity of pathogenic mechanisms. The continued clearly of pathogenic mechanism in animal models should to improved patient treatments, efficient diagnosis assays, and effective vaccines. Leptospirosis is characterized by the development of vasculitis, endothelial damage, and

inflammatory infiltrates composed of monocytic cells, plasma cells, histocytes, and neutrophils. On gross examination, petechial hemorrhages are common and may be extensive, and organs are often discolored due to the degree of icterus. The histopathology is most marked in the liver, kidneys, heart, and lungs, but other organs may also be affected according to the severity of the individual infection. Hypertrophy and hyperplasia of Kupffer cells is evident, and erythrophagocytosis has been reported. In the kidneys, interstitial nephritis is the major finding, accompanied by an intense cellular infiltration composed of neutrophils and monocytes. Leptospirosis can be seen within the renal tubules by electron microscopy.

Pathological finding in the heart include interstitial myocarditis with infiltration of predominantly lymphocytes and plasma cells, petechial hemorrhages, mononuclear infiltration in the epicardium, pericardial effusions, and coronary arteritis. In skeletal muscles, particularly of the leg, focal necrosis of isolated muscle fibers occurs, with infiltration of histocytes, neutrophils, and plasma cell. This evidence of myositis correlates with the intense myalgia reported by some patients. Leptospirosis commonly involves the central nervous system. Aseptic meningitis commonly involves the central nervous system. That the commonest manifestation and estimated to complicate between 5 – 40 % of cases (Plank and Dean, 2000).

2.4 Treatment

Penicillin was found to be significantly more effective than symptomatic management in reducing duration of fever and symptoms, and was most effective when begun within the first 4 days. Penicillin could prevent jaundice, renal involvement, stiff neck, hemorrhage and reduced duration of illness and symptom severity and also prevented leptospiruria. Penicillin is indicated for severe disease (Watt et al., 1988). Erythromycin and doxycycline reduced the duration and severity of illness in anicteric leptospirosis by an average of 2 days (Alexander, 1986; Kelly, 1998; Vinetz, 2001). Doxycycline is recommended for both prophylaxis and mild disease (Guidugli etl al., 2000). Ampicillin and amoxicillin are also recommended in mild disease. Leptospira have been reported as resistant to other aminoglycosides and to vancomycin (Faine et al., 1999). Problems in the determination of susceptibility include the long incubation time required (Ellinghausen, 1983), the use of media

containing serum (Oie et al., 1983; Wylie and Vincent, 1947) and the difficulty in quantifying growth accurately.

It is generally agreed that antibiotic treatment during the first few days of illness is helpful. However, leptospirosis is often not diagnosed until the later stages of illness.

2.5 Prevention

Immunization of animals can prevent disease but not necessarily. Chronic leptospiruria requires the use of biological agents that protect against the serovars endemic in the animal's location. The present challenge is the development of a uniform vaccine applicable for humans. Vaccines containing the serovars canicola, icterohaemorrhagiae, and Pomona have been developed recently in Cuba (Sanchez et al. 1998), Russia (Ikoev et al., 1999), and China (Chen et al., 1985). In Cuba, there was not a single reported side effect in more than 100,000 people vaccinated and protection was reported to be 100% (Sanchez et al., 2000). Several problems have been found in the attempts to develop a vaccine to prevent human leptospirosis. An unacceptable side effect profile of killed bacterial vaccines has often been reported. The killed bacteria vaccines are likely to provide only short-term and possibly incomplete protection, similar to that reported with anti-leptospira vaccines in animals. The locally varying patterns of leptospira transmission may preclude the development of a suitable vaccine. There is potential for inducing autoimmune diseases such as uveitis (Petersen et al., 2001). There is incomplete knowledge of mechanisms of protective immunity against leptospira infection. Physical barriers and various methods of habitat alteration or poisoning may be necessary to limit human exposure to free living animal carriers such as rodents. Protective clothing, appropriate use of surface disinfectants and other hygienic practices are useful in some occupational settings (Edwards, 1986).

2.6 Laboratory diagnosis

2.6.1 Microscopic Demonstration

Leptospirosis may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of bodily fluids such as blood, urine, CSF, and dialysate fluid has been used but is both insensitive and lacking in specificity. Blood microscopy is of value only during the first few days of the acute illness, while leptospiremia is occuring. Direct dark-field microscopy of blood is also subject to misinterpretation of fibrin or protein threads, which may show Brownian motion (Levett, 2001; Turner, 1970). Plasma and serum were used for darkfield microscopy following centrifugation at 1000 xg and 3000 xg, respectively. Darkfield microscopy had low sensitivity (40.2%), low specificity (61.5%) compared with the gold standard and was not recommended as diagnostic procedure for early diagnosis of leptospirosis. Staining methods have been applied to increase the sensitivity of direct microscopy examination. These have included immunofluorescence staining of bovine urine, water, and soil and immunoperoxidase staining of blood and urine. Leptospira were first visualized by silver staining and the Warthin-Starry stain is widely used for histologic examination (Levett, 2001; Plank and Dean, 2000).

2.6.2 Antigen Detection

Detection of Leptospira antigens in clinical material would offer greater specificity than dark-field microscopy while having the potential for greater sensitivity. An evaluation of several methods concluded that radioimmunoassay (RIA) could detect 10^4–10^5 leptospires/ml and an enzyme-linked-immunosorbent assay (ELISA) method could detect 10^5 leptospires/ml.

2.6.3 Isolation or Culture of leptospira

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and has usually finished by the end of the first week of the acute illness (McClain et al., 1957). Therefore, blood cultures should be taken as soon as possible after the patient's presentation. Leptospira can be isolated from blood and CSF samples during the first 7–10 days of illness, and from urine during the 2nd and 3rd week of illness. One or two drops of blood are inoculated into 10 ml of semisolid medium containing 5-fluorouracil e.g. Ellinghausen-MacCullough-Johnson-Haris (EMJH) medium, Neopeptone or Fletcher semisolid (Palmer and Zochrowski, 2002). There are no transport media available, but blood can be collected and shipped at ambient temperature in tubes containing heparin, oxalate, or citrate (Faine et al., 1999). CSF and dialysate may be cultured during the first week of illness. Urine can be cultured from the beginning of the second week. Cultures are incubated at 28-30°C and can be examined by dark-field microscopy for up to 13 weeks before being discarded.

Isolated leptospira can be identified either by serological methods or, more recently, by molecular techniques. Leptospira are susceptible to β–lactams, macrolides, tetracyclines, fluoroquinolones, and streptomycin. Culture identification of leptospira is the gold standard method, but has low sensitivity. It is difficult to practice in most clinical laboratories because it is time consuming and requires the use of specialized media.

2.6.4. Serological Diagnosis

Most cases of Leptospirosis are diagnosed by serology. Antibodies are detected in blood approximately 5-7 days after the onset of symptoms.

2.6.4.1 Microscopic Agglutination Test (MAT)

The reference method for serological diagnosis of leptospirosis is the MAT. Patient sera are reacted with live antigen suspension of Leptospira cells. After incubation, the serum-antigen mixtures are examined for agglutination, and the titers are determined by dark-

field microscopy. The end point is the highest dilution of serum at which 50% agglutination occurs. Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serovars, especially in acute-phase samples. Paired sera are required to confirm a diagnosis with certainly. A fourfold or greater rise in titer between paired sera confirms the diagnosis regardless of the interval between samples. The current CDC case definition is a titer $\geq$ 1: 100 used to define a probable case with a clinically compatible illness. In areas where leptospirosis is endemic, a single titer $\geq$ 1:800 in symptomatic patients is generally indicative of leptospirosis (Faine, S. 1988). The reported high specificity of MAT was 100% and sensitivity of the acute phase was 68.8% in Urban (McBride et al., 2007). However, the MAT uses paired sera and live antigens which are not readily available in the general laboratory.

2.6.4.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA provides highly useful information on class specificity of antibodies, which is of clinical importance. IgM-antibodies are compatible with current or recent disease. The specific anti-leptospira antibodies of the IgM class appeared in approximately six days and decreased subsequently over the months. Specific IgG antibodies were observed later than IgM antibodies. IgG antibodies peaked after a few weeks and then dropped to low level. An IgM specific dot-ELISA technique was developed in which polyvalent leptospira antigens were dotted onto nitrocellulose filter disks in microtiter tray wells, allowing the use of smaller volumes of reagent. The ELISA test appears to be able to detect leptospira antibodies earlier than does the MAT method, beginning around 5 days after symptoms start. Future modifications of this approach have been used to detect IgG and IgA in addition to IgM (Silva et al., 1997). Numerous studies have indicated that the specificity and sensitivity of ELISA and dot-ELISA are sufficient for use in diagnosis of leptospirosis (Terpstra et al., 1980; 1985; Pappas et al., 1985; Adker et al., 1980; Silva et al., 1997; Petchclai et al., 1992).

