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THESIS

MOLECULAR AND IMMUNOGENICITY CHARACTERIZATIONS OF *flaB* AND *lig* AND THEIR APPLICATIONS FOR LEPTOSPIROSIS DETECTION

ANCHALEE LA-ARD

A Thesis Submitted in Partial Fulfillment of
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In this experiment was to test the ability of the specific pair of primers (*rrs*, *flaB* and *lig*) to amplify the DNAs of *Leptospira* and other bacteria and to determine and evaluate the use of nearly full-length 749-bp *flaB* sequencing from well-characterized type strains for species identification of *Leptospira* spp. The result demonstrated that, the primers which were used in this PCR are specific for *Leptospira*. All recognized and unknown species of leptospira can be identified by using standardized *flaB* gene sequencing. Furthermore cloning and expression of target antigen genes from *Leptospira* derived recombinant protein may be useful for developing improved test for rapid diagnosis of leptospirosis. In this study, *L. interrogans* serovar Canicola strain Hond Utrecht IV was chosen to be a target for cloning flaB. Recombinant FlaB (His.FlaB) protein was expressed as an antigen for the production of polyclonal antibodies. The specificity of polyclonal antibodies was confirmed by immunoblot analysis and indirect immunofluorescent antibody technique using purified recombinant FlaB protein and whole-cell antigen preparation from pathogenic and non-pathogenic leptospires and other bacteria as antigens. The result demonstrated that polyclonal antibodies were a potential tool for Leptospira spp. detection. *L. kirschneri* serovar Grippotyposa strain Moskva V was chosen to be a target for cloning the conserved region of lig. The specificity of antibodies against recombinant Lig protein was determined by using immunoblot analysis. The recombinant Lig antigen (GST.Lig) was developed and used as an ELISA antigen for the detection of the anti-leptospiral antibodies. This assay was evaluated with canine sera (n=91) that were microscopic agglutination test (MAT)-negative (at < 1:100 dilution) and sera (n=103) that were MAT-positive (at > 1:100 dilution) using 24 serovars. The ELISA showed a relative sensitivity as compared to MAT was 84.5% whereas the specificity was 76.9%.

In conclusion, the primers which were used in the PCR reaction are specific for Leptospira and flaB sequence has become an accurate tool for discrimination and identification of leptospires. The polyclonal antibodies against recombinant FlaB proteins were a potential tool for Leptospira spp. detection. The recombinant Lig protein based ELISA could be used as a screening test for serodiagnosis of canine leptospirosis. The ELISA described here is suitable for large scale serological examination, routine diagnostics, epidemiology surveys and follow-up investigations for outbreaks. It would be particularly useful in canine with full consideration of clinical picture and vaccination history of animals.

Student's signature Thesis Advisor's signature

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Anchalee La-ard

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LIST OF ABBREVIATIONS

µl	=	microliter
ml	=	milliliter
µg	=	microgram
mg	=	milligram
bp	=	base pair
mM	=	milimolar
M	=	molar
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
PCR	=	Polymerase Chain Reaction
EtBr	=	Ethidium Bromide
UV	=	ultraviolet
SDS-PAGE	=	Sodium Dodecyle Sulfate Polyacrylamide Gel Electrophoresis
rpm	=	round per minute
°C	=	Degree Celsius
E. coli	=	*Escherichai coli*

MOLECULAR AND IMMUNOGENICITY CHARACTERIZATIONS OF *flaB* AND *lig* AND THEIR APPLICATIONS FOR LEPTOSPIROSIS DETECTION

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by infection of spirochetes belongs to the genus *Leptospira*. Leptospirosis causes a wide range of clinical manifestation in mammalian hosts (Faine, 1999). Symptoms of infection vary form subclinical to potentially fatal with multiorgan involvement (Levett, 2001; Bharti *et al*., 2003). The clinical presentation of leptospirosis is variable, and can range from a mild flu-like illness to a severe disease with pulmonary hemorrhage, renal failure, and occasionally death (Levett *et al*., 2001). Consequently, leptospirosis is often misdiagnosed as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis, or gastroenteritis. Timely diagnosis of leptospirosis is essential because prompt, specific treatment, as early in the illness as possible, is important to ensure a favorable clinical outcome (Chin, 2000).

In all animals, the initial symptoms of acute leptospirosis are essentially similar and not characteristic. They are the symptoms of an acute febrile illness such as malaise, depression, anorexia, and conjunctivitis, accompanied by fever, as yet these are not easy to discuss with animals. Clinical signs of leptospirosis may progress to more severe symptoms, such as bleeding and its consequences; jaundice, central nervous system (CNS) involvement, liver and renal failure. Abortion, stillbirth and mastitis in lactating animals occur late in the acute stage. Chronic infection is usually localized in the kidneys and may occur in animals which have passed through the acute stage, with or without detectable clinical evidence of illness. Leptospires are excreted in the urine in the carrier state which may be of short or long duration, and either intermittent or continuous, depending on the animal and the infection serovars. *Leptospira* can survive in surface water, soil and mud as long as conditions are not acid and dry (Faine *et al*., 1999). The animals become infected through the exposure

with water or soil containing urine from infected animals. It is complicated to control this disease, because infected animals often shed leptospires for a week to months following infection and wide rodents may be carrier for more a year (Faine, 1982, Faine *et al.*, 1999). Chronic infection may be detected only by laboratory test if there are no clinical signs. The symptoms, when present, are those of nephritis, with usually the passing of large amounts of urine of low specific gravity (Faine, 1982).

To date, serological testing has been most frequently applied to confirm the clinical diagnosis and to conduct epidemiology studies. Detection of specific anti-leptospiral antibody by microscopic agglutination test (MAT) is the standard reference test for diagnosis of systemic infection, despite the limitations imposed by the need to maintain cultures of several hazardous stock leptospiral serovars, the subjectivity involved in reading the results under dark-field microscopy and relatively insensitive on acute-phase samples. MAT also required analyses of paired sera to verify the seroconversion, which delays diagnosis (Faine *et al.*, 1999). Early diagnosis is the key to the treatment of leptospirosis. There is an urgent need to develop a rapid, sensitive and appropriate diagnostic test that could be used in a routine diagnostic laboratory to detect antibodies against leptospires. To overcome this problem, some potentially useful screening tests for use in all routine laboratories have been proposed (Adler *et al*., 1980).

Information on the nature and identity of leptospiral antigens is important for elucidation of their significance in the immunity leptospirosis and the pathogenesis and diagnosis of Leptospirosis. In 1997, Lin *et al*. identified a 35-kDa protein from *L. interrogans* serovar pomona as a flagellin protein (FlaB). FlaB has been reported as a highly conserved protein among pathogenic *Leptospira* spp. FlaB was included in a group of protein antigens that were targeted to humoral immune response during natural infection (Lin *et al*., 1997). Palaniappan *et al*., 2002 was the first to identify and characterize immunogenic Leptospira proteins that are expressed during infection. The gene for one such immunoreactive immunoglobulin-like 130–kDa protein (LigA) of *L. interrogans* serovars Pomona type kennewicki has been characterized and shown to be expressed *in vivo*, may play a role in host cell attachment and invasive during

leptospiral pathogenesis. Lig can differentiate between vaccinated and naturally infected animals. Therefore, identification of LigA that is expressed only *in vivo* may provide new insights for developing strategies to improve diagnosis, vaccination and treatment protocol (Palanippan *et al*., 2002; Palanippan *et al*., 2004).

Recombinant antigens are brought into attention because of their specificity and reproducibility of quality of prepared antigens. Recombinant antigen based serologic tests are widely used in screening for spirochetal infection such as Lyme disease and syphilis. Recently, the use of recombinant proteins for serodiagnosis of leptospirosis has been widely investigated (Bughio *et al.*, 2003; Dey *et al*., 2004).

Knowledge of the epizootiology of leptospirosis has been important in the design of effective preventive strategies. There is a need for the method that can be used as a tool for diagnostic and epidemiology survey. To control leptospirosis, it is important to establish a rapid identification method and infection-control measures. Thus, cloning and expression of target antigen genes from *Leptospira* derived recombinant protein may be useful for developing improved test for rapid diagnosis of leptospirosis. In this experiments, we expect to evaluate a method that can identification of *Leptospira spp.* and a rapid, simple and sensitive screening test that could be used in any laboratory for leptospirosis detection.

OBJECTIVES

1. To test the ability of the specific pair of primers to amplify the DNAs of *Leptospira* and other bacteria.

2. To determine and evaluate the use of nearly full-length 748-bp *flaB* sequencing from well-characterized type strains for species identification of *Leptospira* spp.

3. To clone and express FlaB as His-fusion protein in *Escherichia coli* from *L. interrogans* serovar Canicola strain Hond Utrecht IV and the conserved region of Lig as GST-fusion protein in *Escherichia coli* from *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V.

4. To generate and develop the utility of polyclonal antibodies against recombinant His.FlaB protein for the IFA test to detect the reference strains of leptospires.

5. To develop and evaluate the utility of the recombinant protein Lig as antigen in an indirect enzyme-linked immunosorbance assay for the detection of antibodies against *Leptospira* in canine sera.

LITERATURE REVIEW

1. Historical aspect

In 1886, Weil published a human disease that caused jaundice, icteric and renal failure but differed from other known infection disease (Weil, 1886). He named the bacterium *Leptospira* (thin spirals) icterohaemorrhagiae, although the human disease from leptospirosis was given the name Weil's disease. In 1970, Stimson isolated the spirochete that caused Weil's disease from a patient having died of "yellow fever" and proposed the name of this organism as *Spirochaete interrogans* since the organism, with its hooked ends, resembled a question mark (Stimson, 1907). A few years later in 1917, Noguchi demonstrated that this spirochete differed from other spirochetes and he proposed to put it in a new genus, *Leptospira* (Noguchi, 1917). At that time, Ido and coworkers studied a carrier of leptospires which was identified in rats (*Rattus norvegicus*) (Ido *et al*., 1917). Leptospirosis in animals was described clinically around 1850, although its etiology was unknown. Subsequently *Leptospira* has been associated with many mammalian hosts, including human, cattle, swine, goats and hamster. However leptospirosis was not identified as a disease in domestic animals until the next century for the first time in dog. Leptospirosis was not recognized as a veterinary problem in food animal production until the late 1930s (Terskich, 1940).

2. Taxonomy and classification

Traditionally, the genus *Leptospira* was divided into two species: the pathogenic *Leptospira interrogans* and the non-pathogenic *Leptospira biflexa*. Pathogenic leptospires are composed of more than 200 serovars and leptospires have been classified on the basis of their antigenic characteristics, called serogroups and serovars. Recently the taxonomy of the genus *Leptospira* has been revised on the basis of DNA hybridization resulting in thirteen proposed pathogenic species (*L. interrogans, L. alexanderi, L. fainei, L. inadai, L. kirschneri, L. meyeri, L. borgpetersenii, L. weilii, L. noguchi, L. santarosai*, genomospecies 1, 4 and 5) and

three non-pathogenic species (*L. biflexa, L. wolbachii* and genomospecies 3) based on at least 70% DNA relatedness by DNA-DNA homology and less than 5% divergence in DNA relatedness (Bharti *et al.*, 2003).

All recognized species have been classified as pathogenic or non-pathogenic species. The differences of the pathogen and non-pathogen are shown in Table 1.

Table 1 Comparison between the pathogenic and non-pathogenic leptospira species (Cinco, 1986).

Characteristic	Pathogenic[a]	Non-pathogenic[a]
Pathogenicity	+	-
Growth at 13 ^{o}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

[a] +, present in more than 90% strains; -, present in less than 10% strains; S, present in 11-89% strains.

3. Biology of Leptospires

Leptospira have the general structure characteristics that distinguish spirochaete from other bacteria. The whole cell structure is surrounded by the outer membrane which consisted of lipopolysaccharide (LPS), protein and phospholipids. The membrane is thick, almost transparent and appears in the darkground as a narrow clear zone or halo. The term protoplasmic cylinder describes the cellular components enclosed by the outer membrane, and consists of a peptidoglycan layer and cytoplasmic membrane. The bacteria contain two periplasmic flagella (PF) which are wound around the protoplasmic cylinder (Figure1). They have characteristic hooked

ends, which are clearly visible during the spinning motility of the bacterium which is caused by PF inserted at each end (Palit *et al.*, 1974).

The outer envelope or outer membrane of leptospira is morphologically similar to those of Gram-negative bacteria but the density of transmembrane outer membrane protein is extremely low (Haake *et al.*, 1991). The outer membrane of pathogenic *Leptospira* species grown in the culture media contains lipopolysaccharide (LPS), a porin (OmpL1), and several liproproteins including LipL36 and LipL41. Various antigens associated with the LPS have located in outer membrane (Jost *et al.*, 1988; Haake *et al.*, 1991; Shang *et al.*, 1995; Barnett *et al.*, 1999).

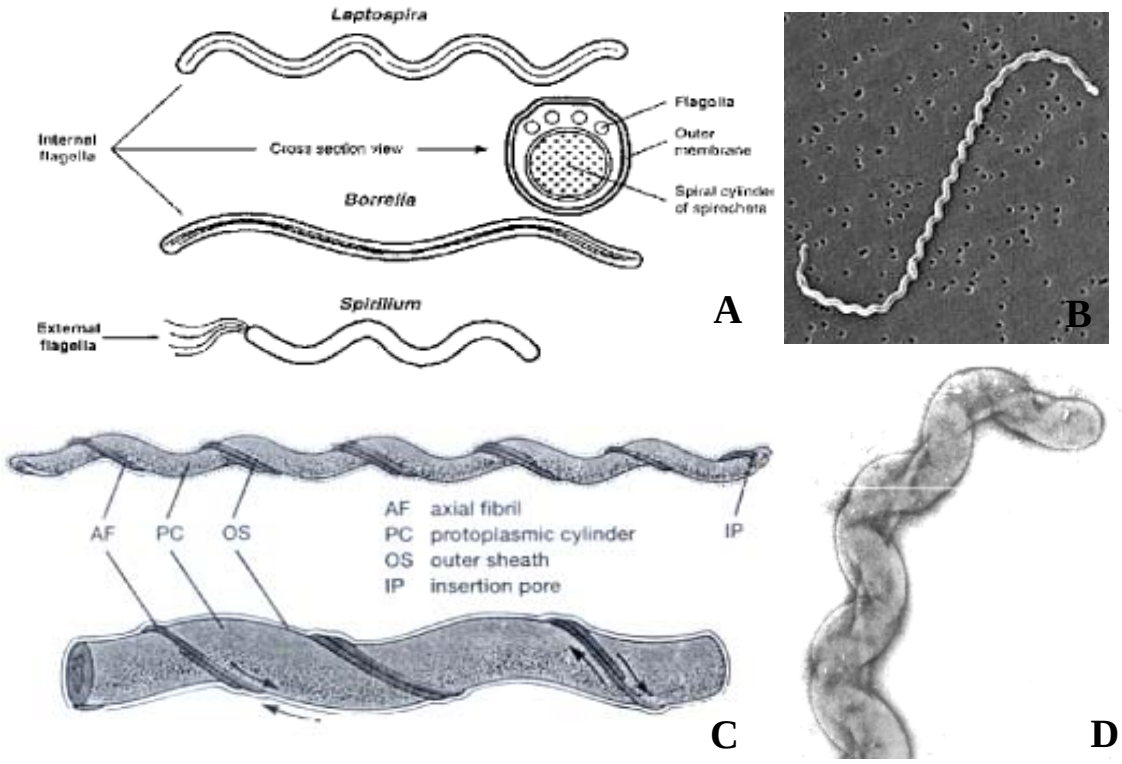


Figure 1 Morphology of the *L. interrogans.* (A) Morphological comparison of *Leptospira, Borellia* and *Spirillum* (Johnson, 1999). (B) Electron micrograph of intact cell showed the spinal shape and hook end (Levett, 2001). (C) Diagrammatic representation of a leptospiral structured as interpreted from electron micrograph (Prescott *et al.*, 1993). (D) Electron micrograph of one end showed the axial filament (Volk *et al*., 1996).

Leptospira species are Gram negative, motile, obligate aerobic, helically coiled rods with a length of 6-20 μm and a width of 0.1-0.5 μm. They are obligate aerobes which grow well in semi-solid medium containing 0.2% agar. Growth characteristics for *Leptospira* differ from those of many other bacteria; the optimum growth temperature *in vitro* is 28-30^{o}C, even though they are pathogenic for mammals with higher body temperatures. Growth of leptospira is often slow on primary isolation, requiring 7-10 days for a normal culture to become dense. They grow in simple media enriched with vitamins (vitamins B_2 and B_{12} and growth factor), long-chain fatty acid, and ammonium salts (Johnson and Faine, 1984).

4. Leptospiral antigenic substances

The cell of leptospira are divided into five parts, the outer membrane, the flagella, the periplasmic space that contain peptidoglycans, the protoplasmic membrane (plasma membrane) and the protoplasmic cylinder. The identification of leptospiral antigens has potentially important implications for the development of serodiagnostic and immunoprotective strategies. Leptospiral antigenic substances are summarized as follow;

4.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is the major surface antigen of leptospires. It plays a critical role in immunity to leptospirosis and forms the basis of serological classification. Leptospiral LPS is known to cause a large number of biological effects such as activation of macrophages, platelets and B cells in susceptible hosts (Isogai *et al*., 1990; Vinh *et al.*, 1994).

4.2 Proteins

There are many leptospiral proteins, they play important role in pathogenesis and transmission of leptospirosis. The important leptospiral proteins are

located at outer membrane, periplasmic membrane, flagella or periplasmic cylinder and other.

Outer membrane proteins (OMPs) are great subject of interest because they are located at the cell surface , where bacterial pathogens interact with the host. OMPs may play a key role in bacterial pathogenesis by acting as (i) adhesins (Kropinski *et al.*, 1978), (ii) porins (McGuinness *et al*., 1990), (iii) target of bacterial antibody (Murphy *et al*., 1988) and (iv) receptor for soluble molecules such as complement proteins (Hoffam *et al*., 1992). The immunodominant proteins recognized during the humoral immune response of the leptospirosis in human include LipL32 and LipL41 of outer membrane proteins and GroEL and DnaK of the cytoplasmic fraction heat shock protein (Barnett *et al*., 1999; Haake *et al*., 2000; Gurreiro *et a*l., 2001).

Leptospires, because of their terminal hooks, are unique among spirochetes. Motility in leptospires depends on the rotation of two periplasmic flagella (PF). Sequence analysis of flagella genes and proteins from several spirochetes shows that there are two distinct classes of proteins, the FlaA and FlaB proteins, in the filament. Sequence alignment revealed of homology in C-terminal and N-terminal region a reduced level of homology in the central region. FlaA proteins are associated with the sheath surrounding a core which is composed of FlaB proteins. The variable central region of FlaB may be expressed and used to assess the immune response (Lin *et al*., 1997). The 35 kDa serovar cross-reactive flagella protein from *L. interrogans* serovar Pomona was identified as a flagellin, a class B polypeptide subunit (FlaB) of the periplasmic flagella. The FlaB is conserved among these serovars, which is believed to contain specific sequence information and which may thus be useful in the design of DNA or synthesis peptide probes suitable for the detection of infection with pathogenic leptospires (Lin *et al.*, 1997; Bughio *et al.*, 1999)

Other immunogenic leptospiral proteins are Leptospiral immunoglobulin-like (Lig) proteins. Lig are surface-exposed outer membrane proteins punctuated by tandem repeats of about 90 amino acids of bacterial immunoglobulin (Ig) like

domains (Palaniappan *et al*., 2002; Matsunaga *et al*., 2003; Koizumi and Watanabe, 2004; Palaniappan *et al.*, 2004) (Figure 2). There are two intact leptospiral genes, *ligA* and *ligB*, and one disrupted gene, *ligC*, which encode domain belonging to the bacterial immunoglobulin-like superfamily. LigA consists only of Ig-like domains, whereas LigB has an additional unique domain at the C terminus. The bacterial immunoglobulin-like domains are found in various adhesion proteins of pathogenic bacteria; for example, the intimin of *E. coli* and the invasin of *Y. pseudotuberculosis* and may function as adhesion molecules (Kelly *et al*., 1999; Hamburger *et al*., 1999; Luo *et al*., 2000). Both LigA and LigB contain an N-terminal lipobox and LigA was lipidated when expressed in *E. coli* (Koizumi *et al*., 2004). Expression of Lig protein and *lig* mRNA was lost after attenuation by culture of *L. kirshneri* and *L. interrogans*, suggesting that Lig proteins are associated with virulence. Lig proteins were not detected in some virulent strains during *in vitro* culture, although specific mRNAs were transcribed (Palaniappan *et al.*, 2002; Palaniappan *et al*., 2004). Recent studies show that expression of Lig proteins, surface exposure of LigB and extracellular release of LigA are all enhanced by physiological osmolarity (Matsunaga *et al*., 2005). Sera from either rats immunized with extracts of *in vitro* cultured low-passage strain or sera from vaccinated dogs failed to react with Lig proteins in Western blot analysis, while sera from captured rat reservoirs or dogs infected with *Leptospira* gave a positive response (Matsunaga *et al*., 2003; Palaniappan *et al*., 2004). Therefore expression of Lig proteins is upregulated during infection of a mammalian host. The *lig* genes are present among various pathogenic, but not non-pathogenic leptospires (Matsunaga *et al*., 2003; Koizumi *et al*., 2004; Palaniappan *et al.*, 2004). Sera from human patients that are infected with different leptospiral serogroup strains reacted with Lig proteins (Koizumi *et al*., 2004). Furthermore, in a mouse model of leptospirosis, the Lig proteins elicited protective immunity against challenges, not only with the homologous serovar Manilae infection but also the heterologous serovar Icterohaemorrhagiae (Koizumi *et al*., 2004). These data suggest that Lig proteins could induce protective immunity against the challenge with strains of various leptospiral serovars. Lig proteins are major antigen recognized during the acute host infection and may play a role in host cell attachment and invasion during leptospiral pathogenesis (Matsunaga *et al*., 2003).

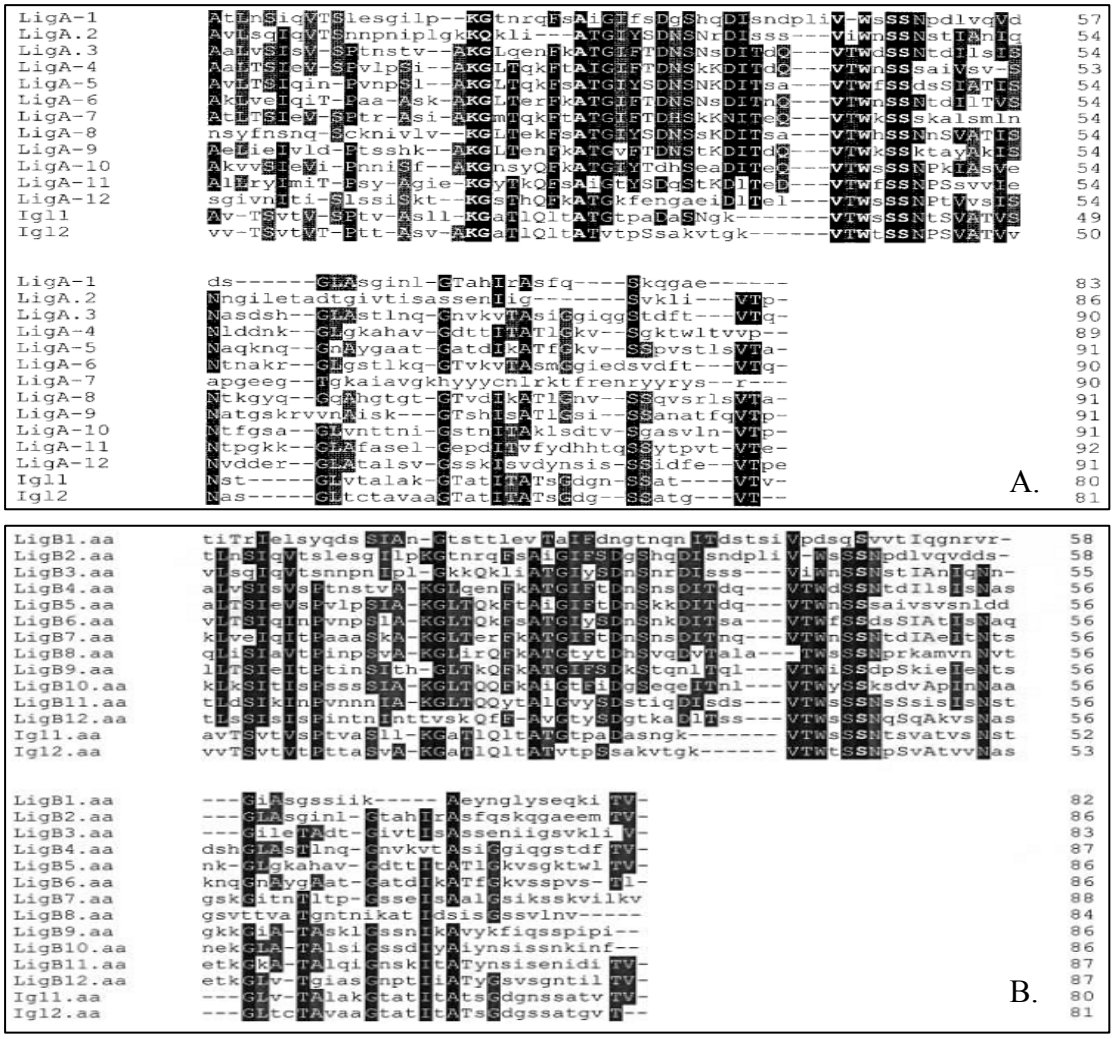


Figure 2 A. Alignment of the predicted amino acid sequences for the 12 tandem repeats and the immunoglobulin-like domain of *E. coli* intimin-binding (receptor) protein (Ig11, CD:pfam02368; Ig12, CD:smart00635). Twelve repeat sequences of a 90-amino-acid sequence include residues from 136 to 218, 224 to 310, 311 to 400, 401 to 489, 490 to 580, 581 to 670, 671 to 760, 761 to 851, 852 to 942, 943 to 1033, 1034 to 1125, and 1126 to 1216 (Palaniappan *et al*., 2002). B. Alignment of 12 repeats of 90 amino acid sequences of LigB and its identity with the bacterial Ig-like domain from Pfam (Ig1 and Ig2). Gaps have been introduced to optimize alignment among the polypeptides (Palaniappan *et al.,* 2005).

4.3 Glycolipoprotein

Leptospira interrogans glycolipoprotein (GLP) has been implicated in pathological and functional derangement in leptospirosis. In 1989, Bao and Dai demonstrated that GLP of *L. interrogans* strain 017 has the cytotoxin effect through a specific attachment, endocytosis in endothelial cells. The endothelial cell probably is a receptor-mediated endocytosis.

The hypothesis of GLP as a toxic factor resulting from *Leptospira* was the enhancement of tissue damage late in the course of disease (Santos *et al*., 1989). Another hypothesis is the putative role of GLP-induced inhibition of Na, K-ATPase as an initial mechanism in the physiopathology of leptospirosis. This glycolipoprotein fraction that present in the infected tissue may induce, through this inhibitor, cellular dysfunctions responsible for the symptoms, in particular those associated with electrolytical disorders such as disturbances of renal electrolyte handling, cardiac arrhythmia or diarrhea (Younes-Ibrahim *et al*., 1995; 1997).

4.4 Peptidoglycans

Peptidoglycan and muramyl peptide derivatives have diverse and complex biological effect in mammals, including stimulation of cells involved in the immune response, activation of the complement cascade, adjuvant effect, induction of inflammation, activation of monocytes and neuropharmacological activity (Stewart-Tull, 1980). Peptidoglycan extracted from *L. interrogans* may play a role in modulating the immuno competent cell functions. It is mitogenic for human peripheral blood mononuclear cells (PBMC) and able to induce complement consumption. Peptidoglycans can contribute to the host response during leptospiral infection to stimulate leucocyte phagocytosis and the metabolic burst of resting as well as phagocytosing leucocytes (Cinco *et al*., 1993). The peptidoglycans from pathogenic leptospires are among the molecules that can directly activate vascular endothelial cells to increase theirs adhesiveness for neutrophilic granulocytes (Dobrina *et al*., 1995).

5. Molecular biology

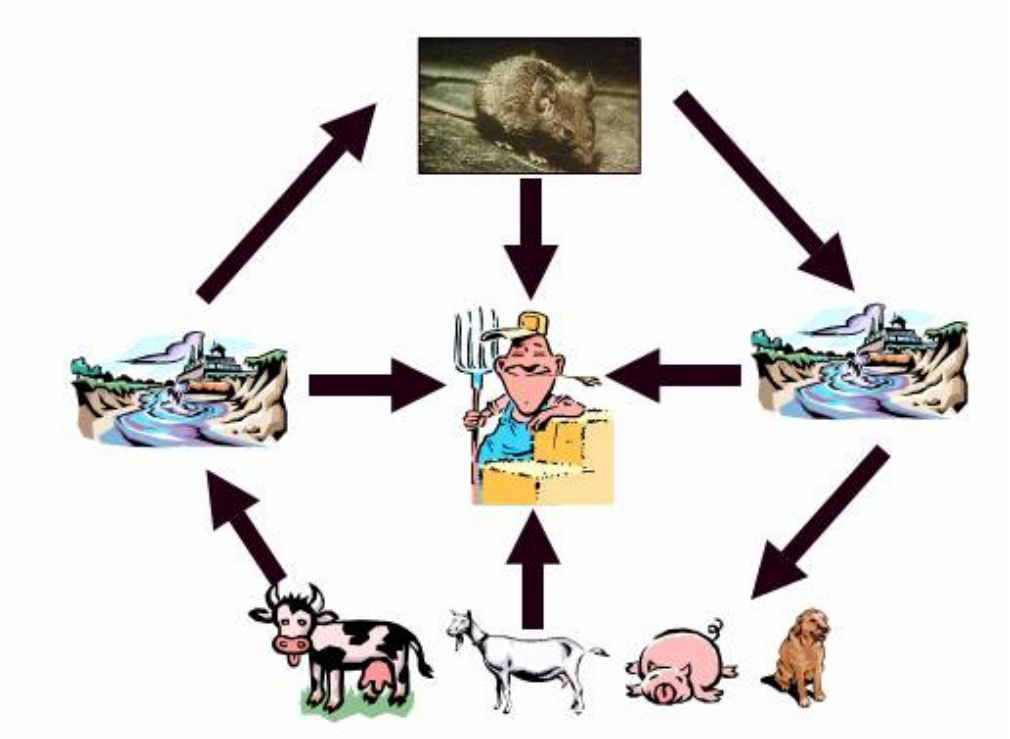
Leptospires are phylogenetically related to other spirochetes. The leptospiral genome is approximately 5,000 kb in size, although smaller estimates have been reported. The genome is comprised of two components, a 4,400-kb chromosome and a smaller 350-kb chromosome. Other plasmids have not been documented. Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene. Several repetitive elements have been identified (Zuerner, 1991; Bergstrom *et al*., 1992).

A number of leptospiral genes have been cloned and analyzed, including several for amino acid synthesis, rRNA, ribosomal proteins RNA polymerase, DNA repair, heat shock proteins, hemolysin, sphingomyelinase, outer membrane proteins, lipopolysaccharide (LPS) synthesis, flagella proteins, and leptospiral immunoglobulin -like proteins (Lig).

6. Epidemiology

Leptospirosis is a widespread zoonosis in the world (Matsunaga *et al*., 2002). The source of infection is usually either direct or indirect contact with the urine of an infected animal. After leptospires are excreted in urine, the organisms may survive for weeks or months in optimum condition. Water-born transmission has been documented; point contamination of water supplies has resulted in several outbreaks of leptospirosis. Inhalation of water or aerosols also may result in infection via the mucous membranes of the respiratory tract (Chan *et al*., 1987). Animals and human can be divided into maintenance hosts and accidental hosts. The most important maintenance host is small mammals, which may transfer infection to domestic farm animals, dogs, and humans. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (Faine, 1999). The rodents can excrete large numbers of leptospires in their urine. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroup icterohaemorrhagiae and ballum, and mice are the maintenance hosts for

serogroup ballum (Bolin, 2002). Domestic animals are also maintenance hosts; dairy cattle may harbor pomona, tarassovi, or bratislava; sheep may harbor hardjo and pomona; and dogs may harbor canicola (Figure 3) (Hartskeerl and Terpstra *et al*., 1996). Some leptospiral serovars are commonly associated with particular animal reservoirs (Table 2). Knowledge of the prevalent serovar and their maintenance hosts is essential to understand the epidemiology of the disease.



Suwanjalern, 2007

Figure 3 The infection pathway of Leptospirosis. It is predominantly an occupational disease which affects humans who come into frequently contact with rodents, wild or domestic animals or polluted water (Hartskeerl and Terpstra ,1996; Levett, 2001; Bolin, 2002; Suwanjalern, 2007).

Table 2 The serovars of *Leprospira* spp. causing human leptospirosis and their reservoir host (Brooks *et al.*, 1995).

Serovar	Source of animals	Disease in human	Clinical findings	Distribution
Autumnalis	Voles, Field mice	Pretibial fever or Ft. Bragg fever	Fever, rash over tibia	USA, Asia
Ballum	House mice	-	Fever, rash, jaundice	USA, Europe
Bovis	Cattle, Voles	-	Fever, prostration	USA, Australia
Canicola	Dogs, Swine	Infectious jaundice	Influenza-like illness, aseptic meningitis	Worldwide
Grippotyphosa	Field mice, Cattle	Marsh fever	Fever, jaundice	Europe, USA, Africa
Hebdomadis	Rats, Mice	Seven day fever	Fever, jaundice	Asia, Europe
Icterohaemorrhagiae	Rats, Dogs	Weil's disease	Jaundice, hemorrhages	Worldwide
Mitis	Swine	Swineherd's disease	Aseptic meningitis	Australia
Pomona	Swine, cattle	Swineherd's disease	Fever, prostration, aseptic meningitis	Europe, USA, Australia

7. Clinical manifestation

The clinical presentation of leptospirosis is biphasic (Figure 4), with the acute or septicemia phase lasting about a week, followed by the immune phase, characterize by antibody production and excretion of leptospires in urine (Levett, 2001). Most of complications of leptospirosis are associated with localization of leptospires within the tissue during immune phase and thus occur during the second week of illness. Most of the recognized cases present with a febrile illness of sudden onset. Multiple clinical reports have indicated that fever is present in most or all cases. A substantial proportion of people infected by leptospira may have subclinical disease or very mild symptoms and do not seek medical attention. Symptomless infection is common and has been reported in several studies (Bovet *et. al*., 1999).

Leptospirosis is an acute, febrile illness occurring in humans or animals all over the world. Symptom infection with *Leptospira* is common in endemic areas. Fever, chills, headache, severe myalgia, conjunctiva suffusion, anorexia, nausea, vomiting, and usually characterize acute leptospirosis. No significant association was seen between any particular leptospiral serovars and the clinical outcome of infection, although in general, infection of a non-maintenance host species result in more severe disease.

8. Pathology

Leptospirosis is characterized by the development of vasculitis, endothelial damage, and inflammatory infiltrates with monocytic cells, plasma cells, histocytes, and neutrophils (Aream, 1962). The pathological changes differ between acute and chronic infection and depend on the virulence of the infection serovar. They also depend on the combination of the infection serovar and the host. The endothelial membrane in blood vessels is damaged during an acute infection. The resulting dysfunction of organs explains clinical signs like jaundice (liver failure), kidney failure, and abortion. In (sub) chronic infection the most important pathological lesion for several animal species is found in the kidney. Independent of the infecting serovar,

an intestinal nephritis is observed with lymphocytic infiltrates (Van den Ingh and Hartman, 1986).

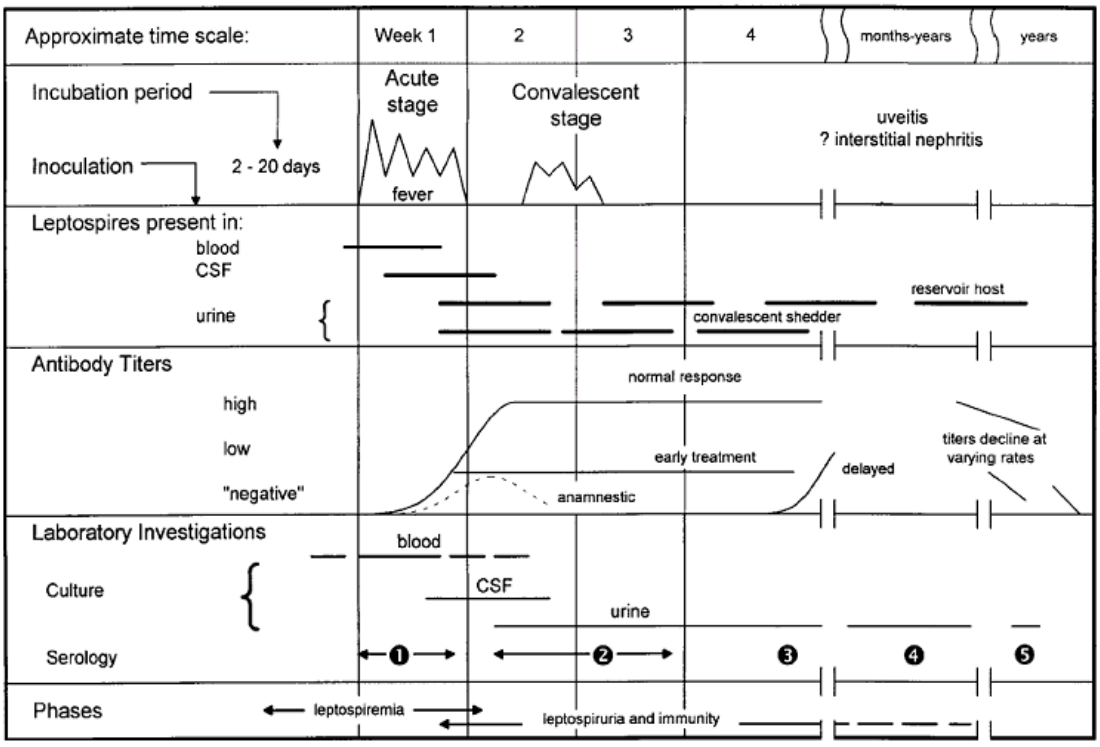


Figure 4 Biphasic nature of leptospirosis and relevant investigations at different stages of disease. Specimens 1 and 2 for serology are acute-phase specimen, 3 is a convalescent-phase samples which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide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

9. Treatment

Leptospirosis is sensitive to most antibiotics except chloramphenical, vancomycin, rifampicin and metronidazole. The antibiotics most usually recommened are penicillin and doxycycline. Doxycyclin is recommended for both prophylaxis and mild disease. Ampicillin and amoxicillin are also recommended in mild disease, whereas penicillin and ampicillin are indicated for severe disease (Watt *et. al*., 1988).

Antibiotics therapy in the leptospiremic phase may effective but treatment during the immune phase appear to be no value.

10. Laboratory diagnosis

Laboratory diagnosis of human leptospirosis has mainly relied on detection of leptospiral specific antibodies in patients' sera. Diagnosis of leptospirosis in animal is difficult and no single diagnostic test provides optimal sensitivity or specificity. Interpretation of leptospiral serologic results is complicated by a number of factors. These factors include: cross-reactivity of antibodies, antibody titers induced by vaccination, and lack of consensus about what antibody titers indicate active infection. Antibodies produced in an animal in response to infection with a given serovar of *Leptospira* often cross-react with other serovars of leptospires. Therefore, an animal infected with a single serovar is likely to have antibodies against more than one serovar in an agglutination test. Patterns of cross-reactive antibodies vary widely between species of animals and between individuals within a species. However, in general, the infecting serovar is assumed to be the serovar to which that animal develops the highest titer. A combination of procedures, including serological assay and tests to detect the presence of leptospires in tissue of body fluids, is recommended for leptospirosis diagnosis in animals.

10.1 Isolation of *Leptospira*

In the acute phase of the disease leptospires can be cultured from blood. Generally the organisms present in blood samples after 3 to 10 days of infection. After this period there is a specific antibody response that results in the clearance of leptospires from the blood. In subacute and chronic infections isolation of leptospires is possible from urine, the genital tract, milk, kidney, and from aborted fetuses (Schonberg *et al*., 1992).

Many different media have been developed for the cultivation of leptospires (Sebek and Janicek, 1964). Cultures are incubated at 28^{o}C to 30^{o}C in the dark and examined weekly by dark-field microscopy for up to 13 weeks before being discarded. Recovery time may be 5-6 weeks or longer, depending on each serovar.

10.2 Microscopic demonstration

10.2.1 Dark field examination

Leptospires can be detected using dark-field examination of urine and thoracic fluid samples. The assay is giving positive result only when high numbers of leptospires are present.

10.2.2 Staining

Leptospirosis in tissue slides can be recognized after being silver-staining. This technique can only be performed on tissue and is not useful for other clinical material (Slee *et al*., 1983). Leptospires may be stained using carbol fuchsin counterstain (Alexander and Rule, 1986). The number of organisms should be high and this method is not *Leptospira* specific.

10.2.3 Detection with labeled antibody

To increase specificity, staining can be performed with the use of labeled monoclonal or polyclonal antibody (Stevens *et al.*, 1985). The antibodies can be labeled with horse radish peroxidase or fluorescein (Scanziani *et al*., 1991).

10.3 Molecular diagnosis

10.3.1 Probe hybridization

Recently, a number of methods have been developed to detect

the leptospires DNA in clinical materials. The assays are based on hybridization of sample DNA with labeled *Leptospira* specific DNA probes. Several kinds of DNA probes are used: digested and labeled chromosomal DNA (Terpstra *et al.*, 1987), cloned DNA fragments and an oligonucleotide probe (Van Eys *et al*., 1988). There are probes that can discriminate between pathogenic and non-pathogenic leptospires. A drawback of the method is its low sensitivity.

10.3.2 Polymerase chain reaction (PCR)

The polymerase chain reaction offers the possibility to detect microorganisms with a very high sensitivity and specificity. Several PCR assays have been described for the detection of leptospiral DNA (Woodward *et al*., 1991). A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovars.

10.4 Serological diagnosis

Serology is the most important diagnostic method for leptospirosis. An infection with leptospires induces an immune response in host. IgM antibodies appear within one week after infection and following by IgG antibodies in the second week. The IgM antibody titer declines rapidly whereas IgG antibodies persist for months or even years (Cousins *et al*., 1985; Hartman *et al*., 1984).

10.4.1 Microscopic agglutination test (MAT)

The reference method for serological diagnosis of leptospirosis is the MAT, in which patient sera are reacted with a live antigen suspension of a leptospiral serovar. After incubation, the serum-antigen mixtures are examined microscopically for agglutination, and the titers are determined.

Although MAT has been accepted as reference test for serological diagnosis of leptospirosis, it has many important drawbacks which make

this test to be performed only in reference laboratory. They are subjective endpoint. MAT requires technical expertise to determine the end titre. The MAT result is extreme tedious and labor intensive to interpret the cut-off value. This leads to error to fatigue following prolonged observation of test end-point. Other drawbacks include the potential for error if the correct serovars are not chosen and dangerous due to the use of live organisms. Moreover, culturing *Leptospira* is labor intensive which involves a health risk for laboratory personnel, and finally there are also problems in antigen standardization (Faine *et al*., 1998; Levett, 2001). Discrimination between IgM and IgG antibodies is impossible as this assay has a very low sensitivity for IgG antibodies (Gowei Priya *et al*., 2003).

10.4.2 ELISA

By using ELISA some of the drawbacks of MAT can be overcome. The major advantages are: 1.the use of inactivated antigen which can be stored for a long time and which enables standardization, 2. the modifications have been used to detect IgG and IgA in addition to IgM, 3. the discrimination that can be made between IgM and IgG antibodies, 4. ELISA is more sensitive than MAT in both the acute and chronic phase of the infection, and 5. ELISA is an objective assay in contrast with the MAT in which the percentage of agglutination has to be determined subjectivity (Thiremann, 1983; Thiremann *et al*., 1985; Trueba *et al*., 1990; Silva *et al*., 1997).

ELISA methods have been applied as a number of modifications. A commercial IgM dot-ELISA dipstick has been shown to be as sensitive as a microtiter plate IgM-ELISA (Levett *et al*., 2001). Another dipstick assay has been extensively evaluated in several populations (Smits *et al.*, 1999; Yersin *et al*., 1999). A dot immunoblot assay using colloidal gold conjugate allowed completion of the assay within 30 minutes (Petchclai *et al.*, 1991).

10.4.3 LEPTO Dipstick®

LEPTO Dipstick® has been developed by the Royal Tropical Institute Amsterdam, The Netherland) using the principle of immunochromatography which composed of a broadly reactive leptospiral antigens and antihuman IgM dye conjugate coated on cellulose strip to detect IgM antibodies in human sera. Smits *et al* (1999) have initiated and evaluated the clinical utility of a dipstick assay for the detection of *Leptospira*-specific IgM antibodies in human sera in different areas. They suggest that the broadly reactive *leptospira* antigen ensure the efficient detection a wide spectrum of *leptospira* infections. The results show that the dipstick assay combines a high specificity (91.6%) with a high sensitivity (88.6%). The Dipstick has been claimed to be simple to perform, requiring neither special equipment nor technical expertise with long shelf–life of reagents at room temperature. Therefore, this method seems to be a suitable method for rapid screening of leptospirosis especially in field study (Levett *et al*., 2001).

10.4.4. Indirect hemagglutination assay (IHA)

Indirect hemagglutination assay (IHA) which used sensitized red blood cells has also been developed as simple immunological tool for serodiagnosis of leptospirosis. The test can be used to detect both IgM and IgG antibodies against leptospires. Centers for Disease Control and Prevention, USA developed IHA® as an initial screening test for the investigation of leptospirosis. The study showed the sensitivity and specificity of 92% and 95%, respectively when compared with MAT. This test is available commercially as the one U.S. Food and Drug Administration-approved product for serological diagnosis of leptospirosis (Levett, 2001). The sensitivity and specificity of IHA among populations has been studied and found the variation among population for example the study in Hawaii showed only 41% sensitivity (Effler *et al*., 2000) whereas the study in Thailand showed 73.8% sensitivity and 97.5% specificity (Naigowit *et al.*, 2000). The variation of sensitivity and specificity among populations was thought to be due to the selection of infecting

serovars found in each specific area for antigen preparation of the test kit. However, IHA has also been recommended as rapid diagnosis for leptospirosis.

10.4.5 Latex agglutination test (LA)

LA has been developed for detection of leptospiral antibodies in serum both from human and animals. The sensitivity of LA test was slightly lower than plate-ELISA both in human and animal sera at 84.8% and 63.1% versus 85.9% and 69.2% respectively (Ramadass *et al*., 1999). The mean overall sensitivity and specificity from the evaluation done in patients with leptospirosis from several regions, which were endemic area of the disease, were 82.3% and 94.6% respectively (Smits *et al*., 2000).

The commercial kit was developed by bioMérieux SA, in collaboration with the Royal Tropical Institute in Amsterdam, The Netherlands. The LeptoTek Dri Dot assay is based on the binding of Leptospira-specific antibodies that present in the serum sample to the Leptospira antigen coated on blue latex particles. Compared to the MAT, the LeptoTek Dri Dot assay showed on agreement rate of 91% for overall sensitivity and 91% for overall specificity. This tool allows laboratories to accurately diagnose leptospirosis within two minutes-quickly, easily and cost-effectively. The assay does not require special skills or equipment and the reagents have a long-shelf, even at tropical temperature.

10.4.6 Macroscopic agglutination test

Macroscopic agglutination test is known as a commercial slide agglutination test (SAT), available called Simple Test (BioManguinhos, FIOCRUZ, Rio de Janeiro, Brazil. It was found to be as sensitive and specific as IgM-ELISA while remaining reactive for a short time after recovery than either the IgM-ELISA or the MAT. This method is easy to performed, inexpensive and rapid for the screening of large numbers of human sera (Brandao *et al*., 1998).

10.4.7 Microcapsule agglutination test (MCAT)

The MCAT was used with the commercial test kit *Leptospira*-MC Test (Japan Lyophilization Laboratory, Tokyo, Japan) for the detection of leptospiral antibodies in the early stage of infection. The test included an antigen mixture of six different serovars of leptospires in reagent A and reagent B. Reagent A was mixed antigen of *L. autumnalis, L. hebdomadis* and *L. australis* which was adsorbed to microcapsule (MC) particles. Reagent B consisted of microcapsule particle sensitized antigen of *L. icterohaemorrhagiae, L. canicola* and *L. pyrogenes*.

The MCAT has been evaluated extensively in Japan and China. In an international multicenter evaluation, the MCAT was more sensitive than either the MAT or an IgM-ELISA in early-acute-phase sample, but failed to detect infections caused by some serovars (Levett, 2001). The MCAT was evaluated in Thailand and showed the similar result as an international evaluation (Suputtamongkol *et al*., 1998).

10.4.8 Indirect fluorescent antibody (IFA)

IFA is one of the popular and widely used techniques for serodiagnosis of leptospirosis. The test is performed on IFA slide covered with intact cells of leptospires. In Thailand, IFA was evaluated with sera from MAT-positive-confirmed cases of leptospirosis, the sensitivity and specificity were reported to be 91.4% and 97% respectively (Appassakij *et al*., 1995). Athough IFA technique gives good sensitivity, specificity and accuracy with capability to examine rather large number of samples in the endemic setting, but this technique needs fluorescent microscope and skilled personnel with limits the use of this technique. Therefore, IFA is recommended to be used only in reference laboratory for leptospirosis.

To control leptospirosis, the knowledge of the epizootiology of leptospirosis is important in the design of effective preventive strategies. There is a need for the method that can be used as a tool for diagnosis and epidemiology survey. Ideally, a

diagnostic test should be easy to perform, rapid and uses only a single specimen. Moreover, a simple method for identification of *Leptospira* species is urgently required, since leptospiral antigens have been more widely characterized. In the present study, the species identification of leptospires is evaluated the use of *flaB* gene sequencing. The recombinant *flaB* and *lig* have been proposed to use as the antigens in diagnostic utility for leptospirosis.

MATERIALS AND METHODS

Materials

1. Animals

1.1 Adult New Zealand white rabbits aged 8-10 weeks old (approx. 1.5 kg. each) were purchased from rabbit farming at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom Province, Thailand.

1.2 Outbred stains of ICR mice aged 6-8 weeks old were purchased from The National Laboratory Animal Centre, Mahidol University, Thailand.

1.3 Inbred strains of BALB/c mice aged 6-8 weeks old were purchased from The National Laboratory Animal Centre, Mahidol University, Thailand.

All experimental procedures with animals were approved by Kasersat University Animal Care Committee. Animals were housed in accordance with The Ethical Principles for the Use of Animals for Scientific Purposes issued by Research Council of Thailand (NRCT).

2. Microorganisms

2.1 Strains of *Leptospira* spp.

The reference strains of *Leptospira* used in this study (as listed in Table 3) were obtained from Department of Veterinary Public Health and Diagnosis Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand and grown in Ellinghausen and McCullough medium as modified by Johnson and Harris supplemented with 1% bovine serum albumin (BSA) in continuous culture.

2.2 Bacterial strains

2.2.1 Competent cell *E. coli* strain JM109 was used as a host cells for cloning. The resulting constructs (pET-28A.*flaB* and pGEX-5X-3.*lig*) were transformed into competent *E. coli* strain BL21 for expression.

2.2.2 All 11 strains of gram negative bacteria and 3 strains of gram positive bacteria *(Escherichia coli, Proteus vulgalis, Enterobacter spp., Pseudomonas aeruginosa, Salmonella* group *E, Serratia marcescence, Vibrio parahaemolyticus, Campylabacter spp., Helicobacter pylori, Listeria monocytogenes, Aeromonas hydrophila, Bacillus cereus, Staphylococcus aureus and alpha-Streptococcus* spp.) were provide by the Department of Veterinary Public Health and Diagnostic Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom province, Thailand. The bacteria were sub-cultured on blood agar medium and cultivated at 37^{o}C for 48 hours before used.

3. Enzymes

Restriction endonuclease (*Bam*HI, *Sac*I, *Sal*I and *Xho*I) and T4 DNA ligase were purchased from Fermentas, Lysozyme and RNase were purchased from Sigma.

Table 3 Reference strains of *Leptospira* used in this work

Genomospecies	Serogroup	Serovars	Strain
L.interrogans	Autumnalis	autumalis	Akiyami A
	Batavia	bataviae	Swart
	Australis	bratislava	Jez Bratislava
	Canicola	canicola	HondUtrecht IV
	Djasiman	djasiman	Djasiman
	Hebdomadis	hebdomadis	Hebdomadis
	Icterohaemorrhagiae	icterohaemorrhagiae	RGA
	Pomona	pomona	Pomona
	Pyrogenes	pyrogenes	Salinem
L. noguchii	Louisiana	louisiana	LSU 1945
	Panama	Panama	CZ 214
L. borgpetersenii	Ballum	ballum	MUS 127
	Javanica	javanica	V. Batavia 46
	Mini	Mini	Sari
	Sejroe	sejroe	M 84
	Tarassovi	tarassovi	Perepelitsin
L. santarosai	Shermani	shermani	1342 K
L. weilii	Celledoni	celledoni	Celledoni
	Manhao	manhao	Li 130
	Sarmin	sarmin	Sarmin
L. kirschneri	Cynopteri	cynopteri	3522 C
	Grippotyphosa	grippotyphosa	Moskva V
L. meyeri	Ranarum	ranarum	ICF
L. biflexa	Samaranga	patoc	Patoc I

4. Plasmid vectors

Table 4 Characteristics of plasmids.

Plasmids	Characteristics	Company
pGEM-T easy	Am^r, cloning vector	Promega
pET-28a	Km^r, expression vector	Novagen
pGEX-5X-3	Am^r, expression vector	Amersham Pharmacia Biotech

5. Primers

All specific primers used in this study were synthesized by DNA Technology Laboratory, BIOTECH, Thailand

6. Serum samples

All serum sample used for antibody testing were obtained from Department of Veterinary Public Health and Diagnostic Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus , Nakhon Pathom.

Table 5 Oligonucleotide primers used in this work.

Primer	Primer sequence (5′--- 3′)	Gene location	Restriction site	Size (bp)
*rrs*F	GGCGGCGCGTCTTAAACATG	16S	-	360[a]
*rrs*R	GTTCCCCCCATTGAGCAAGATT	rRNA	-	
Lig full-F	CGTGTCGACGTGAAGAAAATA TTTTGTATTTCGATTTTCC	*lig* conserve	*Sal*I	1,732[b]
Lig con-R	CCTCGAGGGGATAACGTAGAA ACCGGACTAC	d region	*Xho*I	
Lig1	TCAATCAAAACAAGGGGCT	partial	-	468[c]
Lig2	ACTTGCATTGGAAATTGAGAG	region of *lig*	-	
FlaB full-F	ACAGGATCCATGATTATCACA ACCTGTG	*flaB* full-	*BamH*I	852[d]
FlaB full-R	GAGCTCTCAGATGTGCTGCAG AAGCT	length	*Sac*I	
L-flaB-F1	TCTCACCGTTCTCTAAAGTTAA AC	partial region		793[e]
L-flaB-R2	CTGAATTCGGTTTCATATTTG CC	of *flaB*		
ERIC1	ATGTAAGCTCCTGGGGATTCA C	Repeti-	-	-[f]
ERIC2	CACCGATCCAATGCTAGGTCA	tive motifs	-	

[a]Primers for amplification of 16S rRNA gene of genus *Leptospira* were performed as previous reported by Merien *et al*., 1992.

[b]Primers were designed in this experiments with built-in restriction enzyme sites for amplification of conserved region of *lig* gene based on the *Leptospira kirschneri* Lig sequence (GeneBank accession number AY 190126).

[c]Primers were designed from the conserved amino terminus region of *lig* genes of leptospira (GeneBank accession number AF368236 and AF534640) as previous reported by Palaniappan *et al*., 2005.

[d]Primers for amplification of full length *flaB* gene of genus *Leptospira* were performed as previous reported by Bughio *et al.*, 1999.

[e]Primers for amplification of *flaB* fragment was reported by Kawabata *et al*., 2001.

[f]ERIC primer has been used for generate DNA fingerprinting of *Leptospira* spp. Primer used in this study as previous reported by Weijtens *et al*., 1999.

6. Chemicals and substances

All chemicals used in this experiment were analytical grade. Name and source of chemicals were listed below.

Chemical	Company
Absolute ethanol (C_2H_5OH)	Fisher Scientific
Acetic acid (C_3COOH)	Fisher Scientific
Acrylamide	Research Organic
Adjuvant complete/incomplete's Freund	Difco
Agarose	FMP Bioproduct
Ammonium persulfate $(CNH_4)_2SO_8$	Research Organic
Ampicillin	Sigma
Bacto® Tryptone	Difco
Bacto® Yeast extract	Difco
Bovine serum albumin	Sigma
Calcium chloride	UNIVAR
Chlorofrom	Merck
Coomassie brilliant blue	Fisher Biotech
Dimethyl sulfoxide (DMSO)	EMS
Disodium hydrogen phosphate	UNIVAR
DNA ladder	Fermentas

Chemical	Company
dNTPs	Fermentas
Ethanol absolute	Merck
Ethidium bromide	Promega
EDTA	Sigma
Fetal bovine serum	PAA Laboratories
5-Fluorouracil	Phamachemie
Glycerol	Research Organic
Goat anti-mouse (rabbit) immunoglobulins	Zymed
Guanidine thiocyanate	Biobasic Inc
Hydrochloric acid	Fisher Scientific
Isopropanol	Pacific Science
Isopropyl-ß-D-thiogalactopyronoside	Sigma
Kanamycin	Difco
Leptospira EMJH base	Difco
Methanol	LAB-SCAN
NucleoSpin®	MACHEREY-NAGEL GmbH
PCR buffer	Fermentas
Phenol	Amresco
Phenol red	Amresco
Potassium dihydrogen phosphate	UNIVAR
Potassium acetate	UNIVAR
Primers	DNA Technology Laboratory
Protein molecular weight	Fermentas
Restriction enzyme	Promega
Sarcosyl	Research Organic
Sodium carbonate	Merck
Sodium chloride	UNIVAR
Sodium citrate	Biobasic Inc
Sodium dodecyl sulfate (SDS)	Biobasic Inc
Sodium hydroxide	LAB-SCAN

Chemical	Company
T4 DNA ligase	Fermentas
Taq polymerase	Fermentas
Tris (hydroxymethyl) aminomethane	US Biological
Triton-X-100	Research Organic
Tryptone	Biobasic Inc
Urea	Research Organic
Yeast extract	Biobasic Inc

7. Instruments

Instrument	Company
Autopipette	LABMATE
Dark-field microscope	Olympus
Dry bath incubator (Major Science)	Major Science
ELISA plate	Nunc
ELISA reader	Sunrise
Fluorescent microscopy	Olympus
Gel document	Major science
Incubator	Forma Scientific
Laminar flow	Boss Tech
Microcentrifuge	Denville Scientific
Refrigerate centrifuge	Jouan
SDS-PAGE gel electrophoresis	Finnzyme
Shaker incubator	Vision Scientific
Thermal cycle	LAB Focus
U.V. Transilluminator	Alpha Innotech Corporation
Vortex mixer	Vision Scientifc
Water bath	Memmert

Methods

I. Polymerase chain reaction and sequencing of *Leptospira* species

A. DNA manipulation

1. Bacterial strains

The reference strains of *Leptospira* spp. used in this study are listed in Table 1. They were cultivated in Ellinghausen and McCullough medium as modified by Johnson and Harris in continuous culture (EMJH) medium enriched with 1% bovine serum albumin at 28^{o}C and protected from light for 7-10 days. The growth of bacteria was observed by dark field microscopy and checked for contamination by plating on blood agar.

2. DNA isolation and purification

The *Leptospira* were grown as above and harvested after 7 days (10^8 cells/ ml). Five ml of pure leptospires were collected by centrifugation at 13,000 rpm for 30 minutes at 4^{o}C. The pellets were washed twice by centrifugation in phosphate buffer saline (PBS; pH 7.2) and resuspended in 100 µl of PBS. DNA was extracted by using guanidinium-thiocyanate, phenol-chloroform and ethanol precipitation (modified by Trochimchuk *et al*., 2003). Briefly, 500 µl of D-solution (Appendix B) was added to 100 µl of pellet suspension and incubated for 5 minutes. Subsequently, the 150 µl of phenol and 150 µl of chloroform were added to the solution. It was centrifuged at 13,000 rpm for 10 minutes and aspirated the upper part to a fresh microtube. The DNA was precipitated by an equal volume of cool absolute isopropanol and washed by 500 µl of 70% ethanol. After centrifuged, the DNA was dried at room temperature. The precipitated pellets were resuspended carefully in 30 µl of TE (10 mM Tris HCl (pH 7.4), 1 mM EDTA (pH 8.0). The solutions were then store at -20^{o}C until used for DNA amplification.