2.6.4.3 Indirect Hemagglutination (IHA)

Sheep or human erythrocytes, coated with genus-specific leptospira antigen, are useful antigens for detecting IgM antibodies to leptospira. They have confirmed the utility of IHA as an initial screening test for the investigation of patients suspected of acute leptospirosis (Levett et al., 1998). The sensitivity of the IHA was 92.2%, its specificity was 94.4%. Trial sera were diluted 1/50 in PBS and, 50 ul of diluted serum was mixed with 25 ul of test cell in two wells of a U bottomed plate. Plates were shaken mechanically for 15-30 seconds, and incubated at RT for 60 minutes. Interpretation: no agglutination, negative; $\geq$1 + agglutination, positive. A scale of 0 to 4+ was read (Zochowski et al., 2001).

2.6.4.4 Latex Agglutination (LA)

The sensitization of available latex particles with specific leptospira antigens is used to react with antiserum to cause agglutination of the particles. This assay is simple, requires no special equipment and rapid - it gives results within 2 minutes. The LA assay reported by Smits et al. (2000) had 82.3% sensitivity and 94.6% specificity. Recently, in Thailand, the overall sensitivity and overall specificity of the test were reported as 94.1% and 97.0% respectively (Pradutkanchana and Nakarin, 2005). However, it has a major problem as crude antigens are used which reduce the sensitivity and specificity of the test.

The card agglutination test consists of blue latex particles activated with a broadly reactive leptospira antigen dried on an agglutination card. The assay is based on the binding of leptospira-specific antibodies, present in the serum sample, to the leptospira antigen. Agglutination due to the interaction of serum antibodies and labeled antigen can be observed two minutes after the test agents are mixed.

2.6.4.5 Immunofluorescent antibody (IFA)

Immunofluorescence is a method of using the specific reactivity of antibodies with antigens to reveal the presence of these antibodies in sera and other bodily fluids or to

identify antigens in tissues in the presence of fluorescent dyes. The IFA test for the diagnosis of human Leptospirosis appears to have high sensitivity and specificity for the initial diagnosis of Leptospirosis, with one study finding sensitivity of 91.9% and specificity of 100% (Kemapunmanus et al., 2004) with serum samples collected from patients who presented with acute fever without localizing signs. Pradutkanchana et al. (2003) used an immunofluorescence assay for detection of IgM specific leptospiral antibodies, and found the sensitivity and specificity of the IFA-IgM was slightly greater than IFA-Igs, leading to the suggestion that the IFA-IgM is another alternative test for the diagnosis of acute leptospirosis.

2.6.4.6 Lepto Dipstick

The human Lepto dipstick assay is a simple method for the detection of Leptospira-specific IgM antibodies in human serum samples. The assay is rapid and requires no special equipment. The ingredients are stable and can be stored at room temperature (20-25°C). The assay is based on the binding of human leptospira-specific IgM antibodies to the leptospira antigen. Lepto Dipstick is performed by making a 1:50 dilution of serum in the detection reagent and incubating a wetted dipstick in this solution. The strength of the staining is important in the interpretation of the test results. A color reference strip is used to compare the staining intensity (Figure 4). The dipstick assay is fast and easy to perform and easy to read, and thus has a potentially high acceptability. One study found that the sensitivity of the IgM-dipstick assay was 98%, its specificity was 90.6%, its positive predictive value was 90.9%, and its negative predictive value was 98%. (Levett et al., 2001)

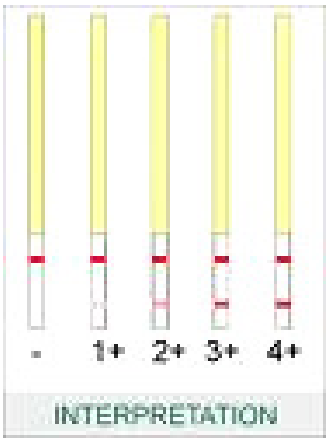


Figure 4. Lepto Dipstick interpretation

2.6.4.7 Lateral-Flow Assay

This one-step, colloidal-gold immuno-assay is based on the binding of specific IgM antibodies to immunobilized antigens from the Patoc I strain (Smit et al., 2001). The bound IgM antibodies are detected using anti-IgM antibodies that are conjugated to mobile, red particles of colloidal gold.

Serum 5 ul was spotted on the pad in the round sample port. Add 130 ul of running buffer. Results were read after 10 min. In the presence of a positive control line, specific anti-Leptospira IgM antibodies are indicated by the appearance of a distinct colored line in the test zone (Wagenaar et al., 2004) (Figure 5).

Figure 5. Lateral-Flow Assay

2.6.5 Molecular Diagnosis

Leptospiral DNA has been detected in clinical material by dot-blotting (Millar et al., 1987) and in situ hybridization (Terpstra et al., 1983). However, this method has a lower sensitivity than PCR, and has not been used extensively for diagnosis since PCR became available.

2.6.5.1 Polymerase chain reaction (PCR)

The PCR method of involves the in vitro amplification of target DNA sequences brought about by thermostable DNA polymerase. A pair of short DNA fragments known as primers is used for specific amplification of DNA fragments. Several primer pairs for PCR detection of leptospira have been described, some based on specific gene targets (Renesto et al., 2000).Most frequently used are the 16S or 23S rRNA genes (Hookey, 1992), while others have been constructed from genomic libraries (Graveamp et al., 1991). Merien et al. (1992) detected leptospira DNA by PCR from clinical specimens. They amplified a 331 bp fragment of the rrs (16S rRNA) gene of both pathogenic and nonpathogenic leptospira. Only PCR and MAT have been subjected to extensive clinical evaluation (Brown et al., 1995; Merien et al., 1995). Brown et al. (1995) examined 71 blood and urine samples from subjects with acute leptospira infection by PCR, culture and MAT. PCR detected 44 cases (62%). PCR and MAT were found to be more sensitive than culture. Merien et al. (1995) studied 200 patients with acute leptospirosis by using PCR, culture and MAT. They found that the PCR was the best method for early diagnosis of leptospirosis during the first 10 days of disease, especially when the clinical symptoms were confusing. They concluded that PCR was rapid, sensitive and specific for diagnosis of leptospira infection, especially during the first few days of the disease. The PCR assay can differentiate between pathogenic and non-pathogenic species. A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovars. While this is not significant for individual patient management, the identity of the serovar has both epidemiological and public health value.

2.6.6 Evaluation, interpretation, and reporting of results

A diagnosis of leptospirosis can be made by isolation of the organism or amplification of leptospira DNA from blood, urine, or other specimens; by demonstration of leptospira in tissues by immunohistochemical staining, or by detection of a fourfold or greater rise in titers between acute and convalescent phase serum samples tested by the same method at the same time. Several assays that detect IgM antibodies give presumptive evidence of recent

exposure to leptospirosis (Cumberland, 2001). Cross-reactive antibodies, sometimes with significant seroconversion, are associated with syphilis or other fever diseases. The isolation of leptospira, the demonstration of leptospira DNA by molecular methods or the detection of leptospira in tissue by immunohistochemistry confirms the diagnosis and differentiates between current infection and past exposure, which may not be clearly differentiated by serology.

Objectives of the thesis

1. To select the antigen preparation and coupling conditions of latex particles.
2. To develop a latex agglutination technique for diagnosis of leptospirosis.

CHAPTER 2

RESEARCH METHODOLOGY

1. Materials

1.1 Bacterial strains

Table 8. Stock list of *Leptospira spp.*

Number	List	Serovar	Strain	Serogroup	Genome Species
1	1	Autumnalis	Akiyami	Autumnalis	*L. interrogans*
2	2	Australis	Ballico	Australis	*L. interrogans*
3	3	Ballum	Mus 127	Ballum	*L. borgpetersenii*
4	5	Bataviae	Swart	Bataviae	*L. interrogans*
5	6	Bratislava	Jez bratislava	Australis	*L. interrogans*
6	7	Canicola	Hond Utrecht IV	Canicola	*L. interrogans*
7	8	Celledoni	Celledoni	Celledoni	*L. weilii*
8	10	Djasiman	Djasiman	Djasiman	*L. interrogans*
9	11	Grippotyphosa	Moskva	Grippotyphosa	*L. kirshneri*
10	13	Hebdomadis	Hebdomadis	Hebdomadis	*L. interrogans*
11	15	Icterohaemorrhagiae	RGA	Icterohaemorrhagiae	*L. interrogans*
12	16	Javanica	Veldrat Bat.46	Javanica	*L. borgpeterseni*
13	18	Pomona	Pomona	Pomona	*L. interrogans*
14	19	Pyrogenes	Salinem	Pyrogenes	*L. interrogans*

Number	List	Serovars	Strain	Serogroup	Genome Species
15	22	Sejroe	M84	Sejroe	*L. borgpetersenii*
16	23	Wolffi	3705	Sejroe	*L. interrogans*
17	24	New	Heusden P2062	Autumnalis	*L. interrogans*
18	25	Ranarum	Iowa City Frgo	Ranarum	*L. meyeri*
19	26	Sarmin	Sarmin	Sarmin	*L. weilli*
20	28	Mini	-	Mini	*L. borgpeterseni*
21	29	Cynopteri	3522C	Cynopteri	*L. kirshneri*
22	30	Saigon	L79	Louisiana	*L. noguchii*
23	31	Panama	CZ214K	Panama	*L. noguchii*
24	32	Shermani	LT821	Shermani	*L. santarosai*

1.2 Chemical reagents

All chemicals and solvents (analytical grade) used in this study were purchased from Pacific science, USA; Sigma, USA; MERCK, Germany; Bio-Rad, USA; LAB SCAN, Ireland; Fluka, Belgium; UNIVAR, Australia; GIBCO, USA; ANALAR, England.