B. Polymerase chain reaction.

The amplifications were performed by using the primers listed in Table 5 (primer: *rrs*F and *rrs*R, Lig1 and Lig2, L-flaB-F1 and L-flaB-R1 and ERIC1 and ERIC2 for amplification of 16S rRNA gene, *lig* gene, *flaB* gene and repetitive motif, respectively). Amplification of 16S rRNA from leptospiral DNA was performed as an internal control. The PCR reactions were carried out in 0.2 ml tube with contained 25 ul of the mixture as follow: 1x PCR buffer, 200 μM dNTPs, 3 mM $MgCl_2$, 100 nmol of each primer, and 0.5 unit of *Taq* polymerase. The amplification of 16S rRNA, *flaB* and *lig* were performed using the following condition: initial denaturation at 94°C 5 min, and then 40 cycles of heat denaturation at 94°C for 30 sec, primer annealing at 56°C for 30 sec, and DNA extension at 72°C for 30 sec. The ERIC-PCR was used a touch-up protocol (Weijtens *et. al*., 1999) with the modifications. The PCR protocol was performed using the following condition: a first step of 5 min at 94°C; consecutively, two cycles of 1 min at 94°C, 1 min at 43°C, and 1 min at 72°C; two cycles of, consecutively, 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C; two cycles of, 1 min at 94°C, 1 min at 47°C, and 1 min at 72°C; two cycles of 1 min at 94°C, 1 min at 49°C, and 1 min at 72°C; two cycles of, 1 min at 94°C, 1 min at 51°C, and 1 min at 72°C; two cycles of 1 min at 94°C , 1 min at 53°C, and 1 min at 72°C; 30 cycles of, 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C and finally, a step of 10 min at 72°C. PCR products were separated on 1.5% agarose gel and stained with ethidium bromide. With this protocol, the presence of the 793-bp, 460-bp 330-bp and fingerprint pattern bands indicated the successful amplification of *flaB, lig* 16S rRNA and repetitive motif respectively.

C. Sequencing and nucleotide analysis

Direct sequencing was performed by using BigDye termination kit (Applied Biosystem Inc., Foster city, CA). The amplified product of *flaB* fragments will be sequenced at Bioservice unit, National Science and Technology Development Agency. The DNA sequences would be then analysed using BioEdit program version 7.0.5.2 (Tom Hall, http://www.mbio.ncsu.edu/BioEdit/ bioedit.html) and deposited in

GenBank. The nucleotide positions 58 to 806 of *fla*B gene were used to construct the phylogenetic tree among various leptospires strains used in this studied and *flaB* gene of *Leptospira* spp. from GenBank (Table 6). Phylogenetic analyses of the *fla*B gene sequences were aligned with those of published leptospiral *fla*B gene sequences available in GenBank using CLUSTAL W (version 1.83; http://www.ebi.ac.uk/clustal/w/) with default parameters. Alignments were optimized manually using a multiple sequence alignment editor BioEdit version 7.0.5.2; gaps were treated as missing data. Sequences data were performed the similarity of phylogenetic tree pattern, identity and divergence percentage. Homogeneity of base frequencies across taxa was tested using algorithms implemented in Mega4 program version 4028 and DNASTAR MegAlign™ analysis software (licensed to Kasetsart University).

Table 6 Strains studied and their *flaB* GenBank accession numbers

Genomospecies	Serogroup	Serovars	Strain	GenBank accession no.
L.interrogans	Autumnalis	autumalis	Akiyami A	This study
	Batavia	bataviae	Swart	This study
	Australis	bratislava	Jez Bratislava	This study
	Canicola	canicola	H.Utrecht IV	This study
	Djasiman	djasiman	Djasiman	This study
	Hebdomadis	hebdomadis	Hebdomadis	This study
	Icterohaemor	icterohaemor	RGA	This study
	Pomona	pomona	Pomona	This study
	Pyrogenes	pyrogenes	Salinem	This study
L. noguchii	Louisiana	louisiana	LSU 1945	This study
	Panama	Panama	CZ 214	This study
L. borgpetersenii	Ballum	ballum	MUS 127	This study
	Javanica	javanica	V. Batavia 46	This study
	Mini	Mini	Sari	This study
	Sejroe	sejroe	M 84	This study
	Tarassovi	tarassovi	Perepelitsin	This study
L. santarosai	Shermani	shermani	1342 K	This study
L. weilii	Celledoni	celledoni	Celledoni	This study
	Manhao	manhao	Li 130	This study
	Sarmin	sarmin	Sarmin	This study
L. kirschneri	Cynopteri	cynopteri	3522 C	This study
	Grippotyphosa	grippotyphosa	Moskva V	This study
L. meyeri	Ranarum	ranarum	ICF	AB030272
L. biflexa	Samaranga	patoc	Patoc I	AF320637

Table 6 (Continued)

Genomospecies	Serogroup	Serovar	Strain	GenBank accession no.
Leptospira spp.	NA[a]	NA	Zanoni	AB027184
	NA	NA	VanTinem	AB027183
	NA	NA	Rachmat	AB027178
	NA	NA	Paidjan	AB027175
	NA	NA	Naam	AB027174
	NA	NA	M20	AB027170
	NA	NA	ManchenC90	AB027171
	NA	NA	Monkarso	AB027178
	NA	NA	LT398	AB027167
	NA	NA	Krematos	AB027166
	NA	NA	Benjamin	AB027162
	NA	NA	Bangkinang1	AB027161
	NA	NA	Ballico	AB027160
	NA	NA	AkiyamiB	AB027158
	NA	NA	AkiyamiC	AB027159
	NA	NA	MitisJohnson	AB027169
	NA	NA	Castellon3	AB027163
	NA	NA	Poi	AB027176
	NA	NA	Yoro-1	AB174774
	NA	NA	Yor0-2	AB174775
	NA	NA	Yoro-3	AB174776
	NA	NA	Yoro-4	AB174777
	NA	NA	Yoro-5	AB174778
	NA	NA	Yoro-6	AB174779
	NA	NA	Yoro-7	AB174780

Table 6 (Continued)

Genomospecies	Serogroup	Serovar	Strain	GenBank accession no.
Leptospira spp.	NA	NA	EP	AB031514
	NA	NA	MC	AB031516
	NA	NA	G	AB031515
	NA	NA	EA	AB031513
	NA	NA	DQ	AB031512
	NA	NA	AB	AB031511
	NA	NA	SP04	AB031510
	NA	NA	UP-PGH	AB031509
	NA	NA	UP-MMC	AB031508

[a]NA- Not available

II. Cloning and expression of recombinant full length of *flaB* and conserved region of a leptospiral immunoglobulin-like protein (*lig*) against *Leptospira*

A. DNA manipulation

1. Media and growth conditions

a) Two strains of *Leptospira* spp. (*L. interrogans* serovar Canicola strain Hond Utrecht IV genomic DNA was used as template for *flaB* gene and *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V was used as template for *lig* gene) were cultivated in EMJH medium supplemented with 1% bovine serum albumin at 28^{o}C and protected from light for 2 weeks.

b) The strains of competent cell *E. coli* were cultivated on LB agar at 37^{o}C. Antibiotics were used at the following concentrations: Ampicillin 100 μg/ml and Kanamycin 25 μg/ml.

2. DNA isolation and purification

The *Leptospira* were grown as above and harvested after 7 days (10^8 cells/ ml). Five ml of pure leptospires were collected by centrifugation at 13,000 rpm for 30 minutes at 4^{o}C. The pellets were washed twice by centrifugation in phosphate buffer saline (PBS; pH 7.2) and resuspended in 100 μl of PBS. DNA was extracted by using guanidinium-thiocyanate, phenol-chloroform and ethanol precipitation (modified by Trochimchuk *et al*., 2003).

A. PCR amplification of *flaB* , *lig*

Two common infecting serovars were used as templates in this experiment, the full-length of the *flaB* gene was amplified from the template DNA of *L. interrogans* serovar Canicola strain Hond Utrecht IV. The gene encoding for the conserved region of LigA and LigB was constructed at the N-terminal 575 amino

acids without the signal sequences. It was amplified from the genomic DNA of *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V. The amplifications were performed by using the primers listed in Table 5. The PCR primers contain the restriction sites incorporated at their 5′ ends (underlined). The PCR reactions were carried out in 0.2 ml tube with contained 25 µl of the mixture as follow: 1x PCR buffer, 200 µM dNTPs, 3 mM $MgCl_2$, 100 nmol of each primer, and 0.5 unit of *Taq* DNA polymerase. The amplification of *flaB* and *lig* were performed using the following condition: initial denaturation at 94°C 5 minutes, and then 40 cycles of heat denaturation at 94°C for 30 seconds, primer annealing using primers listed in the Table 5 for 30 seconds, and DNA extension at 72°C for 1-1.30 minute. After the last cycle, elongation step was extended for 7 minutes. The amplification products were analyzed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. With this protocol, the presence of the 852-bp and 1,728-bp bands indicated the successful amplification of *flaB* and *lig*, respectively.

B. Sequencing and nucleotide analysis

Direct sequencing was performed by using BigDye termination kit. The amplified product were sequenced at Bioservice unit, National Science and Technology Development Agency. The DNA sequences would be then analysed using BioEdit program version 7.0.5.2 and deposited in GenBank.

C. Cloning and *In vitro* expression of recombinant FlaB and Lig protein

1. Construction of cloning vector

The PCR products of *flaB* and *lig* were initially cloned in pGEM-T easy cloning vector according to the manufacturer. The reaction mixer was incubated at 15°C for 18 hours. The ligation products were transformed to competent cell of *E. coli* strain JM109 for amplification of target genes in host cells (Ausubel *et al.*, 1995). The colonies were selected and analyzed by blue-white screening, PCR and restriction endonuclease analysis. The positive clone was isolated for plasmid DNA by a

modification protocol of the method of Sambrook and Russell, 2001 (Appendix C) and sequenced.

2. Construction of expression vector

The positive clones were cultured overnight at 37^{o}C in LB medium containing 100 μg/ml ampicillin and isolated DNA by alkaline lysis method (Appendix C). The 852-bp fragment of *flaB* was digested with *BamH*I-*Sac*I and 1,728-bp of *lig* was digested with *Xho*I-*Sal*I. The fragments were analyzed by electrophoresis in 1% agarose gel and stained with ethidium bromide. The *flaB* and *lig* bands were cut from gel and purified by Nucleospin kit according to the manufacturer's instruction. The purified *flaB* was subcloned into *BamH*I-*Sac*I sites of pET-28a-an expression vector producing a FlaB fusion protein with a polyhistidine tag (His) at the N-terminus was constructed and *lig* was subcloned into *Xho*I-*Sal*I sites of pGEX-5X-3-an expression vector producing a Lig fusion protein with a Glutathione S-transferase (GST) tag at the N-terminus was constructed. In order to construct the plasmid expressing of His.FlaB and GST.Lig protein, the digested fragment genes and the expression vector were ligated with T4 DNA ligase. DNA ligations were performed by incubating both cut expression vectors (pET-28a and pGEX-5X-3) and oligonucletide fragments (*flaB* and *lig*) with 0.1 unit of T4 DNA ligase at 15^{o}C for 18 hours. The recombinant plasmids were transformed to *E. coli* BL21 and to confirm that the protein-coding DNA sequence could be cloned in the proper translation in vector, direct sequence analysis was performed using BigDye Terminator Cycle Sequence Ready Reaction kit (Applied Biosystems).

3. Expression of recombinant proteins

The transformant proven to contain His.FlaB and GST.Lig plasmid were used for protein expression. The single colony of each plasmid was selected and grown in 1 ml of LB broth containing 25 μg/ml of kanamycin for His.FlaB and 100 μg/ml of ampicillin for GST.Lig and incubated overnight at 37^{o}C with shaking. Thereafter 0.1 ml of overnight cultures were inoculated to 5 ml of pre-warm medium

with containing antibiotics, and grown at 37^{o}C with vigorous shaking (~210 rpm) until cells reacted mid-log growth (OD_{600} between 0.5-0.7). The non-induced sample was frozen at -20^{o}C until analyzed by SDS-PAGE. Isopropyl-ß-D-thiogalactopyronoside (IPTG) was added to culture of *E. coli* BL21 for expression of His fusion protein, rFlaB and GST fusion protein, rLig. The optimum condition was first determined using various induction times and various concentration of IPTG.

4. Time course analysis of protein expression

As the same methods for the investigation of recombinant protein expression, after subcultured, the culture was dispensed into 7 aliquots (1 ml each). The sample of 1 tube was taken before induction, centrifuged and pelleted. Each tube of culture was collected by centrifugation at 1, 2, 3, 4 and 5 hours following additional IPTG to final concentration of 1 mM. The cell pelletes were mixed with sample buffer and heat at 100^{o}C for 10 minnutes. The samples were subjected to 12% SDS-polyacrylamide gel electrophoresis (Appendix C), at 8-15 V/cm until the bromphenol blue reached the bottom of resolving gel. Gels were stained with Coomassie brilliant blue and molecular weight of protein was estimated using the standard molecular weight marker.

5. IPTG concentration analysis of protein expression

As the same method for the time course analysis of protein expression, after subcultured, the culture was dispensed into 7 aliquots (1 ml each). Expression of recombinant proteins were induced by vary adding IPTG at final concentrations: no adding IPTG, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM and 1 mM. The samples were collected at 4 hours after induction, centrifuged and pelleted. The cell pelletes were mixed with sample buffer and heat at 100^{o}C for 10 minuets. The samples were subjected to 12% SDS-polyacrylamide gel electrophoresis (Appendix C), at 8-15 V/cm until the bromphenol blue reached the bottom of resolving gel. Gel was stained with Coomassie brilliant blue and molecular weight of protein was estimated using the standard molecular weight marker.

D. Protein purification

1. Determination of recombinant protein purification

The expressed proteins (His.FlaB and GST.Lig) from 1 ml of the initial cultures were determined for protein solubility by resuspending in 200 µl of 1x Sodium chloride, Tris-EDTA (STE) buffer (10 mM NaCl, 10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) and lysozyme was added at a final concentration of 100 µg/ ml. The suspensions were incubated on ice for 30 minutes and then centrifuged at 4,000 rpm for 20 minutes. Both of the soluble and insoluble protein (inclusion bodies) fractions were analyzed on a 12% SDS-PAGE and stained with Coomassie brilliant blue.

2. Partial purification of recombinant protein

After subculture and protein expression, 10 ml of culture were centrifuged, the pellets of recombinant proteins and control antigens were performed by partial purification. Following the insoluble protein fraction analysis, the cell pellets were dissolved in 4 ml of 1M, 2M, 4M, 6M and 8M urea, respectively. The insoluble materials of each urea concentration were broken with sonicator on ice at 5 amplitudes for 10 second and recovered by centrifuge for 30 minutes at 10,000 rpm. Sequentially, the remained insoluble materials were dissolved in 4 ml of 0.1, 0.5, 1 and 2% of N-lauryl sarcosine (sarkosyl). After optimization the solubilizing solution both urea and N-lauryl sarcosine, the proteins were preserved by adding the glycerol to a final concentration of 50% before stored at -20^{o}C.

III. Production and evaluation of the utility of polyclonal antibodies against recombinant His.FlaB protein by immunofluorescent assay (IFA) to detect leptospires.

A. Preparation of recombinant protein antigens for immunization

The recombinant proteins were expressed, partial purified and fractionated by 12% SDS-PAGE. The portion expected size of recombinant proteins of rFlaB was cut and stained with copper chloride staining to visualize the proteins. The portion of the gel that contained the recombinant proteins were crushed in 0.1% sodium dodecyl sulfate (SDS) and sonicated 3 time of 1 minute on ice. The slurry was clarified by centrifugation (14,000 rpm) for 5 minutes. The supernatant was collected and stored at -80^{o}C until utilized.

B. Immunization protocols

A rabbit was used for preparation of hyperimmune sera for rFlaB Rabbits was bled and 5 ml pre-immunization blood samples of rabbits were collected for the determination of leptospiral antibody by microscopic agglutination test (MAT). Five-hundred microliters of the purity of the recombinant FlaB fractions (100 µg/ ml) were emulsified with equal amount of complete Freund's adjuvant (SIGMA) and injected subcutaneously on the dorsal side. Each rabbit was boosted with the same dosage of fusion proteins with incomplete Freund's adjuvant (SIGMA) on the 14th and 28th day. On day 42, the rabbits were bled by heart puncture and the serum was collected and stored at -80^{o}C. The fluid of rabbit's serum was used to detect hyperimmune serum against rFlaB by dot blot, Western blot analysis and also tested for MAT titre against *leptospira* serovars. Rabbit's hyperimmune serums were tested for the specificity by immunoblot analysis and indirect immunofluorescent assay (IFA) with leptospires. Then, they were aliquoted and stored at -20^{o}C.

C. Determination of leptospiral antibodies

Partial purified of His.FlaB and His (control antigen) proteins were absorbed onto 0.45 μm nitrocellulose membrane at the same concentration (4 ng/ ml). After blotting antigens, the membranes were blocked with blocking solution (5% skim milk in PBS-0.05% Tween 20 [PBS-T]) at 37^{o}C for 1 hour. Serial dilutions of the rabbit anti-recombinant FlaB protein were prepared (1:100 to 1:102,400). Each dilution of rabbit serum was incubated with the strips of dotted antigens. The membrane strips were incubated at room temperature for 1 hour. The excess antisera were removed by washing with PBS-T for 5 minutes with gentle agitation. The HRP-conjugated goat anti-rabbit IgG (dilute 1:1,000 (v/v); ZYMED) were applied to each membrane at room temperature for 1 hour. Then the membranes were washed three times with PBS-T. The membranes were developed with 3,3'-diaminobenzidine tetrahydrochloride, and the protein bands were visualized with brown lines and then, the reaction was stopped by soaking the membranes in distill water.

D. Determination of antigenicity of leptospira protein antigens

The *Leptospira* were grown as above and harvested after 7 days (10^8 cells/ ml). Five ml of pure leptospires were collected by centrifugation at 13,000 rpm for 30 minutes at 4^{o}C. The cell pellet was washed once in phosphate-buffer saline (PBS)-5 mM $MgCl_2$ and resuspended in sample buffer consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.25 mM phenylmethylsulfonyl fluoride and 0.1% bromphenol blue in 20% glycerol and boiled for 3 minutes (modified from Matsunaga *et al.*, 2005). Proteins were separated in 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated in blocking solution at 37^{o}C for 1 hour and then incubated with pooled canine sera, dilute at 1:40 (MAT positive and negative sera were adsorbed using lysate from induced *E. coli* BL21 for 1 hour at 37^{o}C). They were then incubated at 37^{o}C for 1 hour with HRP-conjugated protein A (dilute 1:1,000 (v/v); ZYMED). Then washed three times with PBS-T at 37^{o}C for 30 minutes and again wash three times in PBS-T. The membranes were developed with 3,3'-diaminobenzidine tetrahydrochloride, and

the protein bands were visualized with brown lines and then, the reaction was stopped by soaking the membranes in distill water.

E. Determination of the specificity of polyclonal antibodies against FlaB recombinant proteins for detection of leptospires

1. Immunoblot analysis of polyclonal antibodies against leptospires

The *Leptospira* were grown as above and harvested after 7 days (10^8 cells/ ml). Five ml of pure leptospires were collected by centrifugation at 13,000 rpm for 30 minutes at 4°C. The cell pellet was washed once in phosphate-buffer saline (PBS)-5 mM $MgCl_2$ and resuspended in sample buffer consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.25 mM phenylmethylsulfonyl fluoride and 0.1% bromphenol blue in 20% glycerol and boiled for 3 minutes (modified from Matsunaga *et al.*, 2005). Proteins were separated in 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated in blocking solution (5% skim milk in PBS-0.05% Tween 20 [PBST-T] at 37°C for 1 hour and then incubated with rabbit serum against recombinant FlaB protein, dilute 1:1,000 and with canine sera, dilute 1:40 (MAT positive and negative sera were adsorbed using lysate from induced *E. coli* BL21 containing plasmid pET-28a for 1 hour at 37°C). They were then incubated at 37°C for 1 hour with HRP-conjugated goat anti-rabbit IgG (dilute 1:1,000 (v/v); ZYMED). Then the membranes were again wash three times in PBS-T. The membranes were developed with 3,3'-diaminobenzidine tetrahydrochloride, and the protein bands were visualized with brown lines and then, the reaction was stopped by soaking the membranes in distill water.

2. Indirect immunofluorescence assay (IFA)

The twenty-four reference strains of *Leptospira* were prepared as an antigen for IFA. The leptospires were centrifuged at 13,000 x rpm for 30 minutes at 4°C. Then, the sediment was washed with phosphate buffer saline three times and

the supernatant fluid removed. The cells of leptospires and other bacteria *(Escherichia coli, Proteus vulgalis, Enterobacter spp., Pseudomonas aeruginosa, Salmonella group E, Serratia marcescence, Vibrio parahaemolyticus, Campylabacter spp., Helicobacter pylori, Listeria monocytogenes, Aeromonas hydrophila, Bacillus cereus, Staphylococcus aureus and alpha-Streptococcus* spp.) were resuspended with distilled water to final concentration at McFarland nephelometer tube no. 0.5, which was equivalent approximately to $1.5x10^8$ cell/ ml.. The bacterial suspension was fixed in 10% formalin for 10 minutes and then once washed with PBS pH 7.2. Fifty µl of serial dilution of rabbit serum against rFlaB proteins starting with 1: 100 up to 1: 6,400 were added to antigens in the eppendorf tubes and were incubated in moist chamber at 37^oC for 1 hour. After the tubes were 3 times washed, bound antibodies were detected with a 1: 100 dilution goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Kirtegaard and Perry laboratories) applied for 30 minutes at 37^oC. After being 3 times washed, 3 µl of suspension was dropped on the slide and examined under fluorescence microscope; a positive reaction was indicated by a green fluorescence color of the antigens.

IV. Development and evaluation of the utility of the recombinant protein Lig as antigen in an indirect enzyme-linked immunosorbance assay for the detection of antibodies against *Leptospira*.

A. Serum samples

1. Mice serum samples against *leptospira* serovars

a) Preparation of leptospiral antigens for immunization

The 24 strains of *Leptospira* were grown as above and harvested after 7 days (10^8 cells/ ml). Ten ml of pure leptospires were collected by centrifugation at 13,000 rpm for 30 minutes at 4^{o}C. The pellets were washed twice by centrifugation in phosphate buffer saline (PBS; pH 7.2) and resuspended in 100 μl of PBS to a concentration of 10^8 organisms/ 0.1 ml. Before use, these antigens were tested for sterility by determination with dark microscope.

b) Antibodies production

Thirty-five ICR mice and 17 BALB/c mice were used for production of hyperimmune sera against leptospires. Three or four ICR mice were used for each leptospires serovar (Autumnalis, Bataviae, Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, PatocI, Pomona, Sejroe and Tarassovi) and 1 or 2 BALB/C mice were used for each leptospires serovar (Bataviae, Icterohaemorrhagiae, Celledoni, Cynopteri, Djasiman, Hebdomadis, Javanica, Louisiana, Mini, Panama, Ranarum, Sarmin and Shermani). Mice were injected intraperitonealy (IP) with live leptospires of 100 μl of PBS containing $1x10^8$ cell/ 0.1 ml of leptospires. Mice were subsequently injected 2 more times with the same dosage of fresh organisms at two weeks intervals. Two weeks after the third injection, the serovar-specific antisera were collected, aliquoted and stored at -20^{o}C. The antisera were tested for leptospiral antibodies titre by MAT and ELISA using rLig and rFlaB proteins as previous described.

2. Canine serum samples

A total of 194 canine serum samples were provided from Leptospirosis Research Laboratory, Department of Veterinary Public Health and Diagnostic Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus , Nakhon Pathom, Thailand. Ninety-one sera with MAT negative titres at a dilution 1:100 were used to determine the relative specificity of ELISA. For determination of relative sensitivity of the ELISA, 103 canine sera showing MAT titres $\geq$ 100 positive to leptospiral serovars were employed.

B. Microscopic agglutination test (MAT)

MAT was performed as previous described by Cole *et al*., with all 24 serovars of *Leptospira* spp. used as antigens. The level of significant for the MAT was 1:100 by adding 50 µl of serum diluted to 1:50 with PBS pH 7.2. Fifty µl of each live leptospires serovars were added. After incubated at 28^{o}C for 2 hour, 3 µl of suspension was dropped on the slide. The agglutination was observed under a dark field microscope (OLYMPUS model CX31). The titre was the reciprocal of the highest dilution of the serum in which 50% agglutination or lyses of leptospires.

C. Immunoblot analysis for detection of anti-leptospiral antibodies

Partial purified of GST.Lig and GST (control antigen), His.FlaB and His (control antigen) proteins were separated in 12% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated in blocking solution (5% skim milk in PBS supplemented with 0.05% Tween 20 [PBS-T]) at 37^{o}C for 1 hour. Canine sera (primary antibodies from MAT positive and negative sera) were adsorbed using lysate from induced *E. coli* BL21 containing plasmid pGEX-5X-3 and plasmid pET-28A for antibodies detection against GST.Lig and His.FlaB, respectively at 37^{o}C for 1 hour. Then, the membrane were incubated with canine sera (dilute 1:40) at 37^{o}C for 1 hour and wash three times with PBS-T. They were then incubated with Protein A-horseradish peroxidase (HRP) conjugate (ZYMED) for canine serum and HRP-

conjugated goat anti-rmouse IgG (dilute 1:1,000; ZYMED) for mice serum diluted to 1:1,000 (v/v) in 1% skim milk (1% dry skim milk in PBS-T) at 37^{o}C for 30 minutes and again wash three times in PBS-T. The membrane were developed with 3,3´-diaminobenzidine tetrahydrochloride, and the Lig protein bands were visualized with brown lines and then stopped reaction by soaking membrane in distill water.

D. Enzyme –linked immunosorbent assay (ELISA)

The protein purified of GST.Lig and GST (control antigen), His.FlaB and His (control antigen) proteins were diluted in a coating buffer (0.007 M Na_2CO_3, 0.017 M $NaHCO_3$, pH 9.6) at optimum concentration which was established by checkerboard titration. One hundred microliters of the diluted antigen were added into each well of a 96-well microtiter plate (Maxisorb Immunoplates; Nunc). The plates were incubated at 4^{o}C overnight and then wash 3 times with PBS-T. Plates were incubated in blocking solution (5% dry skim milk in PBS-T) at 37^{o}C for 1 hour. Canine sera (primary antibodies from MAT positive and negative sera) were adsorbed as previously. Then, the wells were incubated with 100 µl of canine sera (dilute 1:40) at 37^{o}C for 1 hour. After three washed with PBS-T, the wells were then incubated with 100 µl of Protein A-horseradish peroxidase conjugate (ZYMED) for canine serum and HRP-conjugated goat anti-rmouse IgG (dilute 1:1,000; ZYMED) for mice serum diluted to 1:10,000 (v/v) in 1% skim milk (1% dry skim milk in PBS-T), at 37^{o}C for 30 minutes and again washed three times with PBS-T. Then, plates were added and incubated with TMB (3,3´, 5,5´ tetramethylbenzidine) substrate (ZYMED) for 10 minutes at room temperature. All plates were determined an optical density at 650 nm using an ELISA reader (Rosys anthosb lucy 2). Since Lig recombinant antigens were expressed as GST-fusion protein, GST was used as control and the reactivity rate of GST was deducted for analysis of samples and FlaB antigen was express as His-fusion protein, His was used as control and the reactivity rate of His was deduced for analysis of samples.

E. Data interpretation the cutoff point of ELISA

In canine serum samples, the average absorbance at 650 nm (A_{650}) values $\geq$ 0.177 were considered positive for rLig antigen-based ELISA, whereas A_{650} values < 0.177 were considered negative reaction.

F. Statistical analysis

The relative sensitivity, specificity and accuracy of ELISA for the detection of anti-leptospiral antibodies in dog sera were determined in comparison to the MAT as described below:

- Sensitivity = a/ (a+b) x 100

- Specificity = d/ (c+d) x 100

- Accuracy = [(a + d)/ (a + b + c + d)] × 100

The results obtained from the tests were analysed for the percentage agreement with MAT with the use of the kappa statistics. The kappa statistics is a decimal measure of agreement between two tests, especially in the absence of a standard, and is defined as kappa or κ.

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

Where P = (a + b)(a + c) + (c + d)(b + d); P is the probability, ‘a’ is the number of samples positive by both ELISA and MAT, ‘b’ is the number of samples positive by MAT but negative by ELISA, ‘c’ is the number of samples negative by MAT but positive by ELISA, and ‘d’ is the number of samples negative by both MAT and ELISA. A kappa value greater than 0.81 indicates perfect agreement.

RESULTS AND DISCUSSION

I. Polymerase chain reaction and molecular characterization of *flaB* gene of *Leptospira* species

A. Polymerase chain reaction

Twenty-four reference strains of *Leptospira* and 16 other bacteria as shown in Table 7 were used to analyze 16S rRNA (*rrs*), *lig* and *flaB* PCR. Using three sets of primers, amplification of 331 bp of *rrs* was observed in all 24 *leptospira* strains tested, amplification of 460 bp of *lig* was observed in 22 pathogenic strains tested and amplification of 793 bp of *flaB* was observed in 21 pathogenic strains tested (Figure 5).

A set of primer designed for common region of 16s rRNA gene provided PCR amplification not only in pathogenic leptospires but also in non-pathogenic serovar, *L. biflexa* (PatocI) at 331 bp fragment (Figure 5). PCR assay based on 16S rRNA sequence had been developed by Merien *et al.* (1992). In genereral, rRNA gene conserved among bacterial genes. Therefore, the primer set of *rrs* was the inability to distinguish between the saprophytic *L. biflexa* and pathogenic *Leptospir* spp. It was noteworthy that the PCR assay could readily distinguish between *Leptospira* and other bacteria (Wangroongsarb *et al*., 2005; Merien *et al*., 1992).

A major limitation of PCR-based diagnosis tests is the inability to differentiate pathogenic and non-pathogenic leptospires. Lig1/Lig2 and L-flaB-F1/L-flaB-R1 primers could amplify target DNA from pathogenic leptospires but not saprophytic *L. biflexa*. Lig1/Lig2 primers were designed from the conserved region of *ligA* and *ligB* (Palaniappan *et al*., 2005). These primers amplified genomic DNA from 22 pathogenic strains tested but not an intermediate pathogenic (*L. meyeri* serovar ranarum strain ICF) and *L. biflexa* serovar patoc strain PatocI. The result was similar to the use of L-flaB-F1/L-flaB-R1 for leptospires amplification. On the other hand one strain of pathogenic species *L. borgpetersenii* was not amplified with primer

sets of *flaB* gene (Table 7). The absence of PCR product from *L. borgpetersenii* serovar tarassovi (Perepelitsin) could be due to less specific of primer pairs of *flaB*. An attempt to detect the expression of *flaB* was successful by Southern blot analysis (data not shown). In addition Southern blot hybrization were used to determine the present of DNA fragment of *lig* and *flaB* gene in *L. meyeri* serovar ranarum strain ICF, the hybridization signals were obtained (data not shown). Previous publications have demonstrated that ligA is unique to pathogenic leptospira (Palaniappan *et al*., 2002, Matsunaga *et al*., 2003). Matsunaga *et al*. (2003) found PCR products of *lig* from all pathogenic *Leptospira* except *L. inadai*. Furthermore, *lig* sequences appear to be restricted to pathogenic strains since a pair of degenerate primers was not amplified in the two saprophytic strains. Palaniappan *et al*., (2005) developed a conventional and real time PCR diagnostic assays based on the conserved region of *ligA* and *ligB* of pathogenic leptospires. It is apparent that *lig* genes are conserved at the amino acid terminal of the pathogenic strains. The sensitivity and specificity of the *lig* primers proved to be a useful tool for the early diagnosis of leptospirosis. Specific DNA sequence of *ligA* could not be detected by PCR and Southern hybridization in six studies of *L. borgpetersenii* strains (Srimanote *et al*., 2008). The data was conformed to the result reported by Bulach *et al*. (2006), who sequenced the genomes of two strains of *L. borgpetersenii* and found that the bacteria lacked *ligA* and *ligC*. It has been known that members of this genomospecies are poorly adapted to the environment and are transmitted among mammalian hosts only by direct contact with contaminated tissue or body fluid (Zurner *et al*., 1993; Bulac *et al*., 2006). *L. borgpetersenii* is less pathogenic than *L. interrogans* in both human and hamster models of leptospirosis (Zurner *et al*., 1993; Ren *et al*., 2003; Nascimento *et al*., 2004). Postic *et al*. (2000) suggested that most *L. meyeri* strains actually belong to the pathogenic *Leptospira*. Only *L. meyeri* samaranga should belong to saprophytic species. It is interesting that *L. meyeri* which contained the *ligA* can infect human but may either pathogen or non-pathogen (Levett, 2001; Srimanote *et al*., 2008). The question of which is the actual original ICF has not been unsolved. These result need for a confirmation of the taxonomic status by hybridization studies.