1.3 Media

The EMJH media and Neopeptone media used in this study were purchased from Difo, USA.

1.4 Sera samples

The MAT positive sera titer $\geq$ 1:100 (n = 40) and MAT negative sera (n = 50) sera were obtained from patients at the Tropical Medicine Hospital, Mahidol University,

Bangkok. Positive human (n =30) and pet (n = 30) sera were obtained by LeptoTek Dri Dot (Biomerieux) from patients at the Central Laboratory Hat Yai, Songkhla. Normal control sera of 30 heatlthy individuals were obtained from the Blood Bank unit, Songklanakarin hospital. Control serum samples from thyphiod patients (n=30) were provided by the Department of Pathology, Faculty of Medicine, Songkla nakarin University. All sera were aliquoted and stored at -20°C until used.

2. Methods

2.1 Microscopic agglutination test (MAT)

The MAT tests were performed using the technique described by Galton et al. (1965). The panel consisted of the following 24 serovars of *L. interogans* as live antigens: Australis, Autumnalis, Bratislava, Ballum, Bataviae, Canicola, Celledoni, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Sejroe, Wolffi, New, Ranarum, Sarmin, Mini, Cynopteri, Saigon, Ranama and Shermaniand. 25 ul of diluted serum in sterile 0.85% normal saline was mixed with 25 ul of each live leptospira serovar in a microtiter plate. Then the plate was gently shaken and covered with plastic wrap. The plate was incubated at room temperature for 2-3 hours. 3 ul of the suspension was dropped onto a slide and observed under dark field microscopy (OLYMPUS model BH-2). A sample was considered positive when approximately 50% or more of the leptospira were agglutinationatd at titer $\geq$ 1:100 serum.

2.2 LeptoTek Dri Dot test

LeptoTek Dri Dot is a commercial latex agglutination assay. The assay is intended to be used for the detection of leptospira specific antibodies (IgM and IgG) in sera. The assay was performed according to the manufacturers' instructions. Ten microlitres of a serum was spotted next to the blue dot and within the area marked by the black circle on the card. The blue

dot was mixed with the serum by using a spatula in a quick circular motion until a homogeneous suspension was obtained. Agglutination was read within 30 seconds after the start of mixing.

2.3 Growth of leptospira for antigen preparation

Leptospira interrogans serovars Autumnalis, Pomona and Sejroe were grown in Neopeptone media containing 300 ug/ml of neomycin (Donald and Victor, 1973) and chloramphenicol 5 ug/ml (Schonberg, 1981). The media were shaken at room temperature (RT) or 30°C, 80 rpm and protected from light for 1-2 weeks. After that subcultures leptospira in to EMJH medium usually grow within 1-2 weeks and protected from light for leptospira antigen preparation.

2.4 Leptospira antigen preparation

2.4.1 Whole (crude) cell antigen preparation by sonicated antigens (Adler et al., 1980)

Leptospira 10^{10} cells/ml of serovars Autumnalis, Pomona and Sejroe were grown in EMJH medium for 1-2 weeks. The organisms were washed twice with PBS by centrifugation at 10,000 g, 4°C for 10 minutes, then suspended in PBS, pH 7.2 containing 0.1% sodium azide. The leptospira cells were frozen at -70°C and thawed for 10 minutes at each step for a total of 20 times. Leptospira were sonicated in an ice bath at a setting of 20 kHz, 30 seconds each, for a total time of 5 minutes. Then the leptospira cells were mixed twice with glass beads for 10 minutes. Whole cell extract was aliquoted and kept at -70°C.

2.4.2 Soluble and total membrane fraction preparation using lysis buffer (Guerreiro et al., 2001)

Leptospira cultures of serovars Autumnalis, Pomona and Sejroe containing 10^{10}cells/ml in EMJH medium for 7 days were centrifuged at 10,000 g for 15 min at 4°C. The

pellets were washed in 5mM $MgCl_2$-PBS by centrifugation at 12,000g for 5 min at 4°C and the process was repeated once more. Then the pellets were resuspended in 1 ml of lysis buffer containing 20 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 2 mg lysozyme/ml. The suspension was put through three cycles of freezing and thawing for 20 minutes per cycle. The suspension was then tip sonicated at 20% intensity for 30 seconds per each. The total time of sonication was 5 minutes (Digital Sonifier, Branson). Then the solution was centrifuged at 12,000 g for 45 minutes at 4°C in order to separate the soluble supernatant from the total membrane pellet fraction. The pellet was dissolved with PBS and stored at-20 °C until used.

2.4.3 Outer membrane protein isolation using Triton X-100 (Haake et al., 2000)

OMPs were isolated from *Leptospira spp* serovars Autumnalis, Pomona and Sejroe using 1% Triton X-100 following the method of Haake et al., 2000 with slight modifications. Briefly, $4x10^{10}$ leptospira cells were washed twice in PBS–5 mM $MgCl_2$ and extracted in the presence of 1% Triton X-100 (Calbiochem)–150 mM NaCl–10 mM Tris (pH 8)–1 mM EDTA at 4°C for 30 min. The insoluble material was removed by centrifugation at 17,000 *g* for 10 min. Then the insoluble pellet was resuspended in PBS containing 20 mM $CaCl_2$. Phase separation was performed by warming the supernatant to 37°C for 10 min and subjecting it to centrifugation for 10 min at 1,000 *g* at 4°C. The detergent and aqueous phases were separated. OMPs in the detergent phase were dissolved with PBS and kept at -20°C.

2.5 Protein determination

The protein contents in each antigen preparation were determined by Bradford dye assay based on the equilibrium between three forms of Coomassie Blue G dye. Under strong acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form.

The lyophilized protein standard (Biocon) was reconstituted with deionized water and mixed until dissolved. A working dye reagent was prepared by diluting 1 part of

concentrated dye reagent with 4 parts of distilled or deionized water. The dye reagent was filtered with Whatman no.1 filter paper. The linear range of the assay for protein is 0.1 to 1.2 mg/ml. Pipet 40 ul of sample into 2.0 ml of dry reagent in test tube, mix thoroughly. Then stand the reaction at room temperature for 5 minutes and duplication measure absorbance at wavelength 595 nm, don't read after 1h. Measure the protein concentration by comparison with Protein standard curve 5 concentration that calculation using curves fitting data analysis software from Turner Biosystem Company.

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970 and modification)

The protein suspension obtained through the Bradford assay was fractionated by SDS-PAGE using a Mini-Vertical Slab Gel (C.B.S. Scientific) as described in a standard protocol. The resolving gel consisted of 12.0% (w/v) acrylamide containing 375 mM Tris-HCl pH8.8, 0.1% SDS and 2% glycerol. The polymerization was initiated by the addition of ammonium persulphate (APS, Sigma) and N, N, N, N-tetramethylene-ethylenediamine (TEMED, Sigma) both to a final concentration of 0.05 % (w/v) and 0.005% (v/v) respectively. The 5% acrylamide stacking gel containing 125 mM Tris-HCl pH 6.8 and 0.1% SDS was polymerised by the addition of APS and TEMED to a final concentration of 0.075 % (w/v) and 0.001% (v/v), respectively. The 20 microliters of treated protein sample was loaded in the well of the stacking gel. Electrophoresis was performed in SDS-PAGE running buffer at 80 volts until the samples had fully entered the stacking gel and 120 volts thereafter until the dye reached the bottom of the gel. Following SDS-PAGE, the gels were processed through western blot analysis and incubated in coomassie gel stain at room temperature for one hour with agitation. The gels were then destained in a destaining solution overnight. The gels were dried on 3MM Whatman paper using a vacuum slab gel dryer for 2-3 hours at 60^{0}C.

2.7 Immunoblotting (Coligan et al., 1994)

The standard protocol for western blot was followed. The polyacrylamide gel of protein and blotting paper (MACHEREY-NAGEL) were soaked in the transfer buffer for at least 10 minutes. The nitrocellulose transfer membrane was equilibrated in distilled water for 5 minutes and transfer buffer for 15 minutes. The proteins in the acrylamide gel were transferred to the Nitrocellulose membrane by using a dual cool electrophoresis system (Mini-Vertical Slab Gel/Blotting System, C.B.S. Scientific). Three pre-soaked, thick blotting papers were placed on the platinum anode of the machine. Then the pre-wetted Nitrocellulose membrane was put on the top of the filter paper. The equilibrated acrylamide gel was placed on the center of the nitrocellulose membrane. Another pre-soaked blotting paper was put on the top of the gel, after carefully removing the air bubbles from between the gel and filter paper. The cathode was carefully placed on the stack, and then the power supply was turned on at 90 volts for 3 hours. Following this process, the acrylamide gel was stained with coomasie blue to determine the protein retention on the gel. The Nitrocellulose membrane was gently washed in Tris buffer saline (TBS) tween (0.05%) at room temperature for 5 minutes, then the membrane was blocked in TBS tween-5% skim milk at 25°C for one hour. After washing 3 times for a total of 30 minutes with TBS-Tween, the membrane was incubated with 10 millilitres of absorbed leptospira serum at dilution 1:100 and incubated at room temperature for 2 hours. After washing 3 times with PBS-tween, the membrane was incubated with peroxidase-conjugated goat anti-human IgG, Fab specific (SIGMA) at a 1:2500 dilution for one hour. After washing 3 times with PBS-tween, the ECL reagent (Amershamp) was added to the membrane and the chemiluminescent reaction was detected by using hyperfilm in a darkroom.

2.8 Determining optimal conditions

2.8.1 Antigen optimization

A series of experiments were performed to determine the optimal conditions for appropriate level. Human leptospira serum were reacted with various concentrations of whole cell

antigens (200, 300, 400, 500 and 600 ug/ml), total membrane antigens (100, 200, 300, 400 and 500 ug/ml) and outer membrane proteins (100, 200 and 300 ug/ml) coupled with latex microparticles. The optimal concentration of the antigen was determined to be the concentration that had the highest reactivity with the LAT.

2.9 Latex Agglutination Test

2.9.1 Antigen binding with latex microparticles

A 10% latex microparticle solution (0.88 um) was coated with whole cell, total membrane antigen (300 ug/ml) and outer membrane antigen (200 ug/ml) (using 0.1 M phosphate buffer saline (PBS, pH 7.2) and adjusted to 1 ml with distilled water. Finally, the latex microparticle was made into 1% suspension. The mixture was incubated at room temperature (RT) overnight with constant shaking (Multi Shaker, Bio active). The sensitized beads were centrifuged for 10 minutes at 10,000 g, at 4°C after that washed twice with PBS, pH 7.2. The resulting pellet was resuspended in PBS, pH 7.2 containing 5 mg/ml (w/v) of bovine serum albumin (BSA). The latex beads were left at RT overnight with constant shaking. The latex microparticle were centrifuged and washed twice with PBS at 10,000 g for 10 minutes at 4°C. The resulting pellet was resuspended in PBS containing 0.1% sodium azide. Finally, the sensitized latex beads were sonicated for 1 minute at 20% intensity and stored at 4°C until use.

2.9.2 Latex Agglutination Test (LAT) procedure

The LAT tests were performed on glass slides. Equal volumes of 10 ul of serum sample and 10 ul sensitized latex beads (Table 10) were mixed and placed on the slide, which was rocked gently for 2 minutes. Read the agglutination by eye and confirmed results by microscope.

The serum samples were considered negative if no agglutination was observed within 2 minutes. Positive results were read as + if agglutination was seen within 2 minutes and as ++ if heavy agglutination occurred within 1 minute.

2.10 Stability testing

The sensitized latex microparticle was stored at 4°C. The stability of the latex was tested every 2 weeks for agglutination with MAT positive and negative control sera.

2.11 Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy and kappa value of the LAT was determined by comparing with MAT sera. A kappa value greater than 0.81, LAT indicates perfect agreement test.

Objectives of the thesis

1. To select the antigen preparation and coupling conditions of latex particles.
2. To develop a latex agglutination technique for diagnosis of leptospirosis.

CHAPTER 3

RESULTS

1. Standard curve for leptospira protein determination

Standard bovine serum albumin at concentration 0.1, 0.3, 0.6, 0.9 and 1.2 ug/ml densities of each standard were 0.144, 0.373, 0.612, 0.910 and 1.190 respectively.

2. Protein determination of leptospira

Protein concentrations of various leptospira serovars antigen were determined with standard protein curves that were calculated using curve-fitting data analysis software from Turner Biosystem Company (Table 9).

Table 9. Protein concentrations of *Leptospira spp.* prepared by three extractions. Whole cell extracted by sonication, total membrane extracted by lysis buffer and outer membrane extracted by Triton X-100.

Serovars	**Protein concentration of whole cells (ug/ml)**	**Protein concentration of total membrane (ug/ml)**	**Protein concentration of outer membrane (ug/ml)**
Autumnalis	850	520	300
Pomona	715	456	280
Sejroe	779	450	245

3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB)

3.1 SDS and WB of leptospira serovar Pomona

The protein profile of whole cells (WC) of *Leptospira interrogans* serovar Pomona revealed many protein bands such as 28, 32, 34, 38, 41, 45, 58, 63 and 83 kDa**.** The Western blot analysis of the WC of *Leptospira interogans* serovar Pomona demonstrated many immunoreactive bands, with the major positive bands of 28, 32, 38, 41, 58 and 83 kDa (Figure 6).

The protein profile of total membrane (TM) of *Leptospira interogans* serovar Pomona revealed many protein bands such as 28, 32, 35, 36, 41, 45, 56 and 67 kDa**.** The Western blot analysis of the TM of *Leptospira interogans* serovar Pomona represented major immunoreactive bands 28, 32, 36, 41, 45, 56 and 67 kDa (Figure 6).

The protein profile of outer membrane protein (OMP) of *Leptospira interogans* serovar Pomona revealed major protein bands 28, 32, 41, 45, 56 and 63 kDa**.** The Western blot analysis of the TM of *Leptospira* interogans serovar Pomona represented major immunoreactive bands 28, 32, 45, 56 and 83 kDa (Figure 6).

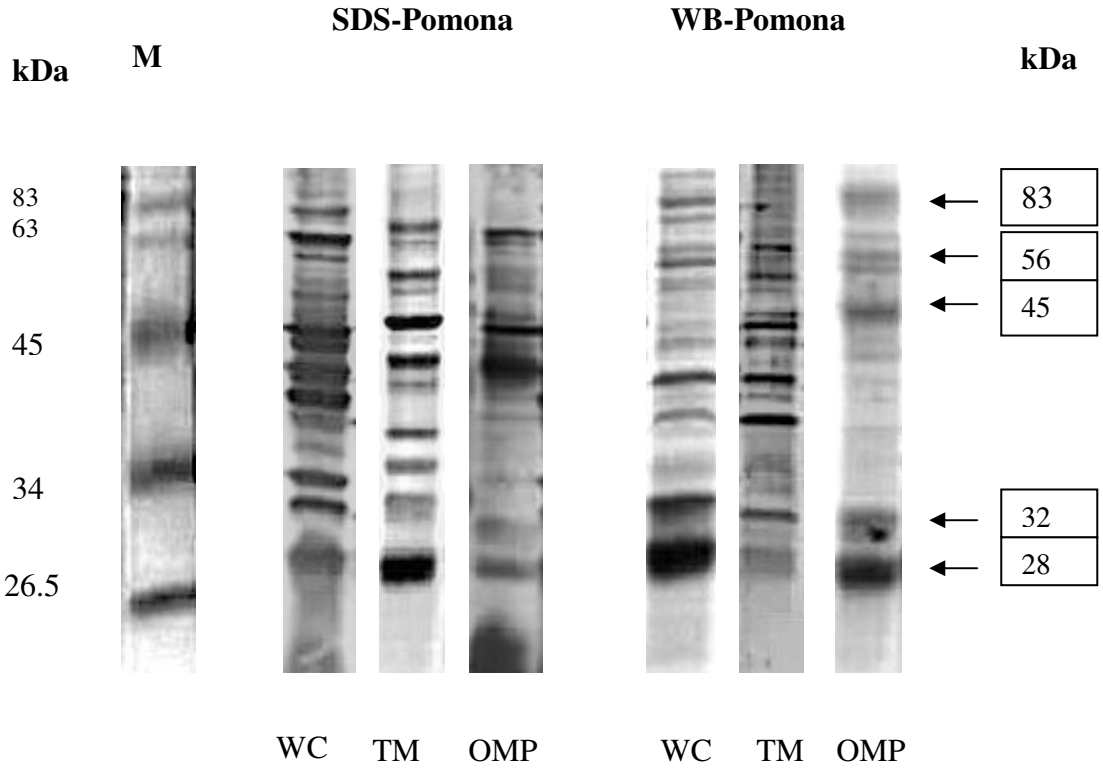


Figure 6 SDS-PAGE profiles of whole cell (WC), total membrane (TM) and outer membrane protein (OMP) of *Leptospira interrogan* serovar Pomona with prestained protein molecular weight marker (M). Western blotting (WB) profile of whole cell, total membrane and outer membrane of *Leptospira interrogans* serovar Pomona reactive with positive serum leptospirosis.