To determine the specificity of the oligonucleotide primers for *Leptospira* spp., a number of pathogenic organisms were tested by using the 3 sets of primers at the same condition for PCR. There was no amplification product with non-*Leptospiral* DNA. *Escherichia coli, Enterobacter spp., Pseudomonas aeruginosa, Serratia marcescence, Listeria monocytogene, Campylabacter spp., Helicobacter pylori, Aeromonas hydrophila, Proteus vulgalis, Salmonella* serogroup E, *Vibrio parahaemolyticus Bacillus cereus, Staphylococcus aureus* and *alpha-Streptococcus* spp. were all negative. Due to the specificity of leptospiral DNA products obtained from this study, these primer sets including *rrs*, *lig* and *flaB* will be further used in clinical samples. Previous publication articles have demonstrated the sensitivity of the PCR for detection of leptospires. The first publication by using 16S rRNA primer for amplification of *rrs* gene of *Leptospira*, the lower limit of detection DNA in the PCR assay was 10 cells/ ml (Merien *et al*., 1992). Wangroongsarb *et al.* (2005) evaluated the application of 16S rRNA and *flaB* PCR for diagnosis of leptospirosis at the sensitivity of 10 cells/ ml. The PCR method is also suitable for the clinical diagnosis of leptospirosis (Wangroongsarb *et al.*, 2005). Moreover, Palaniappan *et al*. (2005) obtained the highest sensitivity in their PCR using Lig1/Lig2 primers with a detection of 6 cells/ ml of leptospiral DNA templates.

Amplification of *Leptospira* strains by ERIC-PCR were shown in Figure 6. The primers used in this study produced characteristic band patterns for different leptospiral strains. For most of strains tested, differentiating bands were observed between 100 and 500 bp. The *L. borgpetersenii* serovar tarassovi (Perepelitsin) and *L. meyeri* serovar ranarum strain ICF produced numerous bands, whereas the other strains produced fewer bands or could not produce DNA band. Fourteen patterns were presented and DNA band patterns were different in each strain. This result demonstrated that this method provided high diversity but low typeability. ERIC-PCR fingerprint was not able to differentiate strains belong to different species and could not classify leptospiral strains into serovar level.

Table 7 PCR amplification of *fla*B of *Leptospira* spp. and other bacteria

Microorganisms	***rrs*** **PCR**	***flaB*** **PCR**	***lig*** **PCR**
L.interrogans**:** sv[a] autumalis (Akiyami A)[b], bataviae (Swart), bratislava (Jez Bratislava), icterohaemorrhagiae (RGA), canicola (Hond Utrecht IV), djasiman (Djasiman), hebdomadis (Hebdomadis), pomona (Pomona), pyrogenes (Salinem)	+	+	+
L. noguchii**:** sv louisiana (LSU 1945), panama (CZ 214 K)	+	+	+
L. borgpetersenii**:** sv javanica (V. Batavia 46), mini (Sari), sejroe (M 84), tarassovi (Perepelitsin)	+	+/-[c]	+
L. santarosai**:** sv shermani (1342 K)	+	+	+
L. weilii**:** sv celledoni (Celledoni), sarmin (Sarmin)	+	+	+
L. kirschneri**:** sv cynopteri (3522 C), grippotyposa (Moskva V)	+	+	+
L. meyeri**:** sv ranarum (ICF)	+	-	-
L. biflexa**:** sv patoc (Patoc I)	+	-	-
***Escherichia coli, Enterobacter spp., Pseudomonas aeruginosa, Serratia marcescence, Listeria monocytogene, Campylabacter spp., Helicobacter pylori, Aeromonas hydrophila, Proteus vulgalis, Salmonella* serogroup E*, Vibrio parahaemolyticus, Bacillus cereus, Staphylococcus aureus, alpha-Streptococcus* spp.**	-	-	-

([a]sv: serovar, [b]strain, [c] *L. borgpetersenii* serovar tarassovi (Perepelitsin) gave negative result for amplification of *flaB* gene)

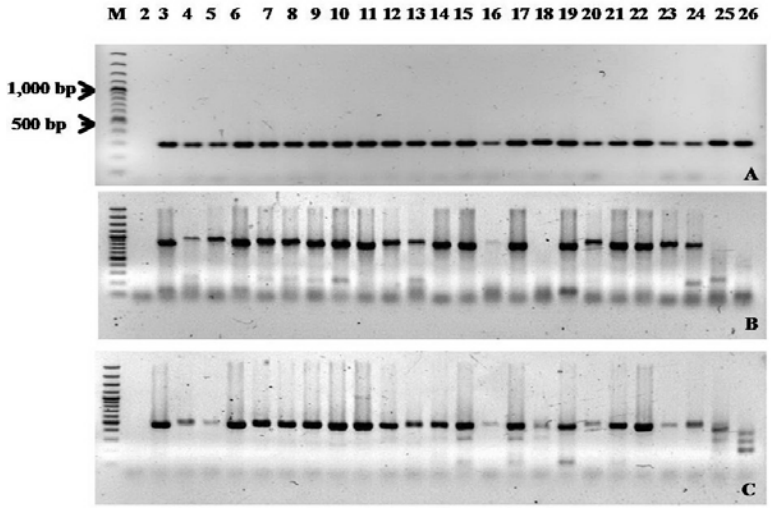


Figure 5 PCR amplification of the target DNA from reference strains of *Leptospira* species on 1.5% agarose gel.(A) 331 bp of common region in 16S rRNA gene, (b) 852 bp of *flaB* gene and (C) 460 bp of *lig* gene
Lanes: 1. 100 bp marker, 2. Negative control (DW), 3. *L. interrogans* serovar autumalis (Akiyami A), 4. *L. interrogans* serovar bataviae (Swart), 5. *L. interrogans* serovar bratislava (Unknown strain), 6. *L. interrogans* serovar canicola (Hond Utrecht IV), 7. *L. interrogans* serovar djasiman (Djasiman), 8. *L. interrogans* serovar hebdomadis (Hebdomadis), 9. *L. interrogans* serovar icterohaemorrhagiae (RGA), 10. *L. interrogans* serovar pomona (Pomona), 11. *L. interrogans* serovar pyrogenes (Salinem), 12. *L. noguchii* serovar louisiana (LSU 1945), 13. *L. noguchii* serovar panama (CZ214K), 14. *L. borgpetersenii* serovar ballum (MUS 127), 15. *L. borgpetersenii* serovar javanica (Veldrat Batavia 46), 16. *L. borgpetersenii* mini (Sari), 17. *L. borgpetersenii* serovar sejroe (M 84), 18. *L. borgpetersenii* serovar tarassovi (Perepelitsin), 19. *L. santarosai* serovar shermani (1342 K), 20. *L. weilii* serovar celledoni (Celledoni), 21. *L. weilii* serovar manhao (Li 130), 22. *L. weilii* serovar sarmin (Sarmin), 23. *L. kirschneri* serovar cynopteri (3522 C), 24. *L. kirschneri* serovar grippotyposa (Moskva V), 25. *L. meyeri* serovar ranarum (ICF), 26. *L. biflexa* serovar patoc (Patoc I).

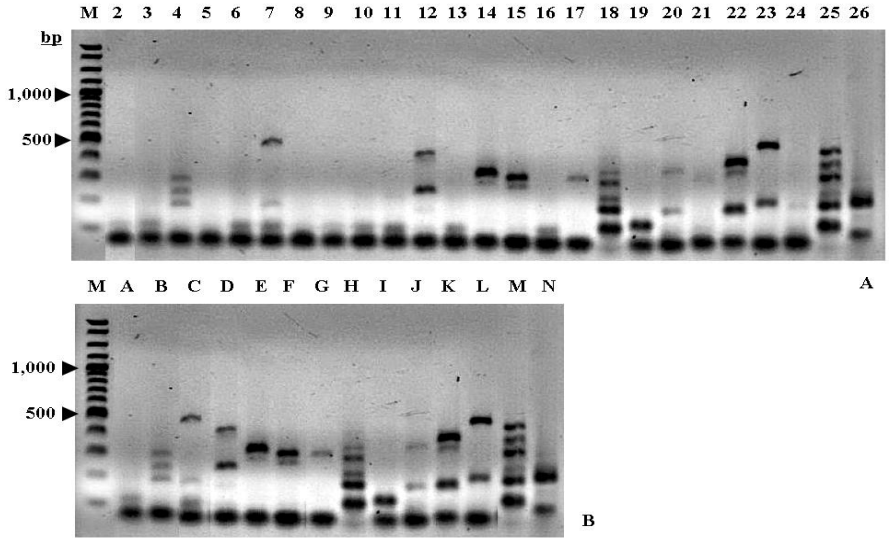


Figure 6 Amplification of reference strains of *Leptospira* species by ERIC-PCR (A) Fingerprints of *Leptospira* (B) Diagram of fourteen amplification patterns.

Lanes: 1. 100 bp marker, 2. Negative control (DW), 3. *L. interrogans* serovar autumalis (Akiyami A), 4. *L. interrogans* serovar bataviae (Swart), 5. *L. interrogans* serovar bratislava (Unknown strain), 6. *L. interrogans* serovar canicola (Hond Utrecht IV), 7. *L. interrogans* serovar djasiman (Djasiman), 8. *L. interrogans* serovar hebdomadis (Hebdomadis), 9. *L. interrogans* serovar icterohaemorrhagiae (RGA), 10. *L. interrogans* serovar pomona (Pomona), 11. *L. interrogans* serovar pyrogenes (Salinem), 12. *L. noguchii* serovar louisiana (LSU 1945), 13. *L. noguchii* serovar panama (CZ214K), 14. *L. borgpetersenii* serovar ballum (MUS 127), 15. *L. borgpetersenii* serovar javanica (Veldrat Batavia 46), 16. *L. borgpetersenii* mini (Sari), 17. *L. Borgpetersenii* serovar sejroe (M 84), 18. *L. borgpetersenii* serovar tarassovi (Perepelitsin), 19. *L. santarosai* serovar shermani (1342 K), 20. *L. weilii* serovar celledoni (Celledoni), 21. *L. weilii* serovar manhao (Li 130), 22. *L. weilii* serovar sarmin (Sarmin), 23. *L. kirschneri* serovar cynopteri (3522 C), 24. *L. kirschneri* serovar grippotyposa (Moskva V), 25. *L. meyeri* serovar ranarum (ICF), 26. *L. biflexa* serovar patoc (Patoc I).

B. Molecular characterization of *flaB* gene

Molecular characterization of *flaB* was studied using amplification and sequencing of the nearly full-length *flaB* gene sequence (with primer L-flaB-F1/L-flaB-R1), multiple-sequence alignment, calculation of percent similarity, and construction of phylogenetic tree.

The nucleotide sequence of *flaB* from 21 strains tested and 37 strains provided data from GenBank were determined using a multiple sequence alignment editor BioEdit version 7.0.5.2. The alignments of *flaB* gene sequences were demonstrated in Figure 7. The position of insertion or deletion, as well as differing and mixed based, were shown in Figure 8. A total of 108 dissimilar base positions distinguished the type strain of *flaB* sequence of pathogenic *Leptospira* species. Different serovars of the same species showed highly identical *flaB* sequence. There was only an average of 0.2 base different of about 749 bp to each other (data not shown).

Multiple alignments of the DNA sequences allowed phylogenetic comparison between species. The *flaB* sequences of 58 strains, representing 8 species using *E. coli* as out group, were determined. The length of the sequences ranged from 746 to 749 bp. The sequence similarity in the 749 bp segment of *flaB* among species tested varied between 50.6% and 96.1% (Figure 10). Analysis of *flaB* sequence demonstrated 3 clades including two pathogenic clades and one non-pathogenic clade as shown in Figure 9. A group of *L. interrogans*, *L. kirschneri* and *L. noguchii* have closely phylogenetic relationship. The percentage similarity of sequence within this clade was high at $\geq$ 93.5%. The highest *flaB* sequence similarity between the type strains of species was between those of *L. interrogan*s and *L. kirschneri* (18/749 bp different). These two species may be confused with each other because they share some serological properties and are frequently demonstrated the same clinical symptoms as caused in human and animals (Haake *et al*., 2004). The close phylogenetic relationship among type strains of *L. interrogans* and *L. kirschneri* was similar to the use of the 16S rRNA sequencing as previous reported by Morey *et al*. (2006). A third

clade, *L. biflexa*, was clearly separated from the pathogenic clade. The *flaB* gene was developed to discriminate among the reference *leptospira* strains tested. It has been found that *flaB* has strong discriminative power and enabled the identification of eight species based on three clades inferred by the phylogenetic analysis of *flaB*. Moreover *flaB* gene restriction fragment length polymorphism (RFLP) has been demonstrated by using BioEdit analysis software. The result showed that some restriction enzyme, i.e. *Hae*III and/or *Taq*II, could be used to characterized and differentiate between pathogenic *Leptospira* spp. (Table 8).

The analysis of nearly full-length of *flaB* offers two additional advantages. Firstly, a 749 bp fragment of *flaB* could be simple amplified and sequenced rather than the longer nucleotide fragments such as *rrs*. Secondly, compared with *rrs*, the degree of polymorphism is higher in *fla*B for the identification of *Leptospira* species, for examples, only 2 of 1,430 bp nucleotides of *L. interrogans* and *L. kirschneri* are different (Morey *et al*., 2006). In contrast, for the same species, the positions of 18 nucleotides are different in 748 bp fragment of *flaB*. It is doubtful that these species would have been distinguished by relying on 16s rRNA gene sequence analysis alone without the previous hybridization studies (Brenner *et al*., 1999). Therefore the *flaB* gene was greater variability than the 16S rRNA gene and greater discriminative value.

Numerous studies used PCR based-sequencing for species identification of *Leptospira* spp (Morey *et al*., 2006; Scola *et al*., 2006; Wangroongsarb *et al*., 2007). Morey *et al*., (2006) demonstrated the usefulness of *rrs* sequence analysis to differentiate *Leptospira* down to the species level. Scola *et al.* (2006) demonstrated the usefulness of partial *rpoB* sequence in the identification of *Leptospira* down to the species level. Wangroongsarb *et al.* (2007) compared the *rrs* and *wzy* sequencing of reference strains of *Leptospira* serovar and leptospiral cultures isolated from Thai patients. The phylogenetic tree of *wzy* sequence was more differentiation power than 16S rRNA gene. The limitations of *wzy* sequences were inability to amplify some strains tested (*L. kirschneri* and *L. borgpetersenii*) and the long size of *wzy* nucleotide sequence (ranged from 1 to 1.5 kb) (Wangroongsarb *et al*., 2007). The 16S rRNA gene sequence were only able to discriminate to nearly the species level, i.e. isolates

could be classified into a group containing *L. interrogans* and *L. kirschneri* type strains, but the *flaB* based typing could be used to infer a greater number of reliable clades, allowing the discrimination between *L. interrogans* and *L. kirschneri* strains.

Several molecular techniques have been evaluated for identification of *Leptospira* species or serovars. These include random amplified polymorphic DNA, arbitrarily primed PCR, use of insertion sequences in PCR-based assays, restriction length polymorphism, specific probes, variable number tandem repeat analysis and pulsed-field gel electrophoresis (Barocchi *et al*., 2001; Levett, 2001). The scarcity of reproducible and distinguishable characters frequently limits phenotypic characterization and phenotype-based identification in routine clinical microbiology laboratories. The development of gene amplification and sequencing, especially that of *flaB* nucleotide sequences, has simplified the taxonomy and identification of bacteria, particularly those lacking distinguishable phenotypic characteristics.

The *flaB* molecular typing scheme has clear methodological advantages over conventional serology and, with the widespread availability of sequencing, as an epidemiological tool that could foreseeable replace serological techniques. The use of this routine typing scheme in conjunction with PCR analysis of samples could be used as a means of rapidly identifying the animal reservoirs responsible for outbreaks of leptospirosis. The results have demonstrated that all recognized and unknown species of *Leptospira* can be identified by using standardized *flaB* sequencing. The *flaB* gene is believed to contain specific sequence information. Michison *et al*. (1991) suggested that the *flaB* gene may be a recent addition in evolutionary terms to the leptospiral gene pool. It may be useful in the design of DNA or synthesis peptide probes suitable for detection of the infection with pathogenic leptospires.

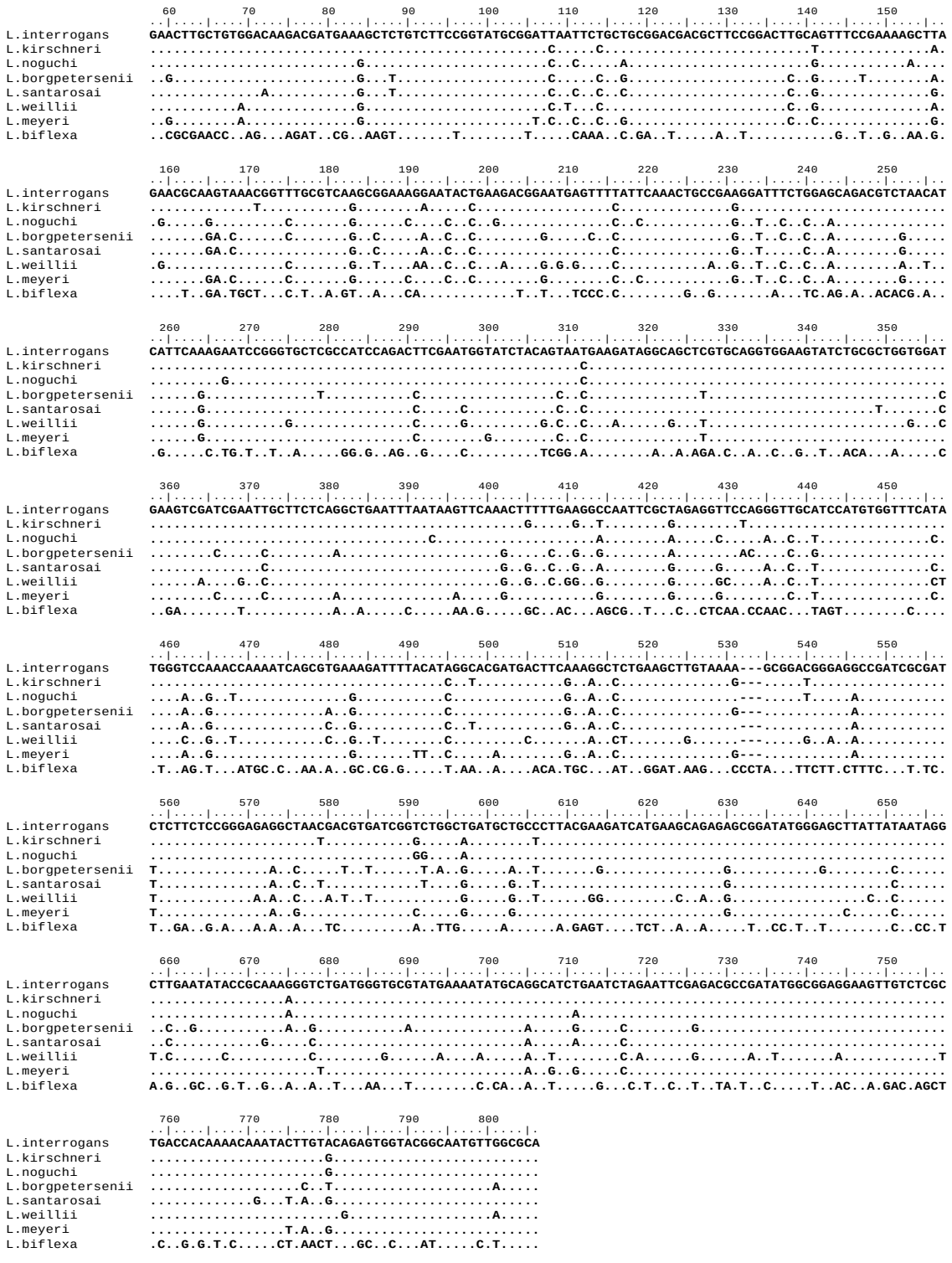


Figure 7 Nucleotide sequence alignment of *flaB*.

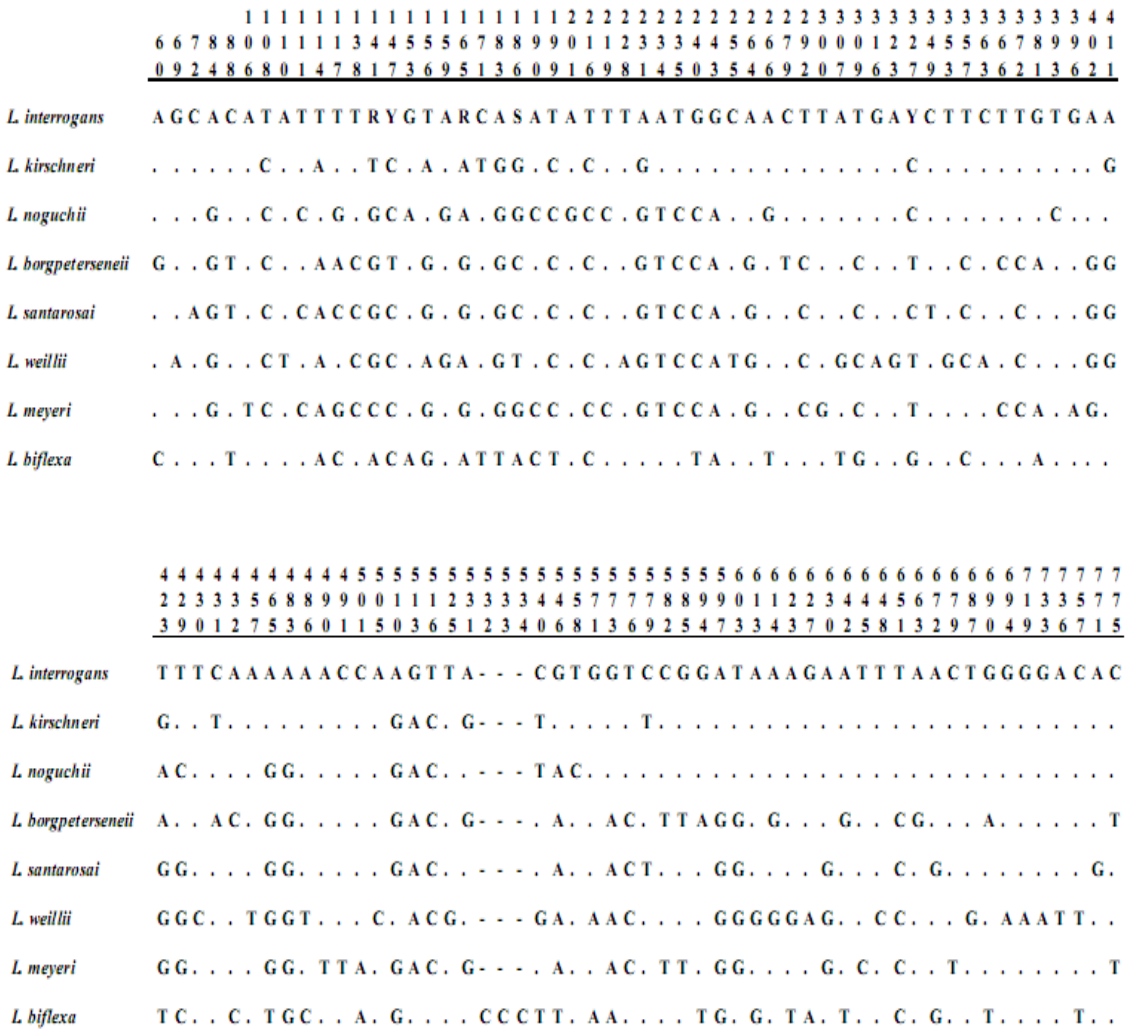


Figure 8 Base differences in the *flaB* genes (position 58-806) of type strains formatted to emphasize the differences within the genus *Leptospira*. Center dots indicate the same bases as *L. interrogans*; a dash indicates a gap.

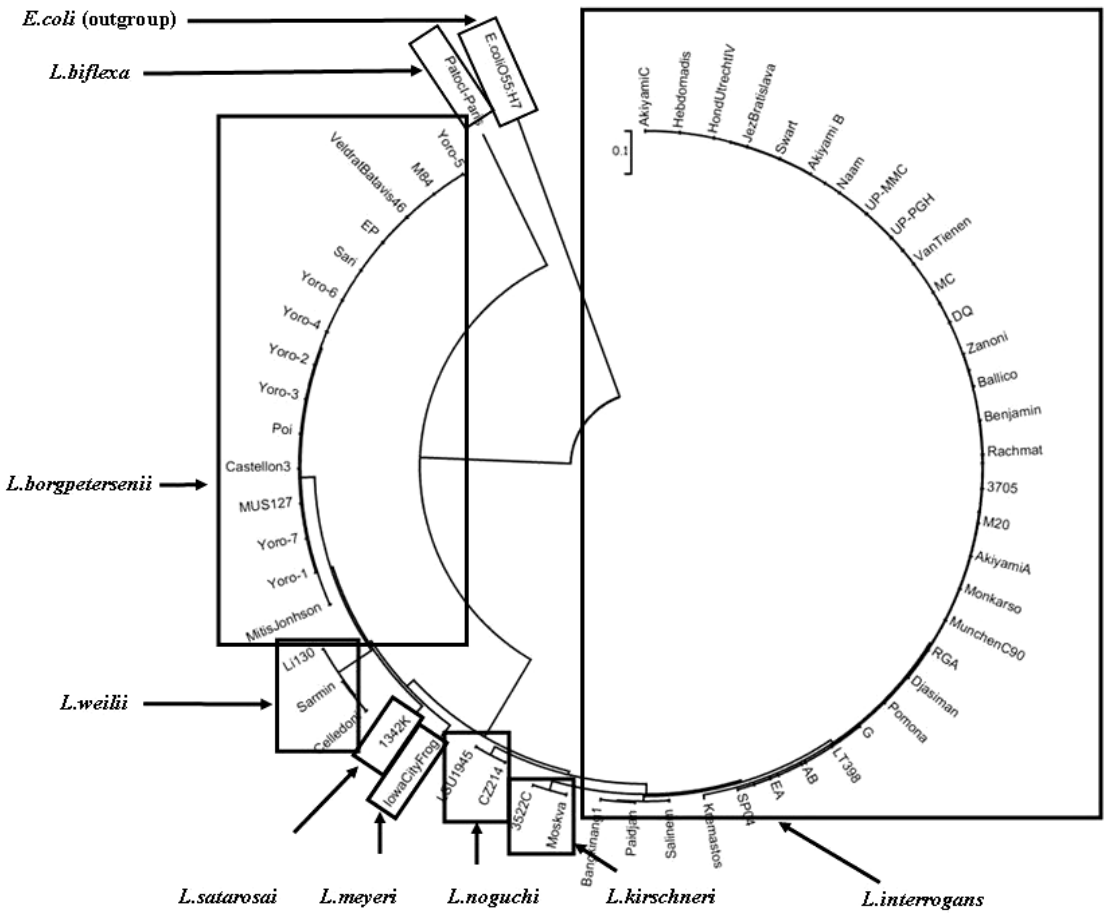


Figure 9 Phylogenetic tree of *flaB* of 58 *Leptospiraceae* based on nucleotide position from 58 to 806 with *E.coli* as outgroup using MEGA 4 version 4028 software (neighbor joining method). The scale bar represents base substitution per site.

Figure 10 Percent similarity observed for partial *flaB* sequences among *Leptospira* species

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Swart	1	100																						
AKiyamiA	2	99.7	100																					
Djasiman	3	99.7	100	100																				
Hebdomadis	4	100	99.7	99.7	100																			
HondUtreth IV	5	100	99.7	99.7	100	100																		
JezBratislava	6	100	99.7	99.7	100	100	100																	
Pomona	7	99.7	99.7	99.7	99.7	99.7	99.7	100																
RGA	8	99.7	100	100	99.7	99.7	99.7	99.7	100															
Salinem	9	97.2	97.5	97.5	97.2	97.2	97.2	97.4	97.5	100														
Moskva	10	96.0	96.0	96.0	96.0	96.0	96.0	96.1	96.0	95.3	100													
3522C	11	95.8	95.8	95.8	95.8	95.8	95.8	96.0	95.8	95.1	99.9	100												
LSU1945	12	93.7	93.7	93.7	93.7	93.7	93.7	93.9	93.7	93.6	94.2	94.0	100											
CZ214	13	93.6	93.6	93.6	93.6	93.6	93.6	93.7	93.6	93.5	94.0	93.9	99.9	100										
V.Batavis46	14	88.5	88.3	88.3	88.5	88.5	88.5	88.5	88.3	90.5	90.0	89.8	89.8	89.7	100									
M84	15	88.5	88.3	88.3	88.5	88.5	88.5	88.5	88.3	90.5	90.0	89.8	89.8	89.7	100	100								
MUS217	16	88.5	88.3	88.3	88.5	88.5	88.5	88.5	88.3	90.5	90.0	89.8	89.8	89.7	100	100	100							
Sari	17	88.5	88.3	88.3	88.5	88.5	88.5	88.5	88.3	90.5	90.0	89.8	89.8	89.7	100	100	100	100						
1342K	18	89.8	89.7	89.7	89.8	89.8	89.8	89.8	89.7	91.1	91.7	91.5	91.9	91.8	93.5	93.5	93.5	93.5	100					
Sarmin	19	82.9	83.0	83.0	82.9	82.9	82.9	82.9	83.0	83.9	84.0	83.9	84.4	84.6	87.1	87.1	87.1	87.1	87.9	100				
Celledoni	20	82.9	83.0	83.0	82.9	82.9	82.9	82.9	83.0	83.9	84.0	83.9	84.4	84.6	87.1	87.1	87.1	87.1	87.9	100	100			
Li130	21	82.9	83.0	83.0	82.9	82.9	82.9	82.9	83.0	83.9	84.0	83.9	84.4	84.6	87.1	87.1	87.1	87.1	87.9	99.3	99.3	100		
ICF	22	89.7	89.6	89.6	89.7	89.7	89.7	89.8	89.6	90.7	90.3	90.1	92.5	92.4	92.6	92.6	92.6	92.6	93.9	85.7	85.7	85.7	100	
PatocI	23	55.8	55.8	55.8	55.8	55.8	55.8	55.5	55.8	56.5	54.9	54.9	55.1	54.9	54.8	54.8	54.8	54.8	55.2	50.9	50.9	50.6	54.9	100

Twenty-three *Leptospira* strains used in this study; 9 strain of *L. interrogans* (Swart, AkiyamiA, Djasiman, Hebdomadis, Hond Utreth V Jez Bratislava, Pomona, RGA and Salinem), 2 strains of *L. kirschneri* (Moskva and 3522C), 2 strains of *L. noguchii* (LUS1945 and CZ214), 4 strains of *L. borgpetersenii* (Veldrat Batavis46, M84, MUS217 and Sari), 1 strain of *L. santarosaii* (1342K), 3 strains of *L. weilii* (Sarmin, Celledoni and Li130), 1 strain of *L. meyeri* (IowaCityFrog) and 1 strain of *L. biflexa* (PatocI).