3.2 SDS and WB of leptospira serovar Sejroe

The protein profile of whole cell (WC) of *Leptospira interrogans* serovar Sejroe revealed many protein bands such as 22, 25, 28, 30, 32, 36, 41, 43, 45, 56, 58 and 83 kDa**.** The Western blot analysis of the WC of *Leptospira interogans* serovar Sejroe demonstrated many immunoreactive bands, with the major positive bands at 28, 30, 32, 38, 41, 43, 56 and 58 kDa (Figure 7).

The protein profile of total membrane (TM) of *Leptospira interogans* serovar Sejroe revealed many protein bands such as22, 28, 35, 38, 41, 45,56 and 83 kDa**.** The Western blot analysis of the TM of *Leptospira interogans* serovar Sejroe represented the major immunoreactive bands at 22, 28, 38, 41, and 56 kDa. (Figure 7).

The protein profile of outer membrane protein (OMP) of *Leptospira interogans* serovar Sejroe revealed major protein bands at 36, 38, 41 and 45 kDa**.** The Western blot analysis of the OMP of *Leptospira interogans* serovar Sejroe represented major immunoreactive bands at 28, 38, 41 and 45 kDa (Figure 7).

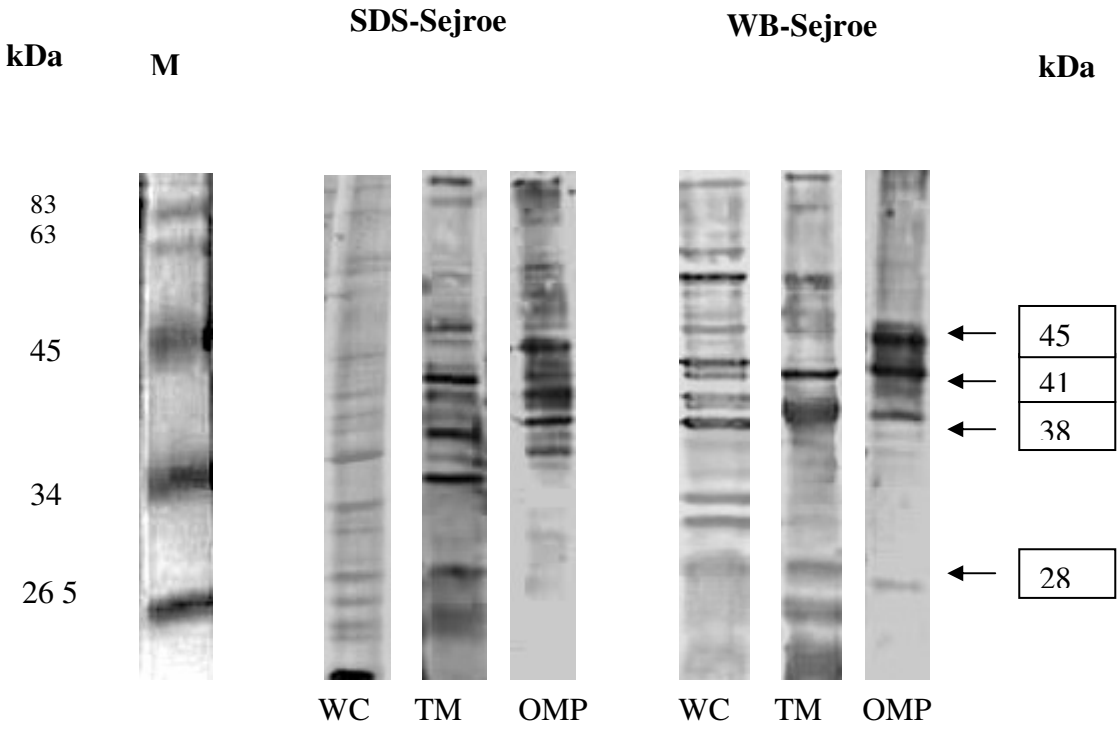


Figure 7 SDS-PAGE profiles of whole cell (WC), total membrane (TM) and outer membrane protein (OMP) of *Leptospira interrogans* serovar Sejroe with prestained protein molecular weight markers (M). Western blotting (WB) profile of whole cell, total membrane and outer membrane of *Leptospira interrogans* serovar Sejroe reactive with positive serum leptospirosis.

3.3 SDS and WB of *Leptospira* serovar Autumnalis

The protein profiles of whole cell (WC) and total membrane (TM) and outer membrane protein (OMP) of *Leptospira interrogans* serovar Autumnalis revealed many protein bands similar to the *Leptospira interogans* serovars Pomona and Sejroe**.** The Western blot (WB) analysis of the WC, TM and OMP of *Leptospira interogans* serovar Autumnalis demonstrated many immunoreactive bands similar to *Leptospira interogans* serovars Pomona and Sejroe.

4. Antigen optimization

4.1 Whole cell antigen optimization

The WC antigen of serovar Sejroe was diluted to final concentrations at 200, 300, 400, 500 and 600 ug/ml. The diluted antigen was tested with 20 leptospilosis serum. The optimal whole cell antigen concentration coupled with latex microparticle was 300 ug/ml (Table 10).

Table 10. Determination of whole cell antigen concentrations of leptospira serovar Sejroe for the Latex Agglutination Test (LAT).

	Concentration of whole cell antigen (ug/ml)				
	200	**300**	400	500	600
Positive - LAT (n = 20)	14	**19**	19	19	19

4.2 Total membrane antigen optimization

The TM antigen of serovar Sejroe was diluted to final concentrations at 100, 200, 300, 400 and 500 ug/ml. The diluted antigen was tested with 20 leptospilosis serum. The optimal total membrane protein antigen concentration coupling with latex micropartical was 300 ug/ml (Table 11).

Table11. Determination of total membrane antigen concentration of leptospira serovar Sejroe for Latex Agglutination Test

	Concentration of total membrane antigen (ug/ml)				
	100	200	**300**	400	500
Positive - LAT (n = 20)	12	15	**19**	19	19

4.3 Outer membrane antigen optimization

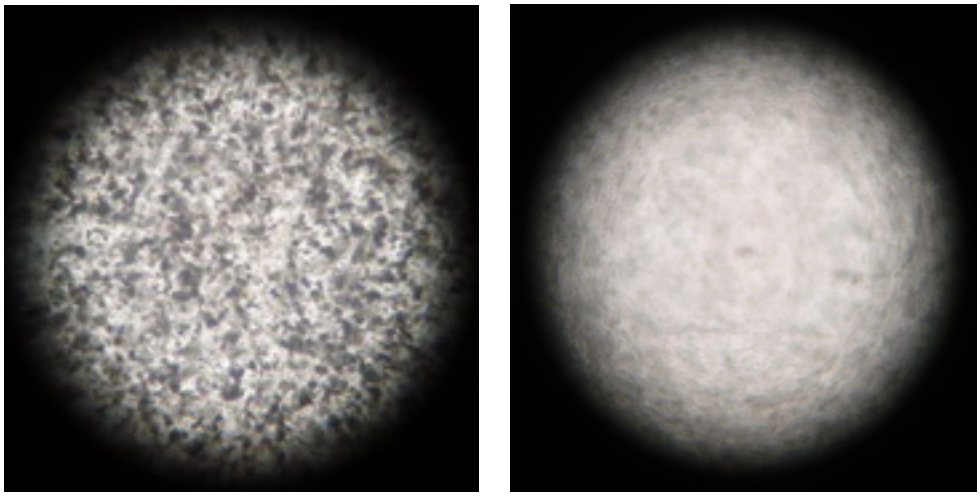
The OM antigen of serovar Sejroe was diluted to final concentrations at 100, 200 and 300 ug/ml. The diluted antigen was tested with 20 leptospilosis serum. The optimal outer membrane protein antigen concentration coupling with latex microparticle was 200 ug/ml (Table 12).

Table12. Determination of outer membrane antigen concentration of leptospira serovars Sejroe for Latex Agglutination Test.

	Concentration of outer membrane antigen (ug/ml)		
	100	**200**	300
Positive - LAT (n = 20)	12	**19**	19

5. Latex Agglutination Test (LAT)

The results of the latex agglutination tests were read within 2 minutes. A test score was considered positive if agglutination occurred, and negative if no agglutination was observed (Figure 8).



A: Positive result **B: Negative result**

Picture 8 Latex agglutination test -positive if agglutination occurred, negative if no agglutination (40x).

5.1 OMP-Autumnalis-LAT

40 positive MAT serums were tested by LAT from outer membrane protein serovar Autumnalis, 35 samples were positive and 5 samples were negative. 50 negative MAT serum were tested by LAT, 48 samples were negative and 2 samples were positive (Table 13). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 87.5%, 96.0%, 93.3%, 94.5%, 90.5% and 0.98, respectively. (Table 16).

Table 13. Latex Agglutination Test (LAT) with MAT sera, OMP antigen extracted from serovars Autumnalis

		MAT		
		+	**-**	**Total**
LAT OMP-Autumnalis	+	35	2	37
	-	5	48	53
	Total	40	50	90

5.2 TM-Autumnalis-LAT

40 positive MAT serum were tested by LAT from total membrane serovar Autumnalis, 37 samples were positive and 3 sample were negative. 50 negative serum MAT were tested by LAT, 46 samples were negative and 4 sample were positive (Table 14). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 92.5%, 92.0%, 92.2%, 90.2%, 93.8% and 0.97, respectively. (Table 16).

Table 14. Latex Agglutination Test (LAT) with MAT sera, TM antigen extracted from serovars Autumnalis

		MAT		
		+	**-**	**Total**
LAT TM-Autumnalis	+	37	4	41
	-	3	46	49
	Total	40	50	90

5.3 WC-Autumnalis –LAT

40 positive MAT serum were tested by LAT from whole cell serovar Autumnalis, 37 samples were positive and 4 samples were negative. 50 negative serum MAT were tested by LAT, 46samples were negative and 4 samples were positive (Table 15). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 95.0%, 82.0%, 87.7%, 80.8%, 95.3% and 0.98, respectively. (Table 16).

Table 15. Latex Agglutination Test (LAT) with MAT sera, WC antigen extracted from serovars Autumnalis

		MAT		
		+	-	**Total**
LAT WC-Autumnalis	+	38	9	47
	-	2	41	43
	Total	40	50	90

Table16. Comparison of OMP, TM and WC (serovar Autumnalis) in LAT using MAT sera

Statistical parameter	LAT (Serovar Autumnalis)			
	OMP	**TM**	**WC**	**Z test**
Sensitivity (%)	87.5	92.5	95.0	P > 0.05, Z= 0.4
Specificity (%)	96.0 *	92.0	82.0 *	P > 0.05, Z =1.5 * P < 0.05, Z =2.2
Accuracy (%)	93.3	92.22	87.7	P > 0.05, Z =0.9
PPV (%)	94.5	90.2	80.8	P> 0.05, Z =1.2
NPV (%)	90.5	93.8	95.3	P > 0.05, Z =0.4
Kappa	0.98	0.97	0.98	P > 0.05, Z =0.4

5.4 OMP-Pomona-LAT

40 positive MAT serum were tested by LAT from outer membrane protein serovar Pomona, 35 samples were positive and 5 samples were negative. 50 negative MAT serum were tested by LAT, 47 samples were negative and 3 samples were positive (Table 17). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 87.5%, 94.0%, 91.1%, 92.1%, 90.3% and 0.93, respectively. (Table 20).

Table 17. Latex Agglutination Test (LAT) with MAT sera, OMP antigen extracted from serovars Pomona

		MAT		
		+	**-**	**Total**
LAT OMP-Pomona	+	35	3	38
	-	5	47	52
	Total	40	50	90

5.5 TM-Pomona-LAT

40 positive MAT serum were tested by LAT from total membrane serovar Pomona, 38 samples were positive and 2 samples were negative. 50 negative MAT were tested by LAT, 41 samples were negative and 9 samples were positive (Table 18). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 95.0%, 82.0%, 87.7%, 80.8%, 95.3% and 0.98, respectively. (Table 20).

Table 18. Latex Agglutination Test (LAT) with MAT sera, TM antigen extracted from serovars Pomona

		MAT		
		+	-	**Total**
LAT TM-Pomona	+	38	9	47
	-	2	41	43
	Total	40	50	90

5.6 WC-Pomona–LAT

40 positive MAT serum were tested by LAT from whole cell serovar Pomona, 39 samples were positive and one sample was negative.50 negative MAT were tested by LAT, 40 samples were negative and 10 samples were positive (Table 19). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 97.5%, 80.0%, 87.7%, 79.5%, 97.5% and 0.97, respectively. (Table 20).

Table 19. Latex Agglutination Test (LAT) with MAT sera, WC antigen extracted from serovars Pomona

		MAT		
		+	-	**Total**
LAT WC-Pomona	+	39	10	49
	-	1	40	41
	Total	40	50	90

Table 20. Comparison of OMP, TM and WC (serovar Pomona) in LAT using MAT sera

Statistical parameter	**LAT (Serovar Pomona)**			
	OMP	**TM**	**WC**	**Z test**
Sensitivity (%)	87.5	95.0	97.5	P > 0.05, Z =0.5
Specificity (%)	94.0 *	82.0	80.0 *	P > 0.05, Z =0.2 * P < 0.05, Z = 2.1
Accuracy (%)	91.1	87.7	87.0	P > 0.05, Z =0.0
PPV (%)	92.1	80.8	79.5	P > 0.05, Z =0.1
NPV (%)	90.38	95.35	97.56	P > 0.05, Z =0.5
Kappa	0.98	0.98	0.97	P > 0.05, Z=0.4

5.7 OMP-Sejroe-LAT

40 positive MAT serum were tested by LAT from outer membrane serovar Sejroe, 34 samples were positive and 6 samples were negative. 50 negative MAT serum were tested by LAT, 48 samples were negative and two samples were positive (Table 21). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 85.0%, 96.0%, 91.1%, 94.4%, 88.8% and 0.98, respectively. (Table 24).

Table 21. Latex Agglutination Test (LAT) with MAT sera, OMP antigen extracted from serovars Sejroe

		MAT		
		+	**-**	**Total**
LAT OMP-Sejroe	+	34	2	36
	-	6	48	54
	Total	40	50	90

5.8 TM-Sejroe-LAT

40 positive MAT serum were tested by LAT from total membrane serovar Sejroe, 37 samples were positive and 3 samples were negative. 50 negative MAT serum were tested by LAT, 45 samples were negative and 5 samples were positive (Table 22). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 92.5%, 90.0%, 91.1%, 88.1%, 93.3% and 0.98, respectively. (Table 24).

Table 22. Latex Agglutination Test (LAT) with MAT sera, TM antigen extracted from serovars Sejroe

		MAT		
		+	**-**	**Total**
LAT TM-Sejroe	+	37	5	42
	-	3	45	48
	Total	40	50	90

5.9 WC-Sejroe–LAT

40 positive MAT serum were tested by LAT from whole cell serovar Sejroe, 38 samples were positive and 2 samples were negative. 50 negative MAT serum were tested by LAT, 42 samples were negative and 8 samples were positive (Table 23). The sensitivity, specificity, accuracy, positive predictive values (PPV), negative predictive values (NPV) and kappa of the latex agglutination test in relation to the standard MAT were 95.0%, 84.0%, 88.8%, 82.6%, 95.4% and 0.98, respectively (Table 24).

Table 23. Latex Agglutination Test (LAT) with MAT sera, WC antigen extracted from serovars Sejroe

		MAT		
		+	-	**Total**
LAT WC-Sejroe	+	38	8	46
	-	2	42	44
	Total	40	50	90

Table 24. Comparison of OMP, TM and WC (serovar Sejroe) in LAT using MAT sera

Statistical parameter	**LAT (Serovar Sejroe)**			
	OMP	**TM**	**WC**	**Z test**
Sensitivity (%)	85.0	92.5	95.0	P > 0.05, Z =0.4
Specificity (%)	96.0 *	90.0	84.0 *	P > 0.05, Z=0.9 * P < 0.05, Z= 2.0
Accuracy (%)	91.1	91.1	88.8	P > 0.05, Z =0.4
PPV (%)	94.4	88.1	82.6	P > 0.05, Z =0.7
NPV (%)	88.8	93.3	95.4	P > 0.05, Z =0.4
Kappa	0.98	0.98	0.98	P > 0.05, Z =0.0

6. Latex agglutination tests with Negative control serum

6.1 LAT with serum healthy blood donor and typhoid fever

Outer membrane protein (OMP) antigen, total membrane (TM) antigen and whole cell (WC) antigen from leptospira serovars Autumnalis, Pomona and Sejroe were coated onto latex beads.

The results of LAT using OMP, TM and WC of Autumnalis with 30 serum samples from healthy blood donors showed false positives in 1 sample (3.3%), 2 samples (6.6%) and 2 samples (6.6%) of tests, respectively. The results of LAT using OMP, TM and WC of Autumnalis with 30 patients with typhoid showed no false positives, 5 samples (16.6%) and 7 sample (23.2%) of tests, respectively (Table 25).

Table 25. The results of latex agglutination tests using OMP, TM and WC antigens from serovar Autumnalis with negative control (healthy blood donors and patients with typhoid fever).

Negative control	**Latex agglutination test results (serovar Autumnalis) (Negative/Positive)**		
	OMP	**TM**	**WC**
Healthy blood donors	**29 / 1**	**28 / 2**	**28 / 2**
Typhoid fever	**30 / 0**	**25 / 5**	**23 / 7**

The results of LAT using OMP, TM and WC of Pomona with 30 serum samples from healthy blood donors showed false positives in 1 sample (3.3%), 2 samples (6.6%) and 3 samples (10%) of tests, respectively. The results of LAT using OMP, TM and WC of Pomona with 30 typhoid patients showed no false positives, 5 samples (16.6%) and 7 samples (23.2%) of tests, respectively (Table 26).