Table 8 Schematic representation of RFLP typing based on *flaB* gene

Enzyme	Frequency position of restriction site						
	L. interrogans	*L. kirschneri*	*L. noguchii*	*L. borgpetersenii*	*L. santarosaii*	*L. weillii*	*L. meyeri*
*Aar*I	323	323	323	323	323	323	323
*Acc*I	-	-	-	364 663	-	70	70 364
*Alw*I	-	114	114 263 276	114 621	114	114 378 621	309
*Apo*I	387 718	387 718	387 718	387 718	387 718	387 718	387 396 718
*Ase*I	109	-	-	-	-	-	-
*Ava*I	-	-	-	657	-	-	-
*Bbs*I	85 208	85 208	85	85 208	85 208	85 548	85 208
*Bce*AI	-	-	-	-	-	323	126
*Bhv*I	104 335 587	104 335 587	129 335 587	335 612	335	-	-
*Bcg*I	388 354	388 354	388 354	-	-	-	-
*Bgl*I	735	735	735	735	735	735	735
*Bmg*BI	581	581	581	-	581	-	581
*Bpm*I	261	261	416	-	-	-	-
*Bpu*EI	-	-	-	364	-	-	364
*Bsa*I	-	-	-	536	536	-	536
*Bsa*BI	552	552	552	-	-	-	-
*Bsa*HI	249 727	249 727	249 727	249 727	249 727	249 727	249 727

Table 8 (Continued)

Enzyme	Frequency position of restriction site						
	L. interrogans	*L. kirschneri*	*L. noguchii*	*L. borgpetersenii*	*L. santarosaii*	*L. weillii*	*L. meyeri*
*Bsa*JI	433	229	199 229	115 229	118 229	272	115 229 433
*Bsa*WI	96 132	96 132	96 132	96 132	96 132	96 132	96 132
*Bsa*XI	234	234	-	-	-	-	-
*Bse*MII	393	393	393	-	147 393	-	147
*Bse*YI	-	-	-	-	-	350	-
*Bsg*I	351	351 798	351 798	351	351 798	351	351 798
*Bsi*EI	368 550	368 550	368 550	368 550	368 550	550	368 550
*Bsl*I	103	103	103	103 205 672 721	103	103 205 672 721	103 205 298
*Bsm*AI	718 755	718 755	536 718 755	536 755	536 718 755	755	536 718 755
*Bsm*BI	718	718	718	-	718	-	718
*Bsp*CI	392	392	392	-	148 392	392	-
*Bsp*EI	132	132	132	132	132	132	132
*Bsp*HI	616	616	616	616	616	616	616
*Bsp*MI	323	323	155 323	323	323	323	323
*Bsr*BI	630	630	630	-	-	-	-
*Bsr*DI	799	799	799	799	799	799	799
*Bss*SI	327	327	327	-	327	-	-

Table 8 (Continued)

Enzyme	Frequency position of restriction site						
	L. interrogans	*L. kirschneri*	*L. noguchii*	*L. borgpetersenii*	*L. santarosaii*	*L. weillii*	*L. meyeri*
*Bst*API	329	329 600	329 600	329	329	-	329
*Bst*BI	294	294 515	294 510	510	510	510	510 214 283 362
*Bst*F5I	283 339 441	283 339 441 542	253 283 362 542	214 283 602	214 602	283 602	602
*Btg*I	-	-	-	115	118	-	115
*Cac*8I	280 703	280 592 703	280 703	64 280 706	280 703	380 703	64 280 703
*Dde*I	156 379	156 379	156 379	156	156 379	156 379	98
*Ear*I	563	563	563	-	-	-	-
*Eci*I	753	201 549 753	201 753	135 549 645 753	104 609 645 753	609 645 753	135 201 549 609
*Eco*571	220 540	540	540	540	540	-	540
*Eco*RI	718	718	718	718	718	-	718
*Fok*I	270 369 428	270 369 428 549	204 270 369 549	221 270 609	270 879	270 609	204 221 270 369
*Hae*III	415 545	545	-	186	186 676	431 463 676	589 603
*Hga*I	135 168 735	135 168 735	135 170 168 735	135 168 430 735	135 168 735	135 170 735	135 168 694 729
*Hin*4I	103 234 577	103 234 510 577	103 478 510 577	158 190 510 577	478 510 536	103 362 545	103 158 190 510
*Hind*III	153 522	522	522	402 522	522	703	402 522
*Hyp*8I	71 170	71	71 170	71 356 432 664	356	71 170 757	71 365
*Mbo*II	213 327 550 623	213 327 550 623	327 550 623	80 213 327 550	85 213 550 623	85 553 550 753	85 213 327 877

Table 8 (Continued)

Enzyme	Frequency position of restriction site						
	L. interrogans	*L. kirschneri*	*L. noguchii*	*L. borgpetersenii*	*L. santarosaii*	*L. weillii*	*L. meyeri*
*Msl*I	446	446	-	-	-	-	-
*Nde*I	457	457	-	457 689	-	-	-
*Nla*IV	431 463	463	270 431 463	463	431 463	431 463	431 463 641
*Nru*I	552	423 552	552	552	423	423 552	423 552
*Ple*I	-	-	444	130	130 444	130 444	130
*Psi*I	649	649	649	-	-	-	-
*Pst*I	-	-	119	-	-	230	-
*Pvu*I	368 550	368 550	368 550	550	368 550	550	550
*Sal*I	-	-	-	363	-	-	363
*Sfa*NI	450 587 714	450 587 714	450 714	587 714	587 714	450 587	587
*Sfc*I	305	305	115 305	305	305	67 226	67 305
*Taq*II	576 672	672	479 560 672	382 479 560	672	560 576	382 479 560
*Tsp*DTI	328 373 633 706	206 373 633 706	328 373 633 706	328 373 444 706	96 206 633 706	96 206 633	96 206 373 633
*Tsp*GWI	148	220	206 220	-	20	794	206
*Xba*I	714	714	714	-	-	-	-
*Xcm*I	294	294	294	294	-	-	294
*Xmn*I	235 374	374	374	374	374	-	374

II. Cloning and expression of recombinant full length of *flaB* and conserved region of a leptospiral immunoglobulin-like protein (*lig*) against *Leptospira*

A. PCR cloning of leptospiral *flaB* and *lig* gene in *E. coli*

The PCR was used to amplify the portion of the *flaB* and *lig* gene. A 852 bp PCR product from DNA of *L. interrogans* serovar canicola strain Hond Utrecht IV and a 1,728 bp product from DNA of *Leptospira kirschneri* serovar grippotyphosa strains Moskva V were analyzed by agarose gel electrophoresis as shown in Figure 11. The PCR products were initially cloned into pGEM-T Easy vector and transformed into *E. coli* JM109 for amplification of target genes in the host cell. The purification of *flaB* gene was then subcloned into *BamH*I-*Sac*I sites of pET-28a (pET-28a.*flaB*) and *lig* gene was subcloned into *Xho*I-*Sal*I of pGEX-5X-3 (pGEX-5X-3.*lig*). The recombinant plasmids were subject to gel electrophoresis to check for presence of insert based on size difference when compared with the vectors plasmid pET-28a for *flaB* gene and pGEX-5X-3 for *lig* gene alone. To confirm whether they were correct clones, pET-28a.*flaB* was digested with *BamH*I and *Sac*I restriction enzyme and pGEX-5X-3.*lig* was digested with *Xho*I and *Sal*I restriction enzymes. The plasmids contained correct insert site as shown in Figure 12. In addition, the correct inserts were also demonstrated by sequencing. The nucleotide sequences obtained were translated to protein using BioEdit program version 7.0.5.2 (Figure 13 and 14). Comparison of the clone *flaB* and *lig* DNA sequences in the database reveal the high degree of sequences conservation, with DNA sequence identity ranged from 95 to 97% and amino acid sequence identity ranged from 97 to 98% for *flaB* gene (compared with GenBank accession number AF064055-AF064056) and DNA sequence identity ranged from 92% to 96% and amino acid sequence identity ranged from 93 to 96% for *lig* gene (compared with GenBank accession number AY190126, AB098516 and AB098517). Amino acid residues of clone *flaB* with other FlaB protein and *lig* with other Lig were compared and aligned in Figure 15 and 16. The correct recombinant plasmids were then transformed into *E. coli* BL21 for further protein expression and also stored as a glycerol stock and kept at -70^{o}C.

B. Expression of the recombinant proteins

Expression of the 6xHis.FlaB (rFlaB) and GST.Lig (rLig) fusion proteins were achieved by isopropyl-ß-D-thiogalactopyranoside (IPTG) induction (Figure 17). The optimal condition was first determined using various concentration of IPTG and various times. As shown in Figure 16, all concentrations of IPTG used gave approximately amount of expressed both rFlaB and rLig proteins but longer induction time allows greater expression. In this experiment the suitable concentration of IPTG was 0.1 mM and induction time was allowed for 4 hours (Figure 18).

C. Characterization and partial purification of recombinant proteins

Upon induction, a strong protein band about 35 kDa of rFlaB and 96 kDa of rLig were observed. Additional work was to determine whether the rFlaB and rLig were expressed as insoluble or soluble protein. Here, after recombinant proteins were incubated with 100 µg/ ml on ice for 30 minutes, the result demonstrated that these two recombinant proteins were produced predominately in the insoluble inclusion body. The expressed proteins were not seen in the culture supernatant or in the soluble cellular fraction, but were present in the insoluble fraction. This suggested that the recombinant proteins had formed inclusion bodies within the cytoplasm of *E. coli* which it would be dissolved in 4M urea and 1% sarkosyl for protein partial purification step both rFlaB and rLig proteins. The results showed that the proteins partial purification were nearly 100%. Successful purifications were demonstrated by subjecting samples into 12% SDS-PAGE as shown in Figure 19.

The recombinant plasmids were transformed and expressed in *E. coli* and were used for nucleotide sequencing. The corresponding amino acid sequences of *flaB* gene and *lig* were deduced. From the results, they were proved that *Leptospira* gene had been clone into the pET-28a and pGEX-5X-3 for *flaB* and *lig*, respectively. The pET and pGEX based vectors are generally used for cloning in *E. coli* basing on its high copy number properly and contain the strong promoter. In addition, its expression could be controlled by IPTG and recombination cells could be easily identified.

The pET expression system (6x-His) contains a high selectivity T7 RNA polymerase for its cognate promoter. The high level of the polymerase and the high translation efficiency mediated by the T7 gene translation signals (Novagen, 2002). In the pET system, the protein coding sequence of interested is cloned to downstream of the T7 promoter and then transformed into *E. coli* strain BL21 (DE3). Protein expression was achieved by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase was expressed from the lacUV5 promoter and then polymerase would be specific binding to the T7 promoter in expression vector (Novagen, 2002).

The pGEX vectors provide a multiple cloning site for fusion gene of interest to the C-terminus of glutathione *S*-transferase (GST) from the parasite *Schistosoma japonicum*. Vector expression is under the control of the *tac* promoter, which is induced by IPTG. All pGEX vectors are also engineered with an internal *lac*I^q gene. The *lac*I^q gene product is a repressor protein that binds to the operator region of the *tac* promoter. It prevents the protein expression until induction by IPTG and maintains a tight control over expression of insert (Amersham phamacia biotech, 2000).

Induction of His.FlaB and GST.Lig with 0.1 mM IPTG at 37^oC resulted in appearance of recombinant proteins as cellular soluble protein and inclusion bodies (Figure 19). The majority of the recombinant proteins both His.FlaB and GST.Lig were in the inclusion bodies fraction. The inclusion bodies are aggregates of inactive protein, and generally produced when that protein is expressed in high level at fast rate that proper folding of the molecule. Intermolecule hydrophobic and ionic interactions of these partially folded proteins with exposed hydrophobic moieties occurred with aggregation. There are advantages aspects of inclusion bodies for making such as high purity concentration protein of interest with few contaminants (mostly from lipoprotein envelope of the cell debris). The insoluble fraction (inclusion bodies) can be further purified by repeated centrifugation and washing steps to yield highly concentrated and relatively pure protein (Novagen, 2000). After

extraction conditions to improve solubilization of inclusion bodies, protein can sometimes be solubilized from inclusion bodies using common denaturants such as 4-6 M guanidine hydrochloride, 4-8 M urea, 0.5-2% Triton X-100, 0.5-2% *N*-luarylsarcosine (Frangioni and Neel, 1993; Frankel *et al.*, 1991; Gentry and Burgess, 1990). Purification methods for target proteins represent adaptations of an existing protocol for solubilizing the actin from crude bacterial lysate and utilize the sodium salt of the alkyl anionic detergent *N*-luarylsarcosine demonstrated that 4M urea and 0.1% *N*-luarylsarcosine could be used to purify both His.FlaB and GST.Lig proteins.

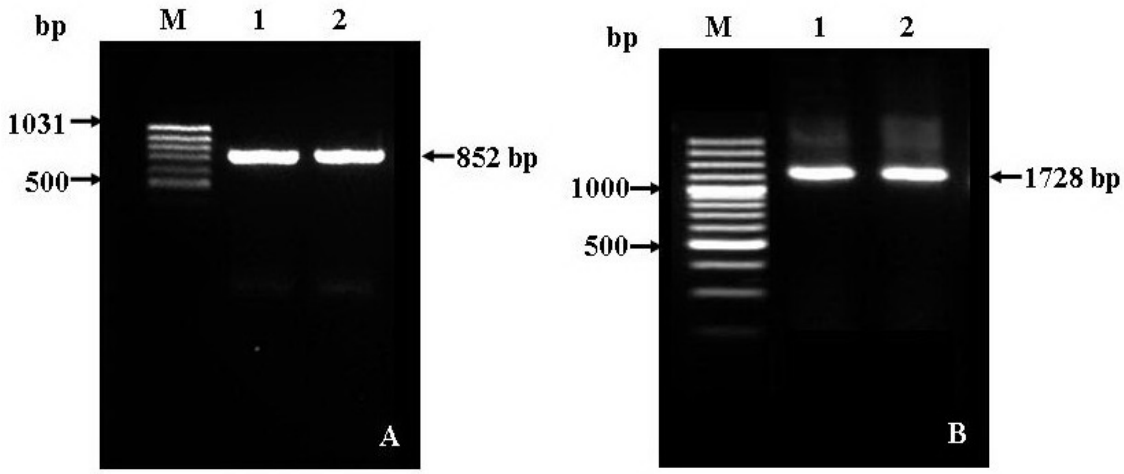


Figure 11 Agarose gel electrophoresis of PCR products using primers for *flaB* (A) and *lig* (B). Figure 11A; Lane 1-2, 852 bp *flaB* amplification products and Figure 11B; lane 1-2, 1,728 bp *lig* amplification products. Lane M, DNA ladder.

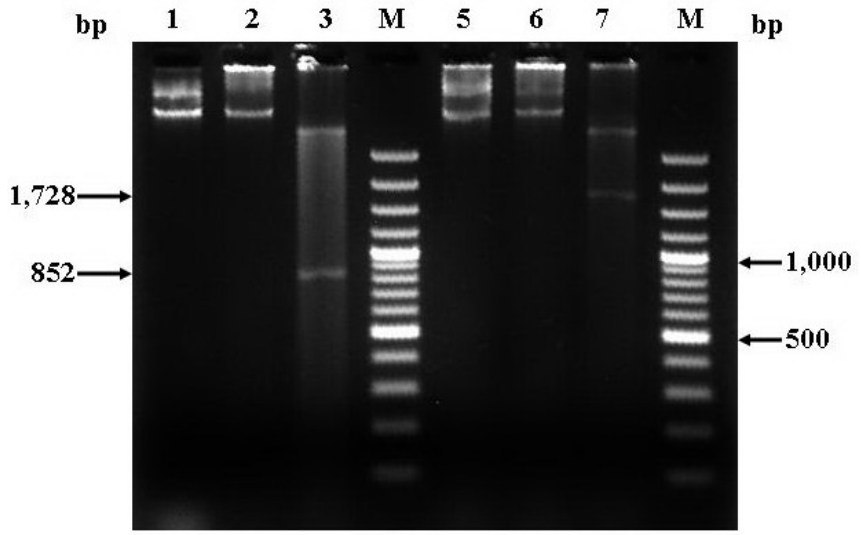


Figure 12 Agarose gel electrophoresis of pET-28a.*flaB* was digested with *BamH*I and *Sac*I restriction enzymes, and pGEX-5X-3.*lig* was digested with *Xho*I and *Sal*I restriction enzymes.

Lane 1- pET-28A plasmid

Lane 2- pET-28A.*flaB* uncut plasmid

Lane 3- pET-28A.*flaB* plasmid digested with *BamH*I and *Sac*I

Lane 5- pGEX-5X-3 plasmid

Lane 6- pGEX-5X-3.*lig* uncut plasmid

Lane 7- pGEX-5X-3.*lig* digested with *Xho*I and *Sal*I

Lane M- 100 bp DNA ladder (Fermentas)

```
1      ATG ATT ATC AAT CAC AAC CTG AGT GCG GTG AAT GCT CAC CGT TCT    45
1       M   I   I   N   H   N   L   S   A   V   N   A   H   R   S    15

46     CTA AAG TTC AAC GAA CTT GCT GTG GAC AAG ACG ATG AAA GCT CTG    90
16      L   K   F   N   E   L   A   V   D   K   T   M   K   A   L    30

91     TCT TCC GGT ATG CGG ATT AAT TCT GCT GCG GAC GAC GCT TCC GGA   135
31      S   S   G   M   R   I   N   S   A   A   D   D   A   S   G    45

136    CTT GCA GTT TCC GAA AAG CTT AGA ACG CAA GTA AAC GGT TTG CGT   180
46      L   A   V   S   E   K   L   R   T   Q   V   N   G   L   R    60

181    CAA GCG GAA AGG AAT ACT GAA GAC GGA ATG AGT TTT ATT CAA ACT   225
61      Q   A   E   R   N   T   E   D   G   M   S   F   I   Q   T    75

226    GCC GAA GGA TTT CTG GAG CAG ACG TCT AAC ATC ATT CAA AGA ATC   270
76      A   E   G   F   L   E   Q   T   S   N   I   I   Q   R   I    90

271    CGG GTG CTC GCC ATC CAG ACT TCG AAT GGT ATC TAC AGT AAT GAA   315
91      R   V   L   A   I   Q   T   S   N   G   I   Y   S   N   E   105

316    GAT AGG CAG CTC GTG CAG GTG GAA GTA TCT GCG CTG GTG GAT GAA   360
106     D   R   Q   L   V   Q   V   E   V   S   A   L   V   D   E   120

361    GTC GAT CGA ATT GCT TCT CAG GCT GAA TTT AAT AAG TTC AAA CTT   405
121     V   D   R   I   A   S   Q   A   E   F   N   K   F   K   L   135

406    TTT GAA GGC CAA TTC GCT AGA GGT TCC AGG GTT GCA TCC ATG TGG   450
136     F   E   G   Q   F   A   R   G   S   R   V   A   S   M   W   150

451    TTT CAT ATG GGT CCA AAC CAA AAT CAG CGT GAA AGA TTT TAC ATA   495
151     F   H   M   G   P   N   Q   N   Q   R   E   R   F   Y   I   165

496    GGC ACG ATG ACT TCC AAG GCT CTG AAG CTT GTA AAA GCG GAC GGG   540
166     G   T   M   T   S   K   A   L   K   L   V   K   A   D   G   180

541    AGG CCG ATC GCG ATC TCT TCT CCG GGA GAG GCT AAC GAC GTG ATC   585
181     R   P   I   A   I   S   S   P   G   E   A   N   D   V   I   195

586    GGT CTG GCT GAT GCT GCC CTT ACG AAG ATC ATG AAG CAG AGA GCG   630
196     G   L   A   D   A   A   L   T   K   I   M   K   Q   R   A   210

631    GAT ATG GGA GCT TAT TAT AAT AGG CTT GAA TAT ACC GCA AAG GGT   675
211     D   M   G   A   Y   Y   N   R   L   E   Y   T   A   K   G   225

676    CTG ATG GGT GCG TAT GAA AAT ATG CAG GCA TCT GAA TCT AGA ATT   720
226     L   M   G   A   Y   E   N   M   Q   A   S   E   S   R   I   240

721    CGA GAC GCC CAT TTG GCG GAA GAA GTT GTC TCG CTG ACC ACA AAA   765
241     R   D   A   H   L   A   E   E   V   V   S   L   T   T   K   255

766    CAA ATA CTT GTA CAG AGT GGT ACG GCA ATG TTG GCG CAG GCA AAT   810
256     Q   I   L   V   Q   S   G   T   A   M   L   A   Q   A   N   270

811    ATG AAA CCG AAT TCA GTT CTC AAG CTT CTG CAG CAC ATC TGA   852
271     M   K   P   N   S   V   L   K   L   L   Q   H   I   *
```

Figure 13 Nucleotide and amino acid sequence of full-length *flaB* gene.

```
1      AAG AAA ATA TTT TGT ATT TCG ATT TTC CTT TCG ATG TTT TTT CAA   45
1       K   K   I   F   C   I   S   I   F   L   S   M   F   F   Q   15

46     GGT TGT ATG TCT TGG CCA CTT TTA ACC GGT CTG GTA GGA TTA ACC   90
16      G   C   M   S   W   P   L   L   T   G   L   V   G   L   T   30

91     GCT GGT AAA AAA AGT AAT GGG CTG TCC TTT TTC CAC CTT CTG TTA   135
31      A   G   K   K   S   N   G   L   S   F   F   H   L   L   L   45

136    GGT AAC TCC AAT CCG ACT ATT ACA AGA ATC GAA CTC AGT TAT CAA   180
46      G   N   S   N   P   T   I   T   R   I   E   L   S   Y   Q   60

181    GAT TCT TCT ATC GCA AAC GGT ACC AGC ACA ACC CTA GGA GTT ACC   225
61      D   S   S   I   A   N   G   T   S   T   T   L   G   V   T   75

226    GCA ATC TTT GAT AAC GGA ACA AAT CAG AAT ATT ACG GAT TTG ACA   270
76      A   I   F   D   N   G   T   N   Q   N   I   T   D   L   T   90

271    TAC ATC GTC CCC GAT TCC CAA TCC GTT GTA ACA ATC GAA GGC AAC   315
91      Y   I   V   P   D   S   Q   S   V   V   T   I   E   G   N   105

316    AGA GTC AGA GGA ATC GCT TCT GGT TCT TCC ATT ATA AAA GCA GAA   360
106     R   V   R   G   I   A   S   G   S   S   I   I   K   A   E   120

361    TAC AAC GGC CTG TAC TCT GAA CAA AAA ATT ACA GTT ACA CCA GCC   405
121     Y   N   G   L   Y   S   E   Q   K   I   T   V   T   P   A   135

406    ATT CTT AAC TCA ATT CAA GTT ACG AGT TTA GAG TCA GGT ATA CTA   450
136     I   L   N   S   I   Q   V   T   S   L   E   S   G   I   L   150

451    CCT AAA GGT ACT AAT CGT CAA TTC TCA GCC ATC GGA ATC TTT TCG   495
151     P   K   G   T   N   R   Q   F   S   A   I   G   I   F   S   165

496    GAT GGT TCT CAT CAA GAT ATT TCC AAC GAT CCA TTG ATC GTT TGG   540
166     D   G   S   H   Q   D   I   S   N   D   P   L   I   V   W   180

541    TCC TCC AGT AAT CCT GAT TTG GTT CAA GTA GAT GAT TCA GGG TTG   585
181     S   S   S   N   P   D   L   V   Q   V   D   D   S   G   L   195
```

Figure 14 Nucleotide and amino acid sequence of conserved *lig* gene.

```
586   GCA TCA GGG ATC AAT TTA GGA ACG GCT CAN ATT CGT GCA TCC TTT   630
196    A   S   G   I   N   L   G   T   A   X   I   R   A   S   F   210

631   CAA TCA AAA CAA GGG GCT GAA GAA ATG ACC GTT GGA GAT GCT GTT   675
211    Q   S   K   Q   G   A   E   E   M   T   V   G   D   A   V   225

676   CTT TCT CAA ATC CAA GTA ACT TCA AAC AAT CTG AAT ATT CCT CTC   720
226    L   S   Q   I   Q   V   T   S   N   N   L   N   I   P   L   240

721   GGA AAA AAA CAA AAA CTA ACA GCT ACG GGA ATC TAC TCG GAT AAC   765
241    G   K   K   Q   K   L   T   A   T   G   I   Y   S   D   N   255

766   TCT AAC AGG GAT ATT TCC TCT TCT GTT ATC TGG AAT TCT TCT AAT   810
256    S   N   R   D   I   S   S   S   V   I   W   N   S   S   N   270

811   TCC ACT ATC GCT AAT ATT CAA AAC AAC GGA ATA TTA GAA ACA GCT   855
271    S   T   I   A   N   I   Q   N   N   G   I   L   E   T   A   285

856   GAT ACT GGA ATC GTC ACT GTT TCT GCT TCT AGC GAG AAT ATA ATC   900
286    D   T   G   I   V   T   V   S   A   S   S   E   N   I   I   300

901   GGA TCC GTA AAA CTA ATC GTT ACT CCA GCA GCC TTA GTT TCT ATT   945
301    G   S   V   K   L   I   V   T   P   A   A   L   V   S   I   315

946   TCT GTT TCT CCG ACA AAT TCT ACA GTT GCA AAA GGT TTA CAA GAA   990
316    S   V   S   P   T   N   S   T   V   A   K   G   L   Q   E   330

991   AAC TTT AAA GCT ACA GGG ATC TTT ACA GAT AAT TCA AAC TCG GAT  1035
331    N   F   K   A   T   G   I   F   T   D   N   S   N   S   D   345

1036  ATT ACC GGC CAA GTT ACT TGG GAT TCT TCT AAT ACC GAT ATT TTC  1080
346    I   T   G   Q   V   T   W   D   S   S   N   T   D   I   F   360

1081  TCA ATT TCC AAT GCA AGT GAT AGC CAC GGG TTA GCT TCC ACA CTC  1125
361    S   I   S   N   A   S   D   S   H   G   L   A   S   T   L   375

1126  AAC CAA GGG AAT GTT AAA GTC ACC GCT TCC ATC GGT GGA ATA CAA  1170
376    N   Q   G   N   V   K   V   T   A   S   I   G   G   I   Q   390
```

Figure 14 (Continued)

```
1171  GGA TCC TCT GAT TTT ACA GTT ACA CAA GCT GCA TTG ACT TCG ATC   1215
391    G   S   S   D   F   T   V   T   Q   A   A   L   T   S   I   405

1216  GAA GTC TCT CCA ACT CGC ACT TCC ATT GCA AAA GGG CTA ACT CAA   1260
406    E   V   S   P   T   R   T   S   I   A   K   G   L   T   Q   420

1261  AAG TTT ACT GCG ATC GGG ATT TTT ACG GAT AAC TCT AAA AAG GAT   1305
421    K   F   T   A   I   G   I   F   T   D   N   S   K   K   D   435

1306  ATT ACG GAT CAA GTC ACT TGG AAC TCT TCT TCA GCA ATC GTA AGC   1350
436    I   T   D   Q   V   T   W   N   S   S   S   A   I   V   S   450

1351  GTG TCT AAC TTA GAC GAC AAT AAA GGT CTG GGA AAA GCT CAC GCT   1395
451    V   S   N   L   D   D   N   K   G   L   G   K   A   H   A   465

1396  GTT GGA GAC ACT ACG ATT ACC GCA ACC TTA GGA AAA GTT TCA GGT   1440
466    V   G   D   T   T   I   T   A   T   L   G   K   V   S   G   480

1441  AAC ACT TGG TTT ACT GTA GTT CCT GCG GTT CTC ACT TCT ATT CAA   1485
481    N   T   W   F   T   V   V   P   A   V   L   T   S   I   Q   495

1486  ATC AAT CCT GTA AAT CCT TCT CTT GCA AAA GGG TTA ACT CAA AAA   1530
496    I   N   P   V   N   P   S   L   A   K   G   L   T   Q   K   510

1531  TTT ACG GCT ACT GGG ATC TAC TCT GAC AAC TCT AAC AAG GAC ATA   1575
511    F   T   A   T   G   I   Y   S   D   N   S   N   K   D   I   525

1576  TCT TCC GCT GTT ACG TGG TTC TCA TCC GAT TCT TCA ATC GCG ACG   1620
526    S   S   A   V   T   W   F   S   S   D   S   S   I   A   T   540

1621  ATT TCA AAC GCC CAA AAA AAT CAA GGA AAC GCT TAC AGA GCA GCT   1665
541    I   S   N   A   Q   K   N   Q   G   N   A   Y   R   A   A   555

1666  ACA GGA ACA ACG GAT ATT AAA GCC ACA TTC GGA AAG GTA AGT AGT   1710
556    T   G   T   T   D   I   K   A   T   F   G   K   V   S   S   570

1711  CCG GTT TCT ACG TTA   1725
571    P   V   S   T   L
```

Figure 14 (Continued)

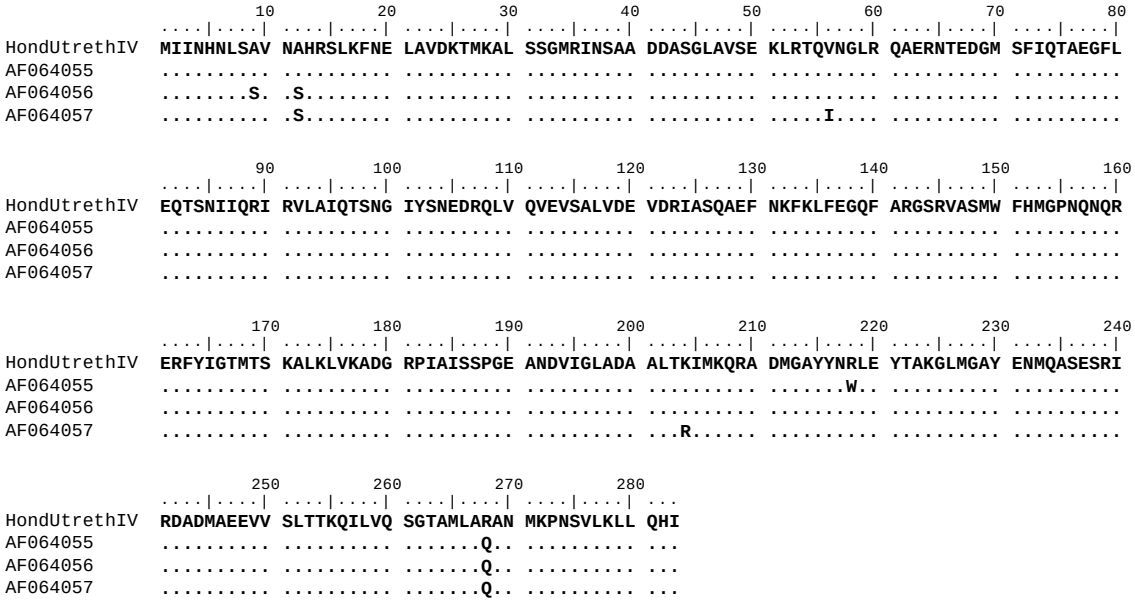


Figure 15 Amino acid sequence alignment of the *flaB* gene compared with the sequences accession no. AF064055, AF064056 and AF064057 from GenBank.

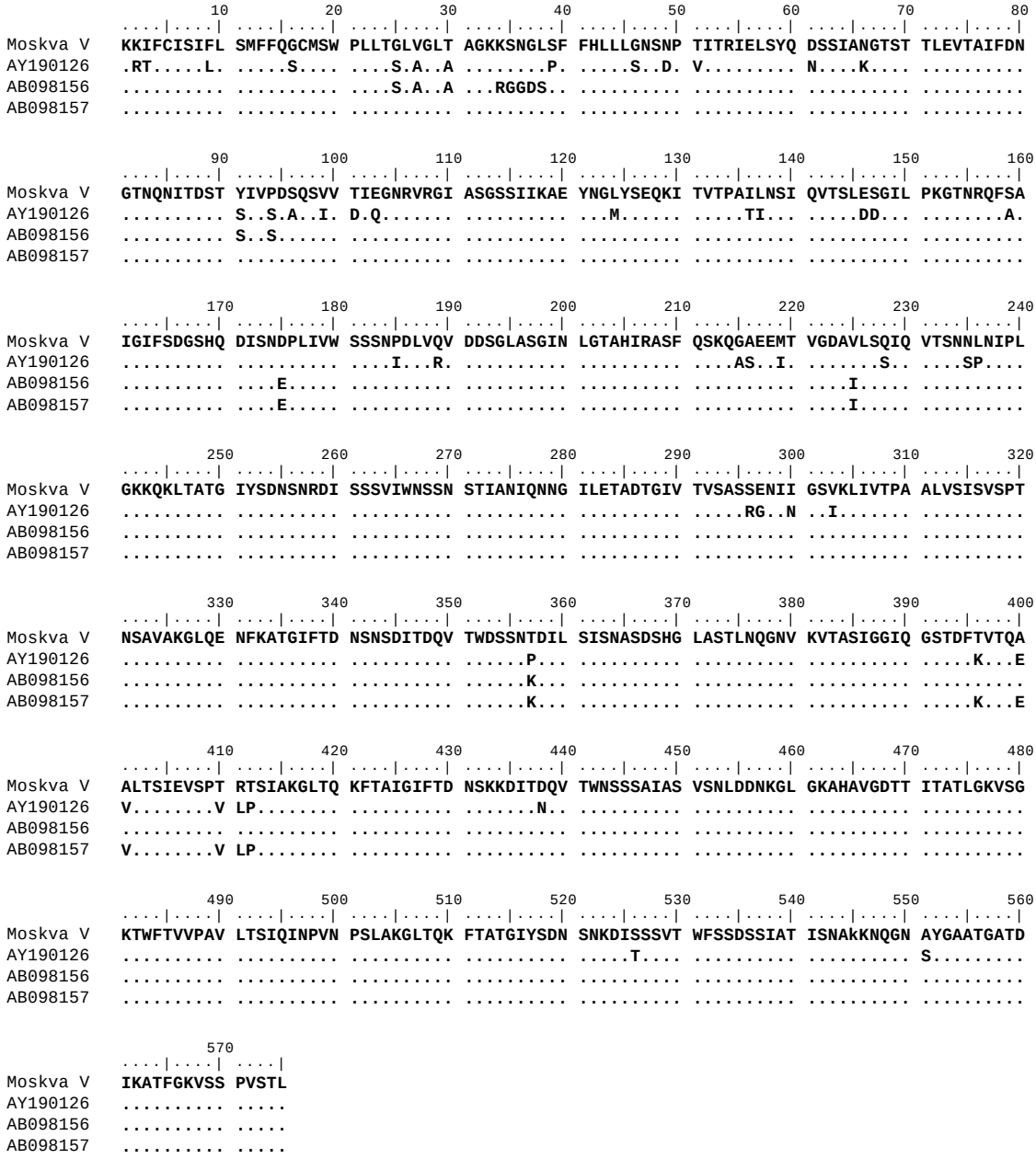


Figure 16 Amino acid sequence alignment of the *lig* gene compared with the sequences accession no. AY190126, AB098516 and AB098517 from GenBank.

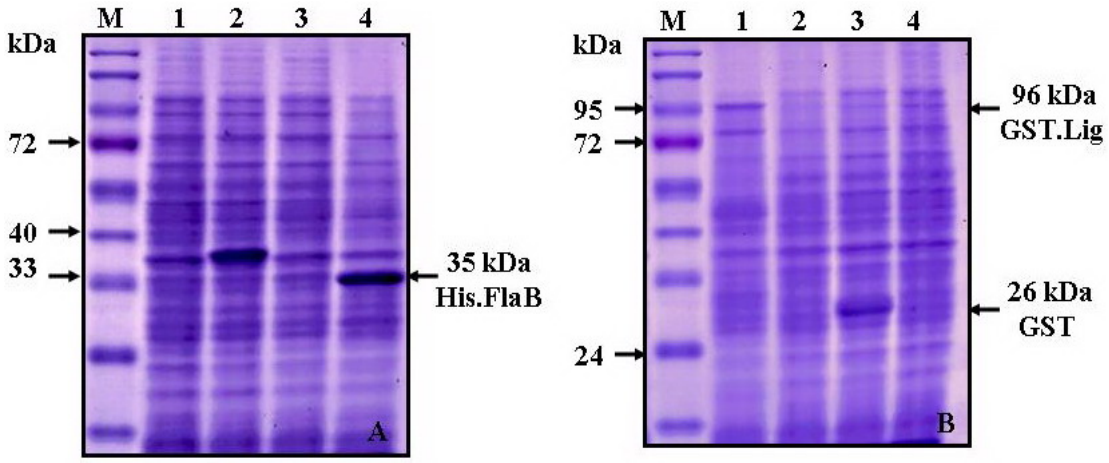


Figure 17 Kinetics of 35 kDa of His.FlaB and 96 kDa of GST.Lig protein expression following IPTG induction. The bacterial lysates were resolved on 12% SDA-PAGE at 90 volt for 2 hours and proteins were visualized by Coomassie blue staining. Figure 17A was SDS-PAGE analysis of recombinant His.FlaB protein about 35 kDa; lane M - prestained molecular weight standard, lane 1 - non-expression of His, lane 2 - expression His, lane 3 - non-expression of recombinant FlaB protein, lane 4 - expression of recombinant FlaB protein. Figure 17B was SDS-PAGE analysis of recombinant GST.Lig protein about 96 kDa; lane M - prestained molecular weight standard, lane 1 - expression of recombinant Lig protein, lane 2 - non-expression of recombinant Lig protein, lane 3 - expression GST (26 kDa), lane 4 - non-expression of GST. Arrow indicates the proteins at expected size.

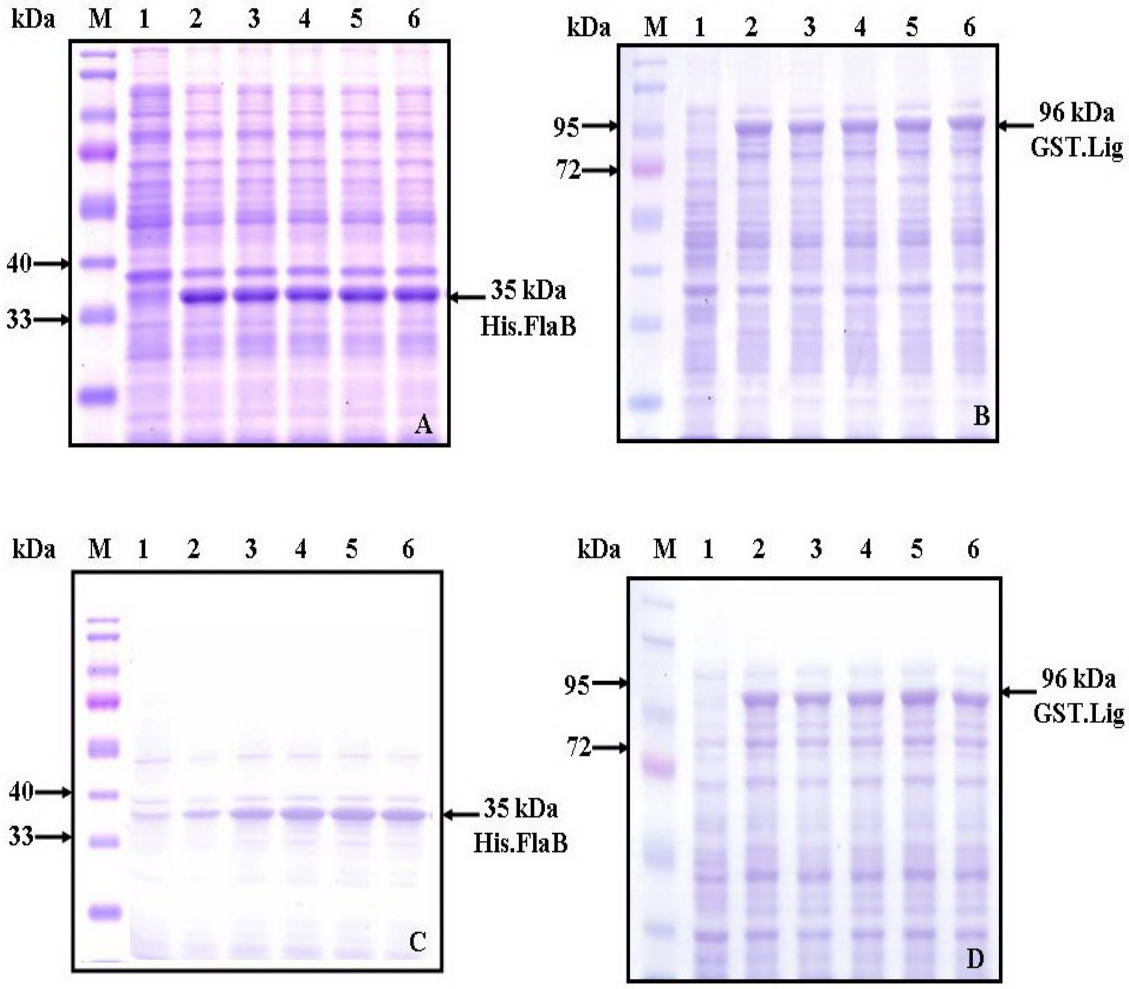


Figure 18 Optimization for IPTG concentration (A and B) and time duration (C and D) after induction of His.FlaB protein about 35 kDa (A and C) and GST.Lig protein about 96 kDa (B and D). Figure 18A and B; lane M - prestained molecular weight standard, lane1 - non-induced lysate cell, another lane represent vary of IPTG concentration for 0.1, 0.2, 0.5,1 and 2 mM. Figure 18C and D; lane M - prestained molecular weight standard, lane 1 - non-induced lysate cell, another lane represented time duration after induction for 1-5 hours.

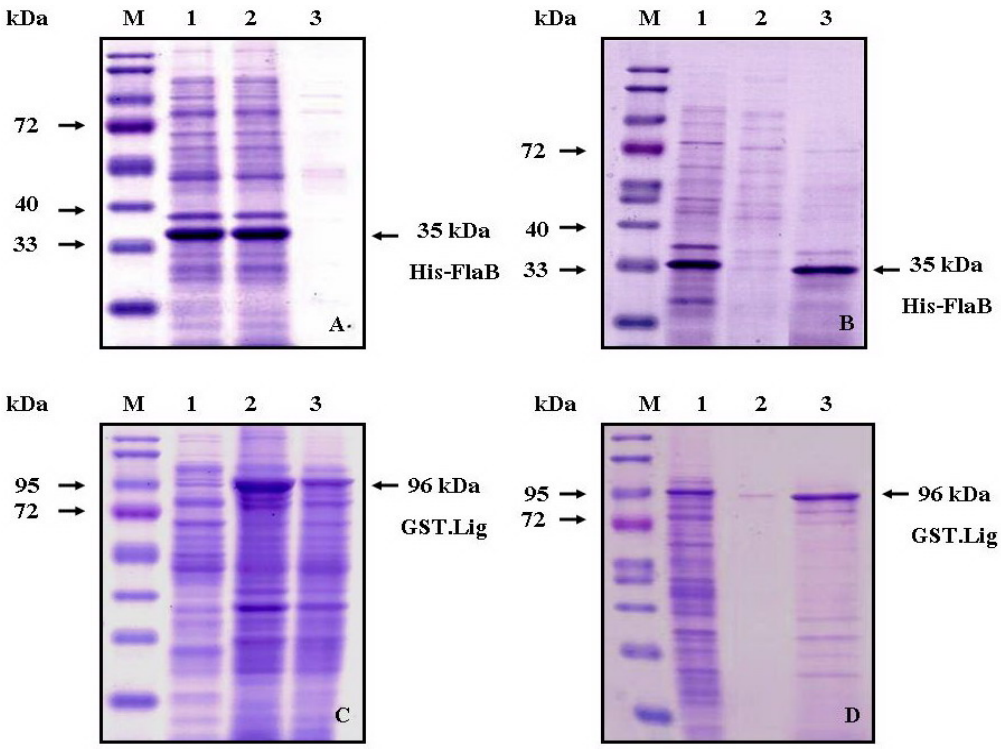


Figure 19 SDS-PAGE analysis of the purified protein of His.FlaB protein (A and B) and GST.Lig protein (C and D). Figure 19A. Characterization of fusion proteins; lane M - prestained molecular weight marker, lane 1 - positive induced cell of His.FlaB, lane 2 - the pellet of His.FlaB, lane 3 - the supernatant of His.FlaB. Figure 19B. Partial purification of target proteins; lane M - prestained molecular weight marker, lane 1 - the pellet of His.FlaB, lane 2 - the supernatant of His.FlaB, lane 3 - the supernatant of recombinant proteins after partial purification by 4M of urea and 1% of sarkosyl. Figure 19C. Characterization of fusion proteins; lane M - prestained molecular weight marker, lane 1 - the supernatant of GST.Lig, lane 2 - the pellet of GST.Lig, lane 3 - positive induced cell of GST.Lig. Figure 19D. Partial purification of target proteins; lane M - prestained molecular weight marker, lane 1 - the pellet of GST.Lig, lane 2 - the supernatant of GST.Lig, lane 3 - the supernatant of recombinant proteins after partial purification by 4M of urea and 1% of sarkosyl.

III. Production and evaluation of the utility of polyclonal antibodies against recombinant His.FlaB protein by immunofluorescent assay (IFA) to detect leptospires.

A. Preparation of recombinant protein antigens for immunization

To determine whether the protein encoded by the isolated *flaB* clone was produced, expression of the protein in *E. coli* was carried out as described. SDS-PAGE analysis of the bacterial taken at 4 hours post-IPTG addition clearly showed the presence of 35 kDa (Figure 17). FlaB was further purified by electroelution. The protein solution was fractioned in SDS-PAGE and was stained with Copper stain solution. A portion of gels that contained the recombinant protein was cut and solubilized in 0.1% SDS. These partial purified proteins (rFlaB protein) were investigated by comparing with non-purified positive recombinant protein. Out of expect, this protein located upper than non-purified protein positive recombinant protein. Suggestion, first, the partial purified will be the lower density than the non-purified positive recombinant protein. Second, the partial purified protein may be changed the conformation during the process. Then, the amino acid sequence in Figure 14 does not present any cysteine (C) or methionine residues that causes of refolding of protein. To archive the biological activity, protein must be refolded in an appropriate condition. Protein refolding requires intramolecular interactions. The simplest refolding method is direct dilution of the solubilized sample in buffer in presence of chemicals such as glycerol that aids refolding (Vallejo and Rinas, 2004). After staining the purified protein and compared with non-purified protein with Coomassie brilliant blue, a purified product represented by a strong single band at 35 kDa position was obtained (Figure 20A). Therefore, the cutting band will be only the expression protein fragment.

B. Production of polyclonal antibodies against rFlaB

A 5 ml pre-immunization blood sample was taken before 1st antigen injection and tested for anti-leptospiral antibodies in serum by MAT. There was no anti-leptospiral antibodies found. Rabbit was immunized with 100 μg/ml of rFlaB protein. After the immunization program was completed, the rabbit was bled by heart puncture and the serum was collected. Antiserum obtained from rabbit displayed very strong immunoreactivities and specificities with Western blot immunoassay, while the normal rabbit antiserum could not recognized with rFlaB (data not shown). When the His and His-FlaB partial purified recombinant proteins were transferred on the nitrocellulose membrane and probed with polyclonal anti-FlaB antibodies, the result showed specific signals for His.FlaB but no signals for His (Figure 20B). The antibodies titre was detected by dot blot analysis. Antibodies titre of hyperimmune serum against rFlaB protein at day 14, 28 and 42 were 1: 6,400, 1: 51,200 and 1: 102,400, respectively. Rabbit's hyperimmune serum was tested for leptospiral agglutination by MAT, the result showed that the rFlaB protein had non-agglutination with *Leptospira*. A 35 kDa genus specific non-agglutination antigen was related to periplasmic material in electron-microscopic immunogold staining (Trueba *et al*., 1992).

C. Determination of the specificity of polyclonal antibodies against FlaB recombinant proteins for detection of leptospires

In order to characterize the specificity of rabbit's hyperimmune serums, Western blot analysis was performed on protein extract isolated from *Leptospira* spp. Cellular protein fragments of *leptospira* were identified according to relative molecular weight after separation by reduced SDS-PAGE and immunoblot with rabbit hyperimmune serum against rFlaB and pooled sera of canine leptospirosis.

The rabbit hyperimmune sera against rFlaB which revealed by immunobloting indicated that rabbit hyperimmune serum could recognized the band appeared within 35 kDa in all serovar of both pathogenic and non-pathogenic strain

tested (Figure 21). Immunoanalysis in Figure 22 showed that canine leptospirosis sera recognized a consistent pattern of immunodominant antigen from leptospiral strains. The major bands of 33-36 kDa proteins were demonstrated in all pathogenic *Leptospira* but did not find in saprophytic one (*L. biflexa*) in this study. No reaction was found with normal canine serum (data not shown). The band appeared within 33-36 kDa region have been previous know to be flagella proteins (Trueba *et al*., 1992; Kelson *et al*., 1988; Chapman *et al*., 1988). The 35 kDa serovar cross-reactive flagella protein from *L. interrogans* serovar Pomona was identified as a flagellin, a class B polypeptide subunit (FlaB) of the periplasmic flagella (Lin *et al*., 1997). It is likely that several *Leptospira* protein antigens have been characterized as minor band on SDS-PAGE and these were clearly recognized when antiserum against a homologous strain was applied (Gitton *et al*., 1992, Zuerner *et al*., 1991). In addition, *Leptospira* species-associated antigens have been recognized and characterized, including flagella components (35 or 33-36 kDa bands). The leptospiral flagella proteins have shown to be antigenically unique and conserved antigens within the genus *Leptospira* (Doungchawee *et al*., 2007; Trueba *et al*., 1992; Julia *et al*., 1991; Kelson *et al*., 1988; Chapman *et al*., 1988).

D. Indirect immunofluorescence assay (IFA) of rabbit hyperimmune sera against rFlaB protein

The existence of anti-FlaB antibodies or the presence of antibodies direct against leptospira FlaB proteins was confirmed by IFA using hyperimmune serum raised against recombinant FlaB protein. All 24 reference strains tested were positive with any titre of rabbit hyperimmune sera (ranged from titre 1: 100 - 1: 6,400) while negative with normal rabbit serum (data not shown). There was a difference in the IFA titre as shown in Table 9. This difference may distribute to the fact that the presence of flagella epitope(s) were recognized vary to each strains tested. The bright green fluorescence of intact cell of *Leptospira* was indicated as positive reaction. The fluorescence of the rabbit serum was specific with *Leptospira* every titres used in this test. On the other hand, negative result (no fluorescence) was observed when other

bacteria were used as an antigen (Figure 23). Polyclonal antibodies against rFlaB by IFA permit simple determination leptospires antigens.

1. Nucleotide sequence accession number

The nucleotide sequence of the full-length of *flaB* gene from *L. interrogans* serovar Canicola strain Hond Utrecht IV has been assigned GenBank accession number EF 517919.

2. Sequence comparison of flagellin proteins

The flagellin protein sequences were used to construct the phylogenetic tree using algorithms implemented in Mega4 program version 4028. The amino acid sequence alignments were optimized manually using a multiple sequence alignment editor using BioEdit version 7.0.5.2. The alignment of flagellin proteins amino acids sequence were demonstrated in Figure 24. Sequences data were performed the similarity of phylogenetic tree pattern, identity and divergence percentage as showed in Figure 25. Protein sequences were analyzed and aligned with a Lasergene software package (DNASTAR, licensed to Kasetsart University). The leptospiral flagellin sequences (*L. interrogans*, *L. borgpetersenii* and *L. biflexa*) were aligned with the sequence of flagellin from several spirochetes and other bacteria. The aligned sequences include all published amino acid sequences of flagellins from GenBank data base. The *L. interrogans* flagellin shows a close relationship with the *L. borgpetersenii*, with a homology (identity) of 98.6% and moderately closes with the *L. biflexa*, with a homology of 64.5%. In comparison to other bacterial species, the *L. interrogans* has an overall 31.6 to 41.8% identity to the flagellin from two spirochetes (*Borrelia spp*. and *Treponema pallidum*), whereas the *Leptospira* flagellins have 59.4 to 64.5% identity to flagellin from *Serpulina hyodysenteriae*. These similarities suggest a common ancestry for this class of protein. In comparison of leptospiral flagellin regions with other bacterial species, they had an overall 17.3 to 38.9% identity (Figure 26).

An amino acid sequence comparison showed that the leptospiral flagellin protein had a considerable degree of overall homology with flagellins from other bacterial species. A similar sequence pattern was also found in other flagellin (Sakamoto *et al.*, 1992; Wallich *et al*., 1990). In previous publication, Lin *et al.*, 1997 demonstrated that the central region of *Leptospira* FlaB protein is highly variable in amino acid sequence and could be species specific. In contrast, the variable central region of a flagellin has not been related to any structural or functional roles in the filament. Based on the structural model proposed by Homma *et al*. (1987) and Namba *et al*. (1989), it may be possible that the variable central region exposed on the outer surface of the filament is involved in an infected host. To test this hypothesis, a truncated polypeptide corresponding to the variable central region of *Leptospira* FlaB may be expressed and used to assess the immune response.

In this study, the polyclonal antibodies against rFlaB protein were tested for immunoreactivity with several *Leptospira* serovars in immunoblotting and indirect immunofluorescent antibody technique. The immunoblot pattern indicated that the hyperimmune sera could recognized the band at 35 kDa region both pathogenic and non-pathogenic strains tested. The implication of the immunobloting data is that the flagellin is a highly cross reactive antigen with in leptospires. Several staining methods such as silver staining and immunostaining including immunofluorescent staining are able to be use to detect leptospires directly in histological section. All immunostaining methods require leptospiral specific antiserum to recognize leptospires in the specimens (Levett, 2001). Appassakiji *et al*. (1995) evaluated the IFA test for the diagnosis of human leptospirosis, they found the IFA test appeared to be sensitive and specific for initial diagnosis of leptospirosis. Amino acid sequences of flagellin were likely to prove useful to differentiate and identify leptospires. Leptospiral flagellin proteins were able to separate genus *Leptospira* from other bacteria.

The results of this study indicate that the IFA with polyclonal antibodies against rFlaB protein can be used as an additional test for detection of *Leptospira* antigens. Although, the limitation of this method is that it needs expensive laboratory

equipment and skilled expertise scientist, it gives some advantages over serologic of diagnosis. Interpretation of MAT titre is difficult, and in some confirmed cases of *Leptospira* infection, titres have not been detected (Levett, 2001). Demonstration of leptospires with the IFA provides definitive evidence of infection. The IFA can be performed within a few hours, whereas culture technique requires 6-8 weeks. The IFA may be useful as a screening test for specimens in laboratory that do not want to invest the time and expense of culturing large numbers of specimen.

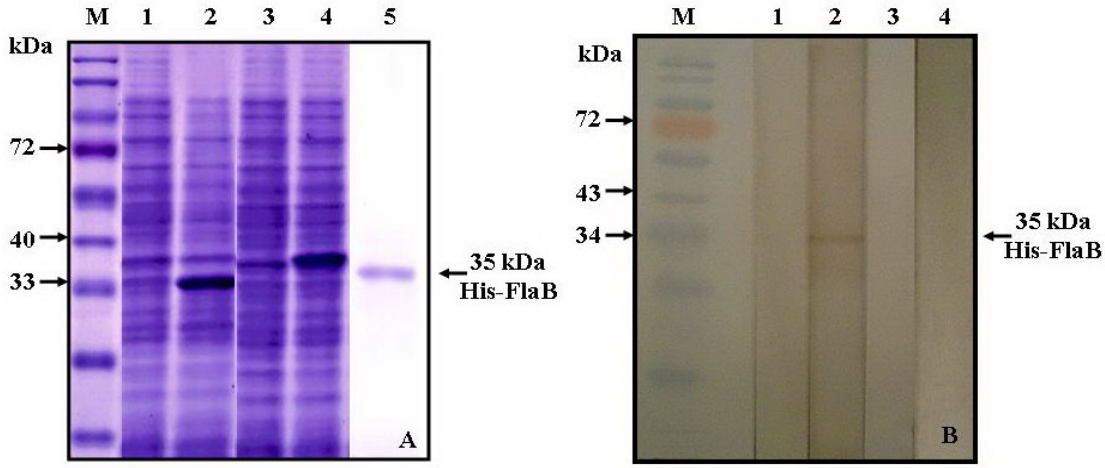


Figure 20 A. Purification of His.FlaB proteins. The molecular mass of the His.FlaB was SDS-PAGE analysis of recombinant His.FlaB protein about 35 kDa; lane M – prestained molecular weight standard, lane 1 - non-expression of recombinant FlaB protein, lane 2 - expression of recombinant FlaB protein, lane 3 - non-expression of His, lane 4 - expression His, lane 5 - gel purification for production of hyperimmune sera against rFlaB protein. B. Immunoblot panel of *Leptospira* probed with rabbit antiserum against recombinant FlaB protein. Western blot immunoassay revealed that the polyclonal antibodies production in this study recognized at 35 kDa. The locations of molecular markers (in kilodaltons); lane M – prestained molecular weight standard, lane 1 - non-expression of recombinant FlaB protein, lane 2 - expression of recombinant FlaB protein, lane 3 - non-expression of His, lane 4 - expression His.

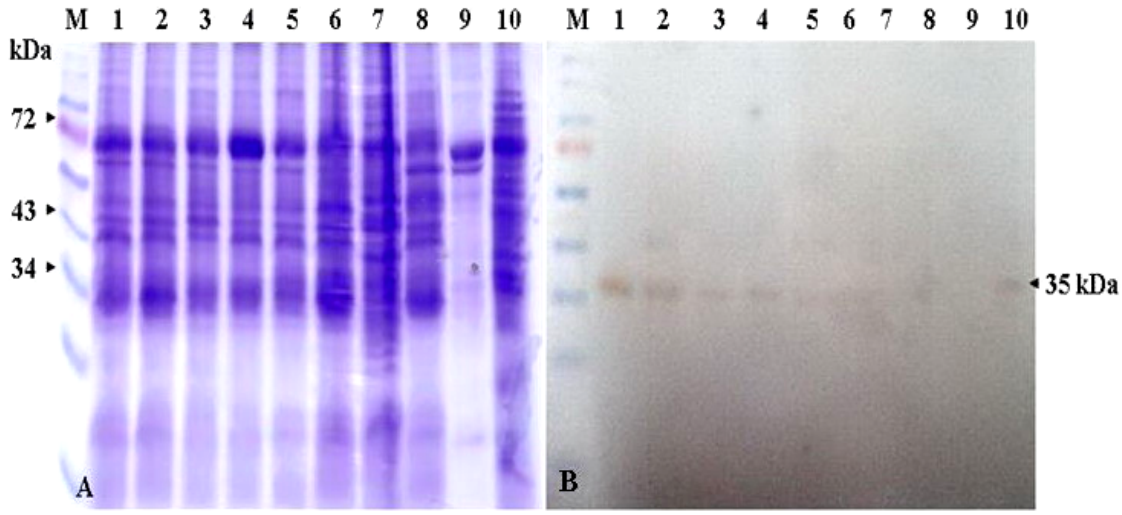


Figure 21 Immunobloting of rabbit hyperimmune sera against rFlaB protein.

A. Cellular protein fragments of leptospira were analysed by 12% SDS-PAGE. B. Immunobloting with rabbit hyperimmune sera against rFlaB protein. Lane M - prestained molecular weight marker, lane 1 - *L. interrogans* sv autumalis (Akiyami A), lane 2 - *L. interrogans* sv bataviae (Swart), lane 3 - *L. interrogans* sv bratislava (Jez Bratislava), lane 4 - *L. interrogans* sv canicola (Hond Utrecht IV), lane 5 - *L. kirschner* sv grippotyposa (Moskva V), lane 6 - *L. interrogans* sv icterohaemorrhagiae (RGA), lane 7 - *L. borgpetersenii* sv sejroe (M 84), lane 8 - *L. interrogans* sv pomona (Pomona), lane 9 - *L. biflexa* sv patoc (Patoc I), lane 10 - *L. borgpetersenii* sv tarassovi (Perepelitsin).

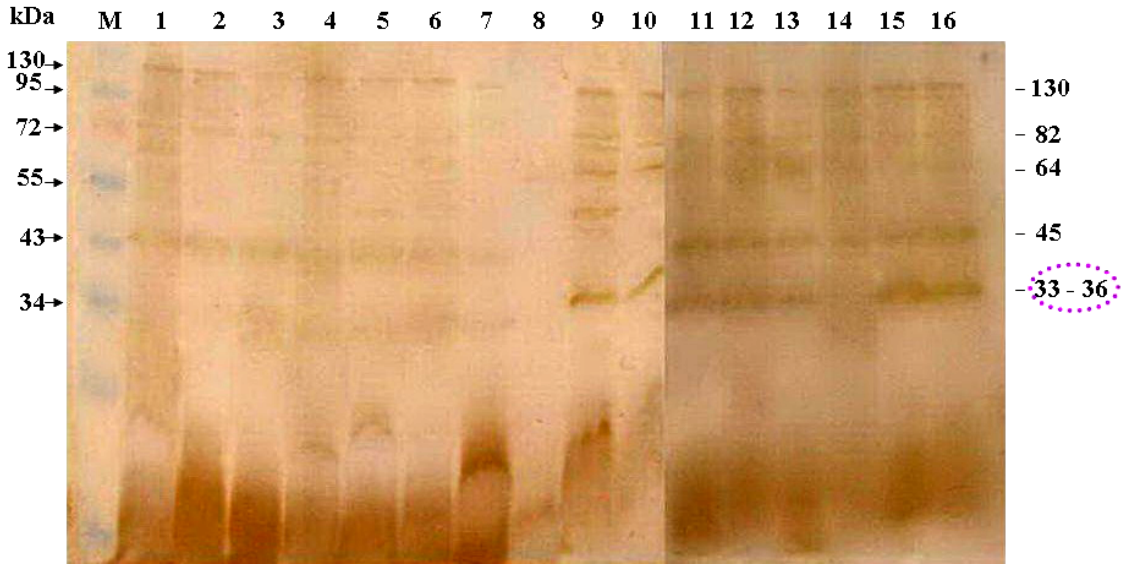


Figure 22 Immunoblot panel of *Leptospira* probed with pooled sera of canine leptospirosis. The locations of molecular markers (in kilodaltons) and *Leptospira* protein are indicated to the right and left, respectively.

Lane M - prestained molecular weight marker, lane 1 - *L. interrogans* sv autumalis (Akiyami A), lane 2 - *L. meyeri* sv ranarum (ICF), lane 3 - *L. interrogans* sv bratislava (Jez Bratislava), lane 4 - *L. interrogans* sv canicola (Hond Utrecht IV), lane 5 - *L. kirschner* sv grippotyposa (Moskva V), lane 6 - *L. interrogans* sv icterohaemorrhagiae (RGA), lane 7 - *L. interrogans* sv pyrogenes (Salinem), lane 8 - *L. biflexa* sv patoc (Patoc I), lane 9 - *L. borgpetersenii* sv sejroe (M84), lane 10 - *L. weillii* sv sarmin (Sarmin), lane 11 - *L. interrogans* sv pomona (Pomona), lane 12 - *L. weillii* sv manhao (L105), lane 13 - *L. interrogans* sv bataviae (Swart), lane 14 - *L. borgpetersenii* sv tarassovi (Perepelitsin), lane 15- *L. noguchii* sv lousiana (LSU1945), lane 16 - *L. santarosai* sv shermani (1342K).

Table 9 Result of polyclonal antibody against rFlaB protein using IFA to detect *Leptospira* spp.

Genomospecies	**Serovars**	**IFA titre of polyclonal antibodies ($1x10^{-2}$)**						
		1:1	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**
L.interrogans	autumalis	+	+	+	+	+	+	+
	bataviae	+	+	+	+	+	+	+
	bratislava	+	+	+	+	+	+	-
	canicola	+	+	+	+	+	+	+
	djasiman	+	+	+	+	+	+	-
	hebdomadis	+	+	+	+	+	+	-
	icterohaemor	+	+	+	+	+	+	-
	pomona	+	+	+	+	+	+	+
	pyrogenes	+	+	+	+	+	+	-
L. noguchii	louisiana	+	+	+	+	+	+	-
	Panama	+	+	+	+	+	+	-
L. borgpetersenii	ballum	+	+	+	+	+	+	-
	javanica	+	+	+	+	+	+	-
	Mini	+	+	+	+	+	+	-
	sejroe	+	+	+	+	+	+	-
	tarassovi	+	+	+	+	+	+	-
	shermani	+	+	+	+	+	+	-
L. santarosai	celledoni	+	+	+	+	+	+	-
L. weilii	manhao	+	+	+	+	+	+	-
	sarmin	+	+	+	+	+	+	+
L. kirschneri	cynopteri	+	+	+	+	+	+	-
	grippotyphosa	+	+	+	+	+	+	-
L. meyeri	ranarum	+	+	+	+	+	+	-
L. biflexa	patoc	+	+	+	+	+	+	-

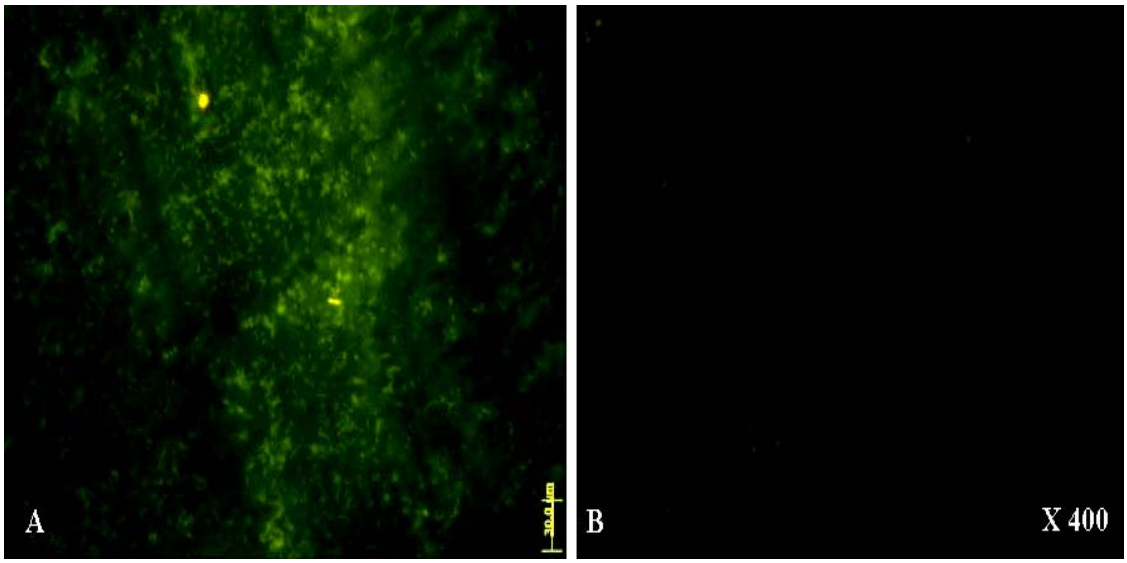


Figure 23 Indirect Immunofluorescence Assay (IFA). The fluorescence of the rabbit serum was a green fluorescence color and specific with *Leptospira* 1:100-1:6,400 titres. No fluorescence was observed at the other bacteria as shown in Figure A and B, respectively.

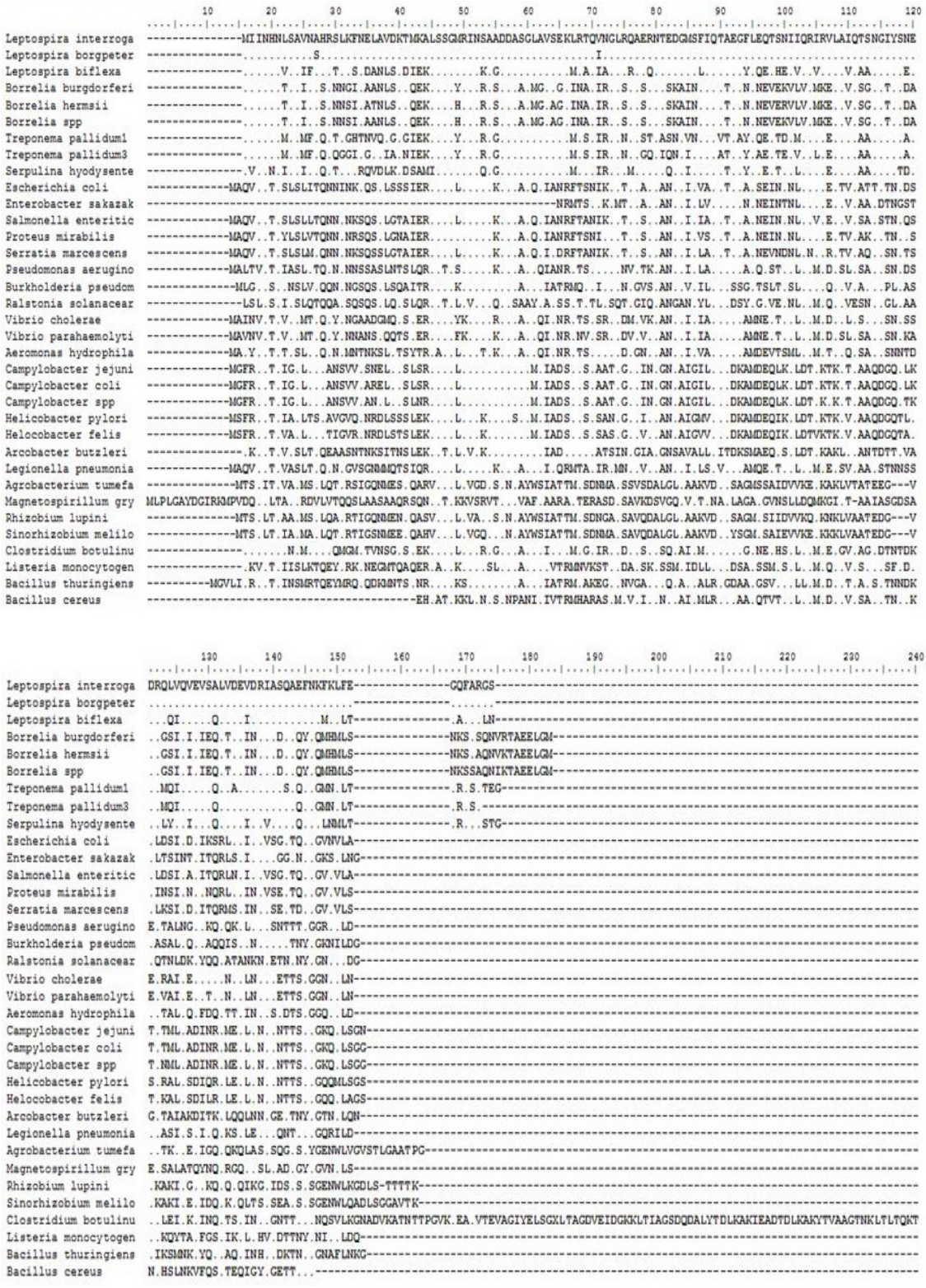


Figure 24 Amino acid sequence alignment of flagellin proteins. The amino acid sequences were obtained from GenBank as described in Figure 24.

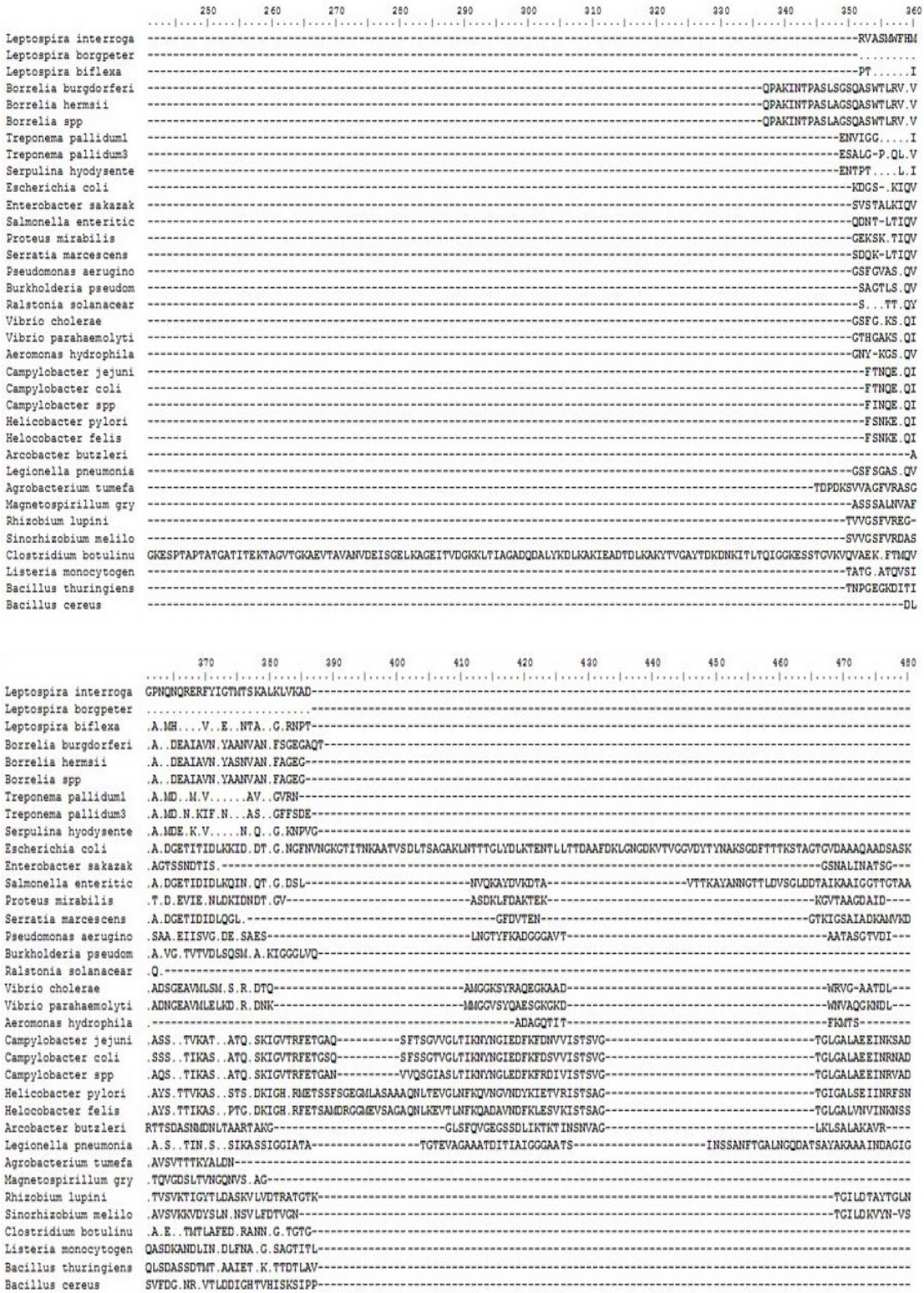


Figure 24 (Continued)

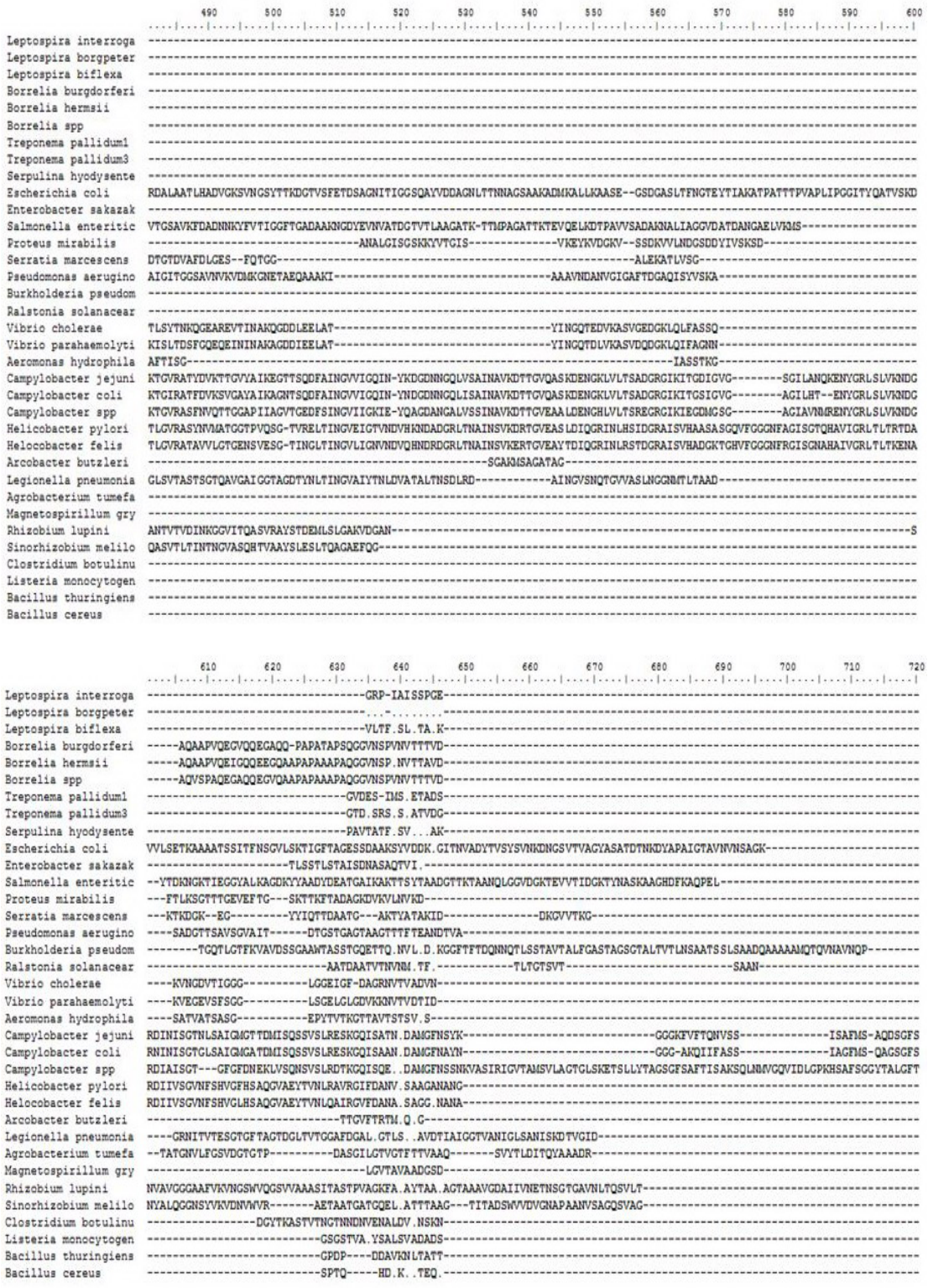


Figure 24 (Continued)

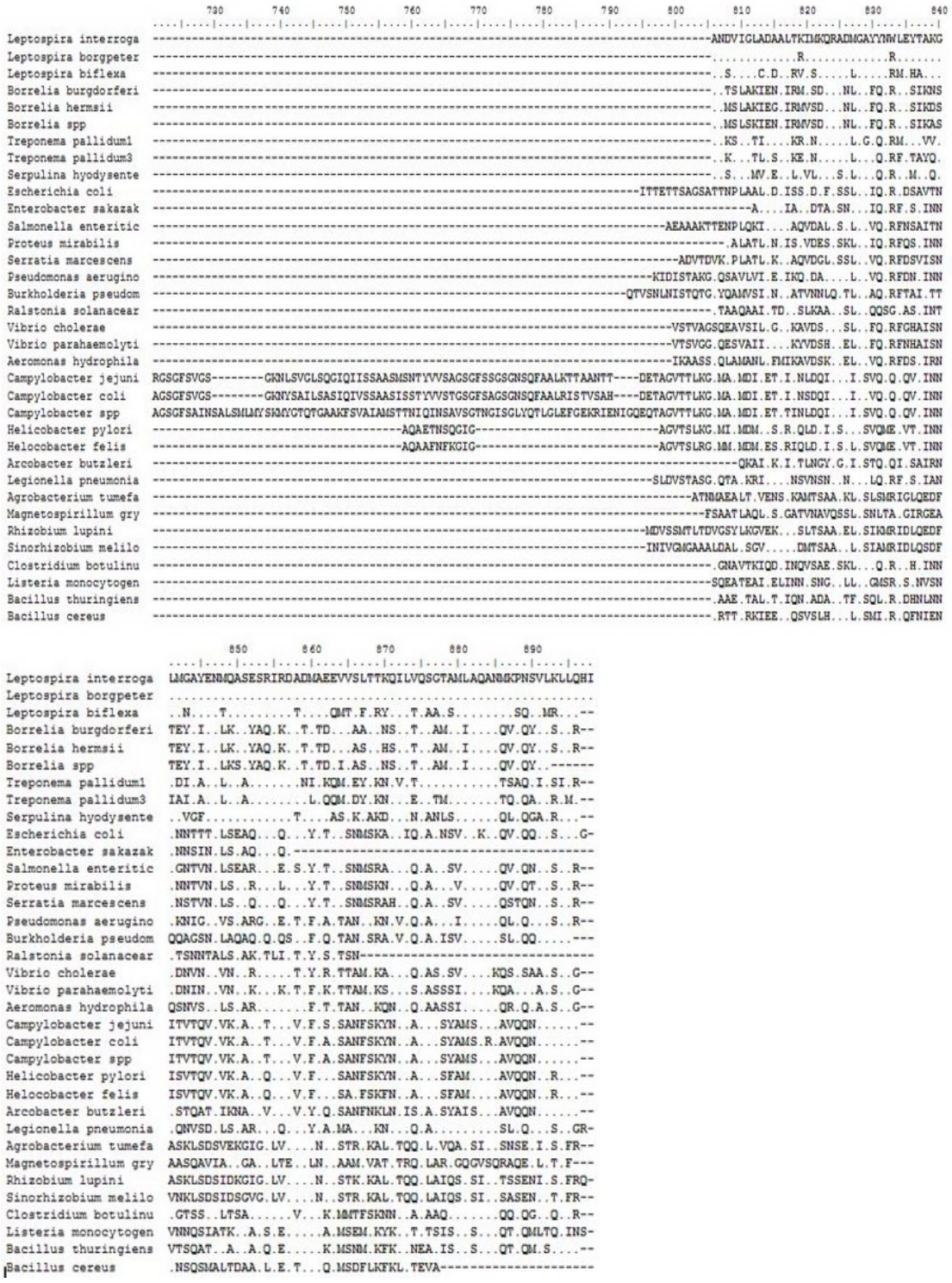


Figure 24 (Continued)

Figure 25 Dendrogram representing phylogenetic relationships of flagellin protein. This dendogram was constructed using the neighbour-joining method. The support each branch, as determined from 1000 bootstrap samples, is indicated by the value at each node (%). The scale bar represents the difference distance in amino acid sequence as determined by taking the sum of all of the horizontal lines connecting two species. Microorganisms studied from *Leptospira interogans* (EF517919, this work), *Leptospira borgpetersenii* (AF064057), *Leptospira biflexa* (AF320637), *Bacillus thuringiensis* (EF595777), *Bacillus cereus* (DQ400661), *Acrobacter butzleri* (EU056204), *Vibrio cholerae* (AF007122), *Vibrio parahaemolyticus* (AF069392), *Borrelia burgdorferi* (AE000783), *Borrelia hermsii* (DQ855535), *Borrelia* spp. (EF688579), *Treponema pallidum* FlaB1 (NC_000919), *Treponema pallidum* FlaB3 (NC_000919), *Campylobacter jejuni* (EF660527), *Campylobacter coli* (M35141), *Campylobacter* spp. (Y11762), *Clostridium botulinum* (DQ658239), *Magnetospirillum gryphiswaldense* (CR354387), *Aeromonas hydrophila* (DQ119104), *Helicobacter pylori* (AY714226), *Helocobacter felis* (Y11602), *Sinorhizobium meliloti* (L49337), *Rhizobium lupini* (AY007306), *Proteus mirabilis* (AF221596), *Serpulina hyodysenteriae* (L34686), *Agrobacterium tumefaciens* (U95165), *Enterobacter sakazakii* (EF442035), *Ralstonia solanacearum* (AY192727), *Pseudomonas aeruginosa* (L81147), *Burkholderia pseudomallei* (AF078153), *Serratia marcescens* (D32256), *Salmonella enteritica* (EU443199), *Listeria monocytogenes* (EU119270), *Escherichia coli* (AB334575), *Legionella pneumoniae* (EU221242).

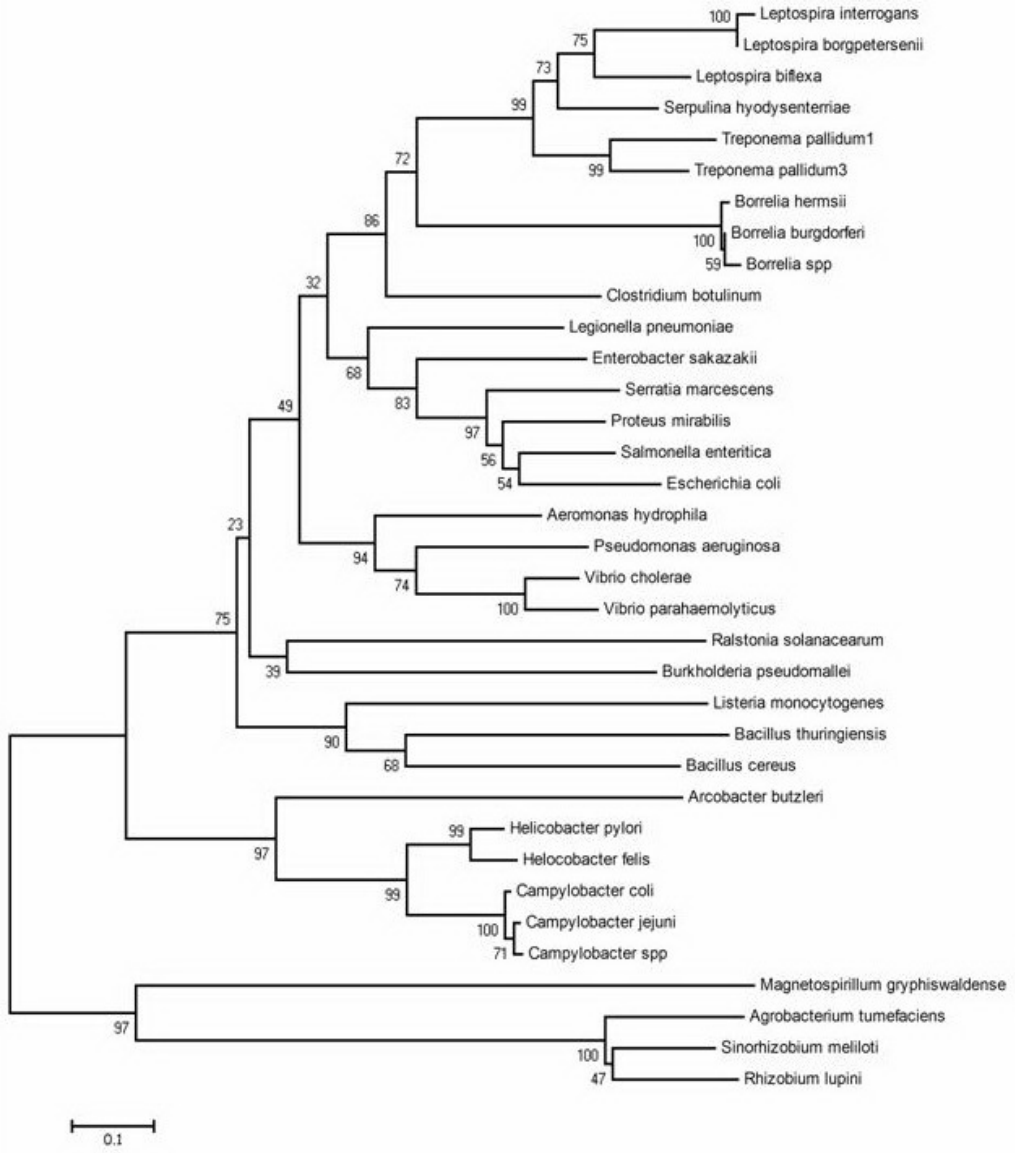

Leptospira interrogans
Leptospira borgpetersenii
Leptospira biflexa
Serpulina hyodysenteriae
Treponema pallidum1
Treponema pallidum3
Borrelia hermsii
Borrelia burgdorferi
Borrelia spp
Clostridium botulinum
Legionella pneumoniae
Enterobacter sakazakii
Serratia marcescens
Proteus mirabilis
Salmonella enteritica
Escherichia coli
Aeromonas hydrophila
Pseudomonas aeruginosa
Vibrio cholerae
Vibrio parahaemolyticus
Ralstonia solanacearum
Burkholderia pseudomallei
Listeria monocytogenes
Bacillus thuringiensis
Bacillus cereus
Arcobacter butzleri
Helicobacter pylori
Helocobacter felis
Campylobacter coli
Campylobacter jejuni
Campylobacter spp
Magnetospirillum gryphiswaldense
Agrobacterium tumefaciens
Sinorhizobium meliloti
Rhizobium lupini
0.1

Figure 26 The identity and percentage of amino acid sequence of flagellin proteins[a]

[a]Flagellin proteins from thirty-four bacterial strains used in this study as mentioned in Figure 25

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Leptospira interogans	1	100																																		
Leptospira borgpetersenii	2	98.6	100																																	
Leptospira biflexa	3	64.5	64.9	100																																
Aeromonas hydrophila	4	35.5	34.6	35.8	100																															
Agrobacterium tumefaciens	5	18.4	18.4	20.2	22.6	100																														
Acrobacter butzleri	6	26.1	25.8	26.6	31.2	19.9	100																													
Bacillus cereus	7	27.9	28.3	31.1	32.0	15.5	23.7	100																												
Bacillus thuringiensis	8	31.8	32.2	31.6	34.4	18.8	29.5	38.4	100																											
Borrelia burgdorferi	9	39.6	39.8	41.8	33.9	18.1	25.7	27.9	29.9	100																										
Borrelia hermsii	10	38.9	39.2	41.1	33.2	18.1	25.7	27.4	29.9	90.4	100																									
Borrelia spp.	11	37.1	37.5	39.7	31.9	16.9	25.1	27.4	28.8	90.6	93.9	100																								
Burkholderia pseudomallei	12	34.3	34.6	34.4	41.5	21.6	29.6	28.3	35.8	28.3	28.1	29.1	100																							
Campylobacter coli	13	34.6	34.3	30.5	36.2	19.7	41.7	26.9	31.2	28.6	28.1	27.3	27.5	100																						
Campylobacter jejuni	14	33.9	33.6	30.1	37.9	20.9	42.7	26.9	32.3	26.8	27.8	27.9	29.3	87.4	100																					
Campylobacter spp.	15	35.3	35.0	31.6	35.2	19.7	41.0	27.9	31.9	27.4	26.6	26.4	28.5	71.0	72.0	100																				
Clostridium botulinum	16	38.9	39.2	44.0	40.2	20.0	30.9	37.9	36.1	38.7	38.9	38.5	29.3	25.9	25.7	25.3	100																			
Enterobacter sakazakii	17	32.8	32.3	33.3	41.3	16.9	25.4	28.0	28.6	34.4	31.7	31.7	31.2	30.7	29.6	28.0	39.2	100																		
Escherichia coli	18	31.8	32.5	34.8	44.2	19.7	30.0	28.8	37.5	31.8	32.0	30.3	37.7	22.7	21.9	22.7	28.1	50.3	100																	
Helicobacter coli	19	31.8	32.2	33.3	36.5	19.4	39.7	25.1	28.5	27.4	28.1	27.9	27.0	52.3	51.9	51.2	27.3	31.2	25.1	100																
Helicobacter pylori	20	31.4	31.8	31.6	34.6	19.7	41.0	25.6	28.8	25.9	26.3	26.4	27.2	53.5	53.5	53.5	25.9	32.3	25.3	80.5	100															
Legionella pneumonia	21	38.9	39.2	41.1	51.2	24.4	32.2	35.6	36.1	33.6	33.2	32.7	40.3	29.5	28.6	30.3	32.0	49.7	33.5	32.0	31.8	100														
Listeria monocytogenes	22	29.0	29.3	33.0	35.2	19.2	26.5	33.8	42.5	30.7	31.0	29.6	33.8	27.9	27.2	29.3	33.1	27.0	33.8	25.4	25.4	36.2	100													
Magnetospirillum gryphiswaldense	23	17.3	17.3	18.1	18.0	22.3	18.4	18.3	16.3	17.3	19.1	18.7	22.6	18.7	17.0	18.0	14.5	19.0	18.7	17.3	17.0	19.4	15.2	100												
Proteus mirabilis	24	35.3	35.7	34.4	45.2	19.4	27.7	30.1	35.4	33.6	32.9	32.1	37.1	29.2	28.9	29.4	35.7	49.2	55.6	29.2	28.6	42.5	32.8	17.7	100											
Pseudomonas aeruginosa	25	37.5	37.5	37.2	53.8	21.6	29.6	34.7	35.1	30.4	29.6	29.1	36.9	34.1	33.1	31.8	32.8	40.7	37.0	31.3	32.0	46.8	37.6	18.7	41.4	100										
Ralstonia solanacearum	26	27.9	27.9	25.4	35.8	17.9	31.2	27.4	31.2	27.1	27.1	25.4	37.5	30.4	30.8	29.6	27.1	29.6	34.2	27.1	29.2	35.4	30.8	20.8	34.8	39.6	100									
Rhizobium lupini	27	18.7	18.7	20.9	21.9	58.1	22.5	15.1	18.8	19.3	20.1	19.7	21.7	18.2	16.8	16.8	17.8	15.9	18.0	20.0	19.7	21.2	17.4	22.6	20.4	19.6	17.1	100								
Salmonella enteritica	28	32.2	32.9	36.2	45.2	20.6	29.0	32.0	33.3	32.4	33.8	32.7	37.7	24.5	24.1	24.3	30.8	48.7	46.8	24.7	23.3	36.4	34.1	17.7	55.9	39.3	33.8	17.0	100							
Serpulina hyodysenteriae	29	59.4	59.4	64.5	37.2	19.3	27.2	28.8	30.6	39.3	38.6	39.0	34.5	30.0	30.0	30.0	43.1	34.4	36.2	32.8	32.4	39.3	29.6	17.0	37.9	36.9	27.1	19.7	37.2	100						
Serratia marcescens	30	31.8	32.2	35.1	46.8	18.8	29.3	29.2	34.4	33.0	32.0	30.9	39.1	29.3	29.3	30.2	35.1	46.6	56.6	30.5	29.0	42.8	32.4	17.0	56.9	39.4	33.3	20.1	62.1	34.1	100					
Sinorhizobium meliloti	31	19.1	19.1	21.3	20.3	59.4	20.5	14.6	20.1	17.6	18.3	16.1	20.4	17.2	16.5	18.7	18.7	18.5	20.5	20.0	20.0	21.8	20.2	22.6	19.6	18.6	17.5	57.2	19.2	21.4	19.0	100				
Treponema pallidum FlaB1	32	53.4	54.1	60.6	36.4	21.0	29.4	26.5	28.0	41.6	41.3	40.2	34.6	29.4	29.4	29.4	42.0	35.4	32.9	31.1	30.1	43.4	31.8	18.4	36.0	37.1	25.0	21.3	33.9	57.3	34.3	19.6	100			
Treponema pallidum FlaB3	33	54.8	55.1	60.3	35.4	17.9	29.8	26.9	30.2	42.5	40.7	40.7	35.8	31.2	31.2	31.2	43.2	36.0	36.8	33.3	31.9	43.5	30.5	19.8	37.2	37.5	25.4	20.4	35.1	55.8	35.1	19.3	68.8	100		
Vibrio cholerae	34	38.2	37.5	39.4	49.5	20.3	26.7	32.4	34.0	32.1	30.8	30.6	33.8	31.1	31.9	30.9	33.0	37.6	35.4	31.4	30.3	42.6	36.2	19.4	38.4	44.9	32.5	18.6	36.7	39.0	38.5	14.9	37.4	39.6	100	
Vibrio parahaemolyticus	35	37.5	37.5	38.7	49.5	19.1	28.0	32.9	33.0	31.0	31.1	30.0	32.3	29.4	30.7	30.2	32.3	36.0	34.1	31.0	29.4	40.7	35.5	16.6	36.2	44.2	32.1	17.7	37.8	37.6	37.1	15.9	36.0	37.5	71.5	100

IV. Development and evaluation of the utility of the recombinant proteins as antigen in an indirect enzyme-linked immunosorbance assay for the detection of antibodies against *Leptospira*.

A. Microscopic agglutination test

MAT is the reference method for serological diagnosis of leptospirosis. The test is performed by mixing serial dilutions of patient's serum while live leptospires and observed 50% agglutination of leptospires under dark-field microscope as the end point of test. The highest dilution of serum which gives the end point is reported as "MAT titre" of the serum sample (Cole *et al*., 1973)

WHO diagnosis criteria of leptospiral infection were used in this study. In case where paired sera can be obtained, a four-fold increase in serum titre was considered current infection. But if a four-fold titre was not achieved, the clinically compatible symptoms must be present to suggest a current infection. For screening of leptospirosis, a 1 : 50 dilution of any serum which gave positive MAT test indicates the likelihood for the presence of leptospiral antibodies. The conformation is done by determining the MAT titre of that serum. The serum with MAT titre of $\geq$ 1 : 100 is positive for leptospiral antibodies. Any single serum with MAT titre of $\geq$ 1 : 400 indicates only recent contact with leptospires which may or may not be the cause of this current illness, therefore only presumptive diagnosis of leptospirosis can be made. Hence, a four-fold rising titre of paired sera is required to confirm the diagnosis of leptospirosis (Faine *et al*., 1999).

The result of MAT for canine serum samples are presented in Table 10. A titre equal to or higher than 1:100 against one or different serovars was considered positive. In most of seropositive cases, multiple titres against different serovars were detected. In seropositive dogs the predominated titres against pathogenic *Leptospira* were serovars; Tarassovi, Icterohaemorrhagiae, Sarmin, Canicola, Ranarum, Bataviae, Shermani, Sejroe and a non-pathogenic strain (Patoc I). Other serovars were almost negative.

The results of the MAT on mice inoculated with leptospires are summarized in Table 11. All mice survived for days 42 after IP inoculation of live lepptosipires. Most animals produced a significant MAT titre against each serovar. No cross-reaction was observed within these mice sera by using MAT. After detection, agglutination titre rose and most animal had maximum titre at $\geq$ 1:6,400 after 42 day inoculation. The highest mice sera titres were demonstrated in sera against *L. biflexa* serovar Patoc I whereas the serological response with one of serova Canicola and one of serovar Pomona were lower than titre in the other serovars. Mice produced wide ranges of antibodies titres, could be due to individuals each ICR mice had the difference immune responses to leptospires antigens.

B. Immunoblot analysis for detection of anti-Leptospiral antibodies

In order to confirm the antigenicity of the recombinant protein, partial purified of GST.Lig and GST (control antigen), His.FlaB and His (control antigen) proteins at the same molar ratio were separated on 12% SDS-PAGE. The immunoblot assay was performed with canine sera. The band with apparent molecular weight about 96 kDa was demonstrated as Lig protein. Figure 27 shows the representative result of immunoblot analysis; three MAT-positive canine sera reacted with the GST.Lig fusion protein but not with GST and three MAT-negative serum did not reacted with both GST.Lig and GST protein. On the other hand, sera from canine did not recognize the previously protein, His.FlaB, which is a prominent component of leptospiral flagella protein.

Our goal for cloning and expression of *lig* is to use recombinant Lig protein as an antigen for detection of *Leptospira* antibodies, in additional to antisera specific to Lig. This experiment also tested this purified protein with MAT-positive (suspected leptospirosis cases) and MAT-negative canine sera to evaluate the immunological activity with recombinant Lig protein. Croda *et al*., 2007 performed an immunoblot analysis for detection leptospirosis sera from patients during urban outbreaks in Brazil. *L. interrogans* serovar Copenhageni was used as a template for cloning and expression of a recombinant fragment LigB. The immunoblot assay detected anti-

LigB IgM antibodies in sera from 92% (95% confidence interval, 85 to 96%) of patients during acute-phase leptospirosis. The assay had a sensitivity of 81% for sera from patients with less than 7 days of illness. Anti-LigB antibodies were found in sera from 57% of the patients who did not have detectable anti-whole-leptospira responses as detected by IgM enzyme-linked immunosorbent assay and microagglutination test. The specificities of the assay were 93 to 100% and 90 to 97% among sera from healthy individuals and patients with diseases that have clinical presentations that overlap with those of leptospirosis, respectively.

C. Determination of ELISA cutoff value

According to checkerboard titration results (data not shown), when IgG antibodies was detected, 1.5 ng/ well of recombinant Lig antigen and GST antigen (used as control), 1:40 dilution of serum and 1:40,000 dilution of Protein A-horseradish peroxidase (HRP) conjugate were used. This condition gave an appropriate value with at least background and non-specific result. The reactivity of anti-rFlaB of canine sera against antigen rFlaB-based ELISA were almost negative. The reactivity of anti-rFlaB was not determined by ELISA.

The frequency distribution of absorbance values for the rLig ELISA of 194 canine sera was shown in Table 12. The cutoff value of an indirect ELISA using rLig antigen was determined from the 91 MAT-negative canine sera. The mean OD_{650} value was calculated to be 0.065 with standard deviation (SD) of 0.028. The cutoff value for estimation of positive and negative samples was determined as the mean plus 4 SD (Ribotta *et al.*, 2002), and calculated to be 0.177. The absorbance values of ELISA were defined as the cutoff value to 0.177 of IgG antibodies response.

Mice serum samples immunized with live leptospires were also used to detect the antibodies against recombinant Lig and FlaB proteins. The results demonstrated that mice sera against serovars batavia and icterohaemorragiae gave the positive signal results with antigen rLig-based ELISA, whereas the other mice sera were negative. It was suggested *Leptospira* serovar batavia and icterohaemorragiae

could induced the antibodies against Lig with antigen obtained from *in vitro* culture. On the other hand the reactivity of anti-rFlaB of mice sera against antigen rFlaB-based ELISA were almost negative.

E. Evaluation of MAT and ELISA

A total of 194 canine serum samples with MAT various titre were used to evaluate the diagnostic potential of conserved region of recombinant Lig as an antigen in the ELISA assay. Considering of a positive cutoff value of 0.177, the sensitivity, specificity and accuracy of ELISA as relative to the MAT were calculated to be 84.5%, 76.9% and 80.9%, respectively (Table 13).

F. Nucleotide sequence accession number

The nucleotide sequence of the *lig* gene from *Leptospira kirschneri* serovar Grippotyphosa strains Moskva V has been assigned as GenBank accession number EF 517920.

As previous report in the part III, the result demonstrated that the application of polyclonal antibodies against rFlaB using IFA can be used for detection of leptospires. It had high specificity for discrimination between leptospires and other bacteria. In this study, Western blot and ELISA were utilized to evaluate the potential of rFlaB as a candidate antigen for diagnosis of leptospirosis. The results showed that rFlaB had poor potential to be used as an antigen to detect leptospiral antibodies, because it had no reaction with several MAT positive cases and it can not distinguish MAT positive from normal cases. Downregulation of antibodies against flagella protein might be an important mechanism for spirochetes to evade host immune response. The hypothesis with this phenomenon possibly evaded mechanism of these spirochetes due to the invasive potential. The ability of *L. interrogans* to cause such a rapid systemic infection after penetration of the skin suggests that it is a highly invasive pathogen (Barocchi *et al*., 2002). The pathogenic strain exhibited significantly faster translocation ability than the nonpathogenic strain which suggests

that this ability of *Leptospira* to rapidly translocate through the cells may be the distinguishing feature of its pathogenicity (Thomas and Higbie, 1990; Merien *et al*., 1997). Barocchi *et al*. (2002) proposed that the rapid translocation of mammalian cells by pathogenic leptospira is a mechanism designed to evade killing by host immune response. Consistent which these hypothesis, there are correlation between decrease of outer membrane proteins (OMPs) and pathogenicity in both *L. kirschneri* and *Borrelia burgdorferi* (Haake *et al*., 1991; Radolf *et al*., 1995). Since, the majority of the recombinant protein His.FlaB was presented in the inclusion bodies fraction. The inclusion bodies are aggregates of inactive protein and misfolded polypeptide (Carrió and Villaverde, 2002). The protein solubilization using concentrated chemical denaturant (typically urea or guanidinium chloride) is resulted to the increase of misfolded and denatured proteins (Rudolph and Lilie, 1996). Middlerberg (2002) demonstrated that protein solubilized in 6M guanidinium chloride or 7.5M urea showed no secondary structure. In this experiment, by using a combination of chemical reagents (4M urea and 1% sarkosyl) to purify rFlaB protein, it was not clear that the rFlaB protein was recovered in a homogeneous and antigenically active form. The purification protocols here were not encountered by the fact that rFlaB protein appeared to retain most of features of its native properties even after the multiple-step purification procedures. The result demonstrated that the rFlaB could not be used as an antigen to detect *Leptospira* antibodies in clinical specimens. This suggested that the purified rFlaB may be non-immunologically active and that the immunogenicity of the epitope(s) was altered by protein purification steps. Moreover, the role of protein FlaB in immune response to *Leptospira* is not yet unknown (Bughio *et al.,* 1999). It is possible to a apply molecular strategies for identification of FlaB regulatory proteins, a key step towards an understanding of the mechanism by which pathogenic *Leptospira* species control their response to the diverse in host and environment encountered during their life cycle.

Previous publication article demonstrated that Leptospires lost their virulence phenotype with sequential *in vitro* culture passage (Haake *et al*., 1991). Therefore expression of virulence factors may be downregulated during culture attenuation. In an earlier report, antisera to LigA of *L. interrogans* type kennewicki

failed to react with antigens obtained from *in vitro* cultured leptospires (Palaniappan *et al*., 2002). However, it was not examined whether the lack of antibody reactivity was due to the absence of detectable LigA expression in virulent strains or due to high-passage culture and virulence attenuation in the study strains. Matsunaga *et al*. (2003) performed the experiment to answer the issue. In experiment with low-passage isolates (*L. interrogans* strain Fiocruz L1-130), antibodies against the LigA and LigB reacted with molecular weight for LigA and LigB, respectively. Loss of LigA and LigB expression correlates with loss of virulence during culture attenuation since antibodies to Lig proteins did not react with a protein band in extracts of high-passage *L. kirschneri* strain RM 52. All the publications mentioned above reported with only 2 leptospiral strains tested for determination of the antisera against Lig obtained from *in vitro* cultured leptospire. Therefore, in this experiment, in order to examine Lig expression during culture attenuated in various *Leptospira* serovars, ICR and BALB/c mice were immunized with high-passage cultured of 21 leptospires serovars. The results demonstrated that mice sera against serovars Batavia and Icterohaemorragiae gave the antibodies to the recombinant conserved region of Lig by using ELISA. Reverse transcriptase PCR with primers of conserved region of *lig* was performed to confirm the expression of Lig protein in high-passage cultures. The extracted RNA from *L. interrogans* serovar Batavia strain Swart and serovar Icterohaemorragiae strain RGA were used as templates to amplify the *lig* region. Amplified products were not observed in control reactions lacking reverse transcriptase, indicating that extract were not contaminated with DNA. The *lig* transcript was not detected in high-passage in these 2 strains when compared the reaction by using primer of *flaB* gene as positive control (data not shown). Interestingly, the antibodies against recombinant Lig protein was found in mice sera induced with antigen obtained from *in vitro* cultured leptospires. This phenomenon due to these two serovars revealed the survival mechanisms. They could be multiplied into host cells which were recognized by host immune response. Immunized mice produced antibodies against Lig protein, which could be detected by using rLig-based ELISA. The mice sera was expressed the Lig protein after immunized with high-passage cultured leptospires. It was demonstrated that the expression of Lig protein is important for the bacterial survival, attachment

and invasive (Matsunaga *et al*., 2003; Srimanote *et al*., 2008). Therefore, the survival of leptospira in host may also play an important for leptospiral virulences.

In immunological methods, detection of IgM and IgG antibodies in ELISA is now widely used in the diagnosis of leptospirosis in specialized laboratories and can easily accommodate a large number of samples. The ELISA is often used as an additional test or alternative to the MAT. The success of ELISA is probably because the method provides highly useful information on class-specificity of the antibodies in clinical importances. IgM-antibodies are compatible with current or recent disease. The specific anti-leptospiral antibodies of the IgM class appeared approximately within 2-10 days after infection and decreased subsequently over the months, depending on the species and immunological competence of animal and the size of the antigen load (Faine *et al*., 1999). Specific IgG antibodies were observed somewhat later than IgM antibodies. IgG antibodies peak also a few weeks and drop off to low levels between 2 and 3 months and can persist long live in host animals (Ratnam, 1994). ELISA has been applied in a number of modifications. IgM-ELISA is more widely used. Several monoclonal antibodies (mAb) were produced for using in competitive or antibody-capture ELISA to detect leptospiral antibodies in bovine serum (Surujballi and Mallory, 2004; Surujballi and Elmgren, 2000; Yan *et al*., 1999). Another modified assays based on enzyme immunoassay principle was dot-ELISA using five sonicated serovars of *Leptospira* spp. for the detection of human IgM, IgG and IgA in the acute phase sera of patients with leptospirosis (Silva *et al.*, 1997). Numerous studies of sensitivity and specificity obtained for the ELISA indicated that this test would be a good alternative to the MAT for detection of leptospirosis (Silva *et al*., 1997; Petchclai *et al*., 1992; Terpstra *et al*., 1985; Adler *et al*., 1980).

The MAT is the reference method for diagnosis and detects antibodies at serovar levels and is used to compare with the other developing technique for evaluating their diagnosis sensitivity, specificity and accuracy. However, the MAT has limitation because it is time-consuming, using live organisms, requiring expertists, and interobserver variations (Plank *et al*., 2000; Cumberland *et al*., 1999; Winslow *et al*., 1997). Moreover, because a fourfold rise in titer between acute and

convalescent phase samples is necessary for serologic confirmation, the MAT is not useful for guiding clinical management early in the course of the patient's illness. (Cumberland *et al*., 1999).

In the present study, recombinant Lig antigen-based ELISA was developed and evaluated for the serodiagnosis of canine leptospirosis. The Lig protein is unique to pathogenic *Leptospira* spp. and that the *Leptospira* infected host produced antibodies to Lig (Matsunaga *et al*., 2003; Palaniappan *et al*., 2002). The recombinant conserved region of Lig (rLig) protein was used as the antigenic preparation. To evaluate the relative sensitivity and relative specificity of ELISA, a total 194 canine serum samples were used. The ELISA test in this study had a sensitivity of 84.5% relative to the MAT when canine serum sample with MAT of $\geq$ 100 to difference serovars were tested. Some MAT negative serum samples were found to be positive by ELISA. Of the 91 MAT-negative sera tested, 21 samples were reacted with recombinant Lig as an antigen in the ELISA. The specificity value of 76.9% that was examined with sera by MAT, may be an underestimation of the true specificity of the indirect ELISA. The results could be inconsistent for many reasons, including the presence of nonspecific components in the serum samples or the presence of antibodies of an isotype(s) not recognized by MAT. One explanation for this result could be that MAT-negative sera may contain non-agglutination leptospiral antibodies which were detected by ELISA but not by MAT. Since MAT can detect only agglutinating antibodies (Ribotta *et al*., 2002). The result demonstrated that, of the 91 MAT-negative sera tested, 21 samples were positive with recombinant Lig as an antigen in the ELISA. Several studies have demonstrated that the agglutinins produced are mainly IgM (Gowei Priya *et al*., 2003; Surujballi *et al*., 1997; Adler *et al*., 1980). Other studies have reported that very few animals and humans with leptospirosis produced IgG agglutinins examined by MAT. The ELISA test can measure IgM and IgG antibody levels in serum. Therefore, the non-agglutinating antibodies could be detected by the ELISA (Moris *et al*., 1974). Previous publication, Croda *et al*. (2007) performed Western blot analysis to detected IgM and IgG antibodies using the recombinant N-terminal Lig as an antigen and found that high proportion of leptospirosis patients had anti-Lig IgG antibodies during the acute phase

of the illness. Srimanote *et al*. (2008) used the C-terminal portion of recombinant LigA (cLigA) as a detect reagent by an indirect ELISA for detecting IgM and IgG in sera of leptospirosis patients. The diagnosis sensitivity was 100% in both the cLigA IgM and IgG and specificities IgM and IgG-ELISA were 98% and 100%, respectively (Srimanote *et al.*, 2008). In this study, the result on the application of the conserved region of recombinant protein Lig for the detection of anti-leptospirosis in canine sera conformed to previous report. Palaniappan *et al*. (2004) evaluated the diagnostic potential of conserved and variable regions of LigA and LigB (Con, VarA and VarB), a kinetic ELISA was performed using MAT-positive canine sera. The conserved regions of LigA and LigB (rCon) showed strong reactivity to MAT-positive canine. This region appeared to be specific for serodiagnosis of leptospiral infection and this antigen can apply for amplification of leptospiral infection despite vaccination (Palaniappan *et al*.,2004).

Commercially available vaccines containing Icterohaemorrhagiae and Canicola serovars are broadly used. In general, vaccination animals develop relatively low agglutinating antibody titres (100-400) to those serovars in the vaccine and these titres persist for one to three months after vaccination. Although, some animals develop high titres after vaccination (particularly those vaccinated several time a year), these high vaccination titres decrease with time and may persist for six months or more after vaccination. Introduction of new vaccines may also change the typical pattern of post-vaccination antibody titres (Scanziani *et al*., 2002; Bolin, 2003). The result of this experiment showed that 16 canine samples lacked in antibodies for recombinant Lig protein using ELISA. The MAT titres might be due to the present of non-specific reactivity or vaccination (8 samples were MAT positive with antibodies titre ranged from 100 to 800 to serovar Canicola and/or Icterohaemorrhagiae). It must also be considered that the antisera used in this study are defined as positive or negative for particular *Leptospira* serovar on the basis of MAT results alone. Due to the difficulty and expense associated with the culture of *Leptospira*, the results obtained from culture samples are not available. The further studies using sera from dogs with both leptospiral vaccination and natural infection are substantial to answer this question. Diagnosis of leptospirosis in animals depend

on a good clinical and vaccination history. Since the vaccinal status of these dogs was unknown, all titres against serovars Icterohaemorrhagiae and Canicola were considered positive in MAT-positive group. Coordination between the diagnostic laboratory and the veterinarian is required to make an accurate diagnosis. Some of techniques such as isolation of leptospires suffer from a lack of sensitivity and a common serological test based-ELISA prone to have specificity problems. Therefore, a combination of procedures allows maximum sensitivity and specificity in establishing the diagnosis (Pamela, 2008; Bolin, 2003; Levett, 2001; Faine *et al*., 1999).

In this study, the ELISA determined positive sera for canine leptospirosis caused by serovas Tarassovi, Icterohaemorrhagiae, Sarmin, Canicola, Bataviae and Ranarum. The reactivity with these serovars is of particular related as are emerging serovars in many parts of the world (Oni *et al*., 2007; Meeyam *et al*., 2006; Bolin, 2003; Scanziani *et al*., 2002).

The results of this experiment suggest that the development and evaluation of recombinant antigen-based ELISA for the detection of canine anti-leptospiral antibodies in this study is a simple, rapid, sensitive and specific test for screening canine sera. The assay involves a recombinant immunoglobulin-like protein of leptospira antigen whose expression is highly associated with pathogenic leptospiral infection. It is easily standardized and is suitable for the examination of a large number of sera It is also safe than MAT since it eliminates the handling of live leptospires. It uses an antigenic preparation that can be routinely prepared in large amounts. Thus, it is suitable for large scale serological examination, routine diagnostics, epidemiology surveys and follow-up investigations for outbreaks. Due to, the diagnosis of leptospirosis based on a single serum sample, the ELISA described here would be particularly useful in canine with full consideration of clinical picture and vaccination history of animals. Further studies are needed to use the appropriate samples which were determined as leptospirosis infection by using the other confirmation methods such as isolation of leptospira organism in order to establish the advantages and are also determined the limitations of the test for leptospirosis

detection. This is only a preliminary study, further evaluation of this antigen in diagnosis of leptospirosis is in progress in routine laboratory.

Table 10 MAT with 24 leptospiral serovars. A titre of ≥ 1:100 dilution of serum was considered positive.

Serovar	No. serum positive for titre					Total
	1:100	1:200	1:400	1:800	1:1,600	
autumnalis	1	0	0	0	0	1
ballum	1	0	0	0	0	1
bataviae	7	1	1	0	1	10
bratislava	2	3	0	0	0	5
canicola	14	1	0	0	0	15
celledoni	0	1	0	0	0	1
cynopteri	1	0	0	0	0	1
icterohaemorrhagiae	11	4	3	2	0	20
pyrogenes	1	1	0	0	0	2
ranarum	9	0	1	0	0	10
sarmin	16	0	0	0	0	16
sejroe	6	0	0	0	0	6
shermani	9	0	0	0	0	9
tarassovi	54	18	3	3	0	78
patoc	17	1	1	0	0	19
Neagative						93
Total						194

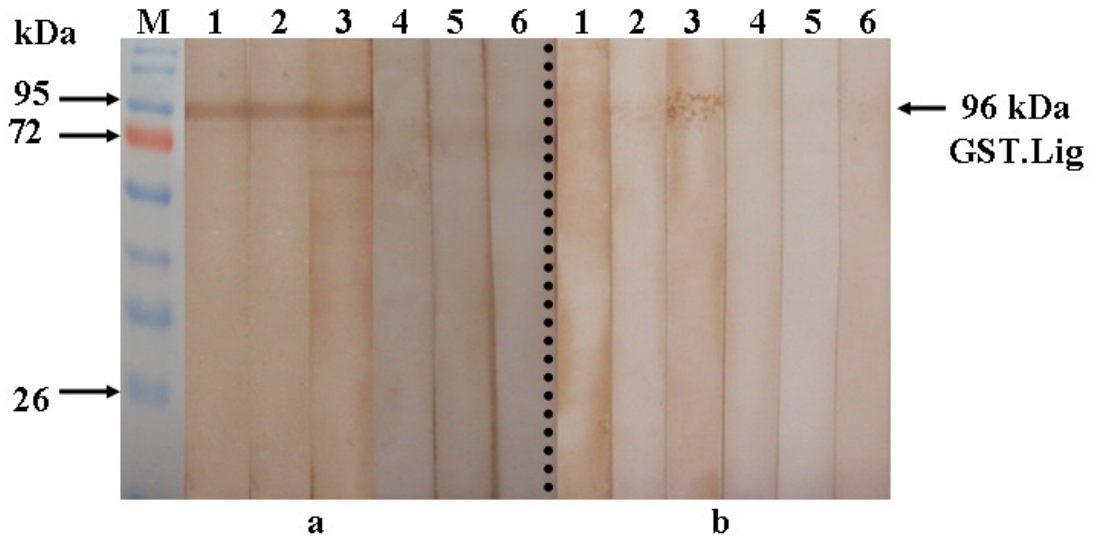


Figure 27 Anti-recombinant Lig antibodies in sera from canine leptospirosis. Membranes were prepared from SDS-PAGE of recombinant protein fragments of *L. kirschneri* serovar Grippotyphosa strain Moskva V Lig conserved region at expected size 96 kDa (a) and GST protein at 26 kDa as control (b) . The membranes were probed at 1: 40 dilution with MAT positive (lane 1 to 3) and MAT negative canine sera (lane 4 to 6) follow by 1: 1,000 dilution of HRP-conjugated protein A. Antibodies binding were visualized by exposure to stable DAB® peroxidase substrate. The relative mobility (in kDa) of molecular mass standards is shown on the left.

Table 11 Microscopic agglutination titre (MAT) and ELISA optical densities (OD) of 52 mice serum samples at 42 days after inoculation with *Leptospira* spp.

Serovar	Serum	MAT titre[a]	ELISA (OD 650)					
			Lig	GST	Δ[b]	FlaB	His	Δ
Autumna	1	1:1,600	0.526	0.521	0.005	0.559	0.577	-0.018
	2	1:3,200	0.426	0.412	0.014	0.434	0.712	-0.278
	3	1:1,600	0.346	0.238	0.108	0.251	0.279	-0.028
Bataviae	1	1:6,400	2.326	0.478	1.848+	0.3	0.525	-0.225
	2	1:6,400	2.002	0.359	1.643+	0.376	0.44	-0.064
	3	1:800	2.113	0.489	1.624+	0.866	0.945	-0.079
	4	1:6,400	2.251	0.536	1.715+	1.604	1.074	0.530
Bratisla	1	1:12,800	0.489	0.712	-0.223	0.502	0.606	-0.104
	2	1:12,800	0.91	0.900	0.010	0.973	0.719	0.254
	3	1:12,800	0.553	0.542	0.011	0.446	0.847	-0.401
	4[c]	1:25,600	0.373	0.238	0.135	0.341	0.53	-0.189
Canicola	1	1:200	0.297	0.186	0.111	0.326	0.427	-0.101
	2	1:12,800	0.344	0.232	0.112	0.374	0.585	-0.211
	3	1:3,200	0.243	0.247	-0.004	0.316	0.555	-0.239
	4	1:6