Table 26. The results of latex agglutination tests using OMP, TM and WC antigens from serovar Pomona with negative control (healthy blood donors and typhoid fever).

Negative control	**Latex agglutination test results (serovar Pomona) (Negative/Positive)**		
	OMP	**TM**	**WC**
Healthy blood donors	**29 / 1**	**28 / 2**	**27 / 3**
Patient of typhoid fever	**30 / 0**	**25 / 5**	**23 / 7**

The results of LAT using OMP, TM and WC of Sejroe with 30 serum healthy blood donors showed false positives in 1 sample (3.3%), 2 sample (6.6%) and 3 (10%) of tests, respectively. The results of LAT using OMP, TM and WC of Sejroe with 30 typhoid patients showed no false positives, 5 samples (16.6%) and 7 samples (23.2%) of tests, respectively. (Table 27).

Table 27. The results of latex agglutination tests using OMP, TM and WC antigens from serovar Sejroe with negative control (healthy blood donors and typhoid fever).

Negative control	**Latex agglutination test results (serovar Sejroe) (Negative/Positive)**		
	OMP	**TM**	**WC**
Healthy blood donors	**29 / 1**	**28 / 2**	**27 / 3**
typhoid fever	**30 / 0**	**25 / 5**	**23 / 7**

6.2 Comparison between WC, TM and OMP antigens of LAT with serum from the commercial Lepto Tek Dri Dot kit

6.2.1 Outer membrane protein (OMP) antigen, total membrane (TM) antigen and whole cell (WC) antigen from leptospira serovar Sejroe and serogroup Sejroe were coated onto latex microparticle. The antigen latex was evaluated with 30 leptospira human serum samples that had tested positive with the commercial Lepto Tek Dri Dot kit (serovar Hardjo and serovar Sejroe) (Table 28).

Table 28. Latex agglutination test (LAT) with human positive leptospirosis serum by commercial kit- Lepto Tek Dri Dot.

Sample	**LAT (Positive / Negative)**		
	OMP	**TM**	**WC**
Human positive serum samples from the Lepto Tek Dri Dot kit	**28 / 2**	**30/ 0**	**30 / 0**

P > 0.05, Z = 1.4

6.2.2 Outer membrane protein (OMP) antigen, total membrane (TM) antigen and whole cell (WC) antigen from leptospira serovar Sejroe and serogroup Sejroe were coated onto latex beads. The antigen latex beads were evaluated with 30 leptospira pet serum that had been tested as positive by using the commercial Lepto Tek Dri Dot kit (serovar Hardjo and serovar Sejroe) (Table 29).

Table 29. Latex agglutination test (LAT) with pet positive leptospirosis serum by commercial kit -Lepto Tek Dri Dot

Sample	**LAT (Positive / Negative)**		
	OMP	**TM**	**WC**
Pet positive serum from Lepto Tek Dri Dot	**29 / 1**	**30 / 0**	**30 / 0**

P > 0.05, Z = 1.1

7. Stability results

Latex particles were bound with OMP from 3 serovars that had been stored at 4°C for up to 10 months; TM and WC samples had been stored up to 7 months.

CHAPTER 4

DISCUSSION

The results from this experiment demonstrate that the protocol developed for isolation of each type antigen from three serovars of leptospira works very well. The highest yields of protein were obtained from the whole cell (WC) preparation, followed by total membrane (TM) and outer membrane protein (OMP). In this study, a latex agglutination test (LAT) was developed using OMP, TM and WC antigens, and the results were analysed with the Microscopic Agglutination test (MAT), which is the gold standard method for leptospirosis diagnosis. The OMP, TM and WC antigens were extracted from the *L. interrogans* serovars Autumnalis, Pomona and Sejroe, which are emerging serovars in many parts of the world including Thailand (Ribotta et al., 2000). The main evidence that supports the validity of our protocol is the number of protein bands in SDS-PAGE and immunoreactive bands in western blot that correlate well with the protein concentrations in each type of antigen The Western blot tests of OMP *Leptospira interrogans* found fewer immunoreactive than in TM and WC. The immunoreactive bands of OMP, TM and WC were the same as previously reported (Amutha et al., 2006). However, the most interesting point may be the number of specific bands of 28 and 45 kDa which can be seen in all three types of antigen preparations. The ratio of each type of protein in each antigen preparation may play a role in the percentage of sensitivity and specificity of the tested latex .Therefore, the highest sensitivity obtained in whole cell coupling latex while highest specificity gained from outer membrane protein coupling latex. In addition, the accuracy, positive predictive value (PPV) and negative predictive value (NPV) of LAT using OMP, TM and WC antigens from the serovars Autumnalis, Pomona and Sejroe were not different ($P > 0.05$).

The outer membrane protein and total membrane protein based latex were developed for the first time to detect leptospira antibodies from human sample. In the present study, the sensitized beads could detect the antibodies against the pathogenic serovars of leptospira. Outer membrane protein coupling latex has been shown to have lower sensitivity than whole cell and total membrane coupling latex .However, a higher specificity was obtained in

OMP-LAT. The percentage of sensitivity and specificity of WC-LAT was similar to a previous report. All of the prepared antigen latex beads have been shown to be perfect agreement well with MAT (kappa value > 0.81). However,results of LAT compared with 40 MAT positive sera showed some negative result of leptospirosis, this may be MAT sera different serovars from LAT. While, results of LAT with 50 MAT negative sera demonstrated some positive result, the caused may be from patient have infected leptospira or cross reacted with other disease. The different positive in each serovar was difficult to understand. People may have contact with the organisms but do not become ill enough to seek medical attention. Leptospirosis antibodies usually appear within 7 days after the onset of symptoms, and the antibodies can persist in detectable quantities for many months (Farr, 1995).

The antigen latex was also evaluated with normal healthy blood donor sera to look for a background of leptospira infection in a community. The results demonstrate some background of antibody response of leptospirosis in the population with highest in WC-LAT of all serovars (Autumnalis serovars: OMP-LAT, TM-LAT, WC-LAT: 3.3%, 6.6% and 6.6% respectively, Pomona serovars: OMP-LAT, TM-LAT, WC-LAT: 3.3%, 6.6% and 10% respectively, Sejroe serovars: OMP-LAT, TM-LAT, WC-LAT: 3.3%, 6.6% and 10% respectively).

The antigen beads have been demonstrated to be crossed reaction with typhoid serum with highest percentage in WC-LAT but none in OMP-LAT in all three serovars of leptospira (OMP-LAT, TM-LAT, WC-LAT: 0%, 16.67% and 23.33% respectively).Typhoid fever is one of the infectious disease in which the clinical symptoms are hard to differentiate from leptospirosis. This is largely due to the broad cross-reactivity between leptospira with other fever diseases, making it difficult to determine the cause.

When our prepared coupling latex was compared with commercial kit lepto tek dri dot, whole cell and total membrane latex bead from the sejroe serovars were shown to be in 100% agreement with 30 human sera that were positive with the commercial kit, while the outer membrane latex beads could detect only 93.3% of them. Two patients sera that positive by WC-LAT and TM-LAT may be the result of cross reaction of the prepared antigen. However, there was no statistically significant difference between them ($P>0.05$, $Z=1.4$). Similar results were also obtained with 30 pet sera that positive with commercial kit ($P>0.05$, $Z=1.1$).

The developed latex test is simple and inexpensive and at the same time requires no specific expensive or sophisticated equipment. In addition, the sensitized beads were highly stable and could be stored in the refrigerator for more than 6 months. According to leptospirosis serology, the antibodies usually appear within 5-7 days after the onset of the symptoms. There is a need to develop new diagnostic approaches such as an antigen capture assay which detect leptospirosis early in the course of illness and can be feasibly applied in point of care settings, with the aim to initiate timely therapeutic interventions and reduce the high mortality from severe disease forms.

The main limitation of this study was the relatively small sample size. Extension of the study period to enroll more patients was not possible due to the considerable resources required. Another limitation was that in the leptospirosis cases of this study, most were MAT single serum. MAT paired sera are useful in investigation of leptospirosis that not enough because it was difficult to get down the patients for a second convalescent sample.

CHAPTER 5

CONCLUSION

Three types of leptospira antigen, sonicated whole cell extract, total membrane protein and outer membrane protein, were prepared from *Leptospira interrogans* serovars Autumnalis, Pomona and Sejroe. SDS-PAGE analysis of each antigen revealed many protein bands such as 22, 28, 32, 36, 38, 41, 45, 56, 58 and 83 kDa. The Western blot analysis of each antigen preparation demonstrated some major immunoreactive bands such as 28, 32, 38, 41, 45 and 56 kDa. The concentration of each antigen type and conditions for coupling with the latex particle were optimized with positive leptospirosis serum. Whole cell extract required the highest concentration of antigen to couple with the latex particles, while the lowest antigen concentration required was found with the outer membrane proteins.

The coupled latex was evaluated with 40 human MAT positive and 50 human MAT negative serum samples. Each coupled latex type from the three serovars gave similar results. WC LAT resulted in the highest sensitivity, however it was not significantly different from either the OMP LAT or the TM LAT. The specificity of the WC LAT was significantly lower than the OMP LAT. The accuracy, PPV, NPV and kappa value of the WC LAT were not significantly different from the OMP LAT and TM LAT. All types of prepared latex were in perfect agreement with the standard MAT test.

The antigen-based latex was also tested with 30 strong healthy blood donor serum samples. The highest percentage of background of leptospira antibody was found in the WC LAT while the lowest percentage was observed in the OMP LAT. The magnitude of the positive reactions of LAT in positive cases was stronger in the WC LAT than in the OMP LAT or the TM LAT.

The results from this experiment demonstrate that the developed protocol works quite well. OMP LAT has been shown to be a good candidate diagnostic test for leptospirosis. However, the test should be further examined with sera which are prospectively collected from patients who present with clinically suspected leptospirosis.

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Appendix A

Buffers and reagents for leptospira antigen preparation

1. Outer membrane protein isolation by triton X-100

1 % Triton X-114

Triton X-114	1	g
DW	100	ml

Mix well, kept at room temperature

0.5 M CaCl

CaCl	2.94	g
DW	40	ml

Mix well

2. Preparation of soluble and total membrane fraction

Lysis buffer (20mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 2 mg Lysozyme/ml)

1 M Tris pH 8	2	ml
1 M NaCl	15	ml
1 M EDTA	0.2	ml
20 mg Lysozyme/ml	10	ml

Mixed the reagent and adjust volume to 100 ml with distilled water. The solution was stored at 4°C.

0.5 M $MgCl_2$ (Stock solution)

$MgCl_2$	4.76	g
DW	100	ml

Mixed well the reagent

3. Whole (crude) cell antigen preparation

0.1% Sodium azide (stock 10 ml)

Sodium azide	10	mg
DW	10	ml

Mixed well and stored at room temperature.

Appendix B

Bradford's reagent for protein measurement

Coomassie Brilliant Blue G250	0.1	g
95 % Ethyl alcohol	50	ml
Phosphoric acid	100	ml
DW to	1000	ml

Appendix C

Reagent for SDS-PAGE

40% Acrylamide/Bisacrylamide solution

Acrylamide	38.9	g
Bis	1.06	g
DW to	1000	ml

Filter and store at 4°C in the dark.

1.5 M Tris-HCl, pH 8.8, 0.4% SDS (resolving gel buffer)

Tris base (USB Co, USA)	18.15	g
SDS (Bio Basic Inc.)	0.4	g

Dissolve in DW; adjust to pH 8.8 with 1N HCl. Bring total volume to 100 ml with distilled water. The solution was stored at 4°C.

0.5 M Tris-HCl, pH 6.8

Tris base (USB Co, USA)	6.05	g
SDS (Bio Basic Inc.)	2.0	g

Dissolve in DW; adjust to pH 6.8 with 1N HCl. Bring total volume to 100 ml with distilled water. The solution was stored at 4°C.

10% Ammonium persulfate (APS)

Ammonium presulfate	100	mg

Dissolve in 1 ml of distilled water.

Separating or resolving gel (12%)

1.5 M Tris-HCl pH 8.8, 4%SDS	2.5	ml
40% Acrylamide gel	3.1	ml
DW	3.24	ml
10% APS	50	ul
TEMED	10	ul

The gel was poured on the vertical gel electrophoresis and overlay with DW. Then the gel solution was allowed to polymerize for 30 minutes.

Stacking gel (4%)

0.5 M Tris-HCl pH 6.8, 2%SDS	1.25	ml
40 % Acrylamide	0.5	ml
DW	3.2	ml
10 % APS	50	ul
TEMED	10	ul

The gel solution was allowed to polymerize for 10 minutes.

2X Sample buffer (SDS reducing buffer)

The sample buffer was prepared as a stock solution by combining the following ingredients:

0.5 M Tris-HCl pH 6.8, 2% SDS	4.0	ml
Glycerol	2.0	ml
10% SDS	1.7	ml
2-merocaptoethanol	1.0	ml
1% Bromphenol blue	1.3	ml
Distilled water	10	ml

This mixture was stored at -20°C in aliquots.

Electrode (running) buffer pH 8.3 (5X)

Tris base	15	g
Glycine	72	g
SDS	5	g

Dissolved in distilled water adjust to a final volume of 1000 ml. The solution was stored at 4°C.

Appendix D

Reagent for gel staining

Coomassie brilliant blue R-250 (protein buffer)

Coomassie brilliant blue R-250	1.25	g
Methanol	250	ml
Glacial acetic acid	50	ml

Dissolve reagents and adjust the volume to 500 ml with distilled water. Fitter the solution with a Whatman No. 1 paper for remove a particular matter and keep at RT.

Destaining solution

Methanol	200	ml
Glacial acetic acid	50	ml
Glycerol	25	ml

Dissolve reagents and adjust the volume to 1000 ml with distilled water. The solution was stored at RT.

Appendix E

Reagents and buffers for immunoblot analysis

Towbin's buffer (transfer buffer)

Tris base	0.3	g
Glycine	1.44	g
SDS	0.01	g
Methanol	15	ml

Dissolve and adjust the volume to 100 ml with distilled water.

0.15 M Tris buffer saline (TBS) pH 7.4

Tris base	18.18	g
DW	700	ml

The pH was adjusted to 7.4 with 1N HCl. The final volume was adjusted to 1000 ml with DW.

Blocking buffer (5% skim milk in TBS-T)

Skim milk powder	2.5	g
TBS-Tween	50	ml

The solution was heated on a hot plate. The solution was cooled down to room temperature before use.

Washing buffer (0.05% Tween-20 in TBS pH 7.4)

This solution was prepared by adding 0.5 ml of Tween-20 in 1 L of TBS ph 7.4 and mixed well.

Conjugate solution (secondary antibody)

This solution was prepared by diluting anti-human immunoglobulins peroxidase conjugate with blocking buffer to make the desire dilution.

Appendix F

Leptospira media and antibiotic

1. Neopeptone medium

The base medium consisted of the following ingredients:

Neopeptone	2	g
NaCl	1	g
Distilled water (DW)	1000	ml

All of ingredients were mixed, dissolved by boil and adjusted pH to 7.2 before adjusted the volume. This medium was then sterilized by autoclave at 121°C. for 15 minutes. After inactivated at 56°C for 1 hr. that 80 ml of rabbit serum containing 0.4 ml of rabbit blood was added in 100 ml of the medium. The medium was heat inactive 56°C for 30 minutes, RT overnight. Inactive 56°C for 30 minutes again. The neopeptone medium was aliquoted and kept at 4°C until used.

2. Fletcher media

Fletcher medium base	2.5	g
H2O (DW)	920	ml

All ingredients were boiled and then autoclaved at 121°C, 15 pound 15 mins and pH was adjusted to pH 7.4. After the temperature of the solution was declined to with 55°C, Inactive 80 ml of rabbit serum (Gibco) containing 400 ul of rabbit blood were added to the solution. The solution of 5 ml was aliquoted into a screw capped tube and then heat inactivated at 56°C for 1 hr. for 2 days consecutively.

3. Ellinghausen and McCullough modified Johnson and Harris medium (EMJH)

100 ml of EMJH medium consisted of 90 ml of EMJH base solution and 10 ml of 10X stock.

EMJH base solution

EMJH	2.3	g
DW	900	ml

Adjust to pH 7.4, and autoclave at 121°C. for 15 minutes. The solution was kept at 4°C until used.

100 ml of 10X stock are prepared by adding the following ingredients.

Bovine serum albumin (BSA)	10	g
Sodium pyruvate	0.2	g
Sodium acetate anhydrous	0.1	g
Tween 80	1.25	g
$FeSO_4.7H_2O$	0.01	g
1.5% CaCl2	0.7	ml
1.5% $MgCl_2.2H_2O$	0.7	ml
0.4% $ZnSO_4.7H_2O$	1	ml
0.3% $CuSO_4.5H_2O$	0.1	ml
0.1% vitamin B_{12}	0.2	ml
20% glycerol	0.5	ml
0.1% $MnSO_4.4H_2O$	1	ml

3 ml of rabbit serum and 50 ul of rabbit blood was inactivated 56°C, 1 hr. before added in 100 ml of EMJH medium.

4. Antibiotic

5 ug/ml chloramphenicol

Chloramphenicol	0.05	mg
DW	10	ml

Mix and kept at -20°C

300 ug/ml neomycin

Neomycin	0.3	mg
DW	10	ml

Mix and kept at -20°C

Appendix G

Reagent for Sensitization of latex beads

100 mg/ml Bovine serum albumin (BSA)

Bovine serum albumin	1	g
DW	10	ml

Mixed the reagent and stored at 4°C

1% Latex bead (prepare before use)

10% Latex bead	100	ul
PBS	900	ul

Mix solution

0.1% sodium azide

Sodium azide	1	g
DW	10	ml

Mix solution and kept at room temperature.

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List of Publication and Proceeding

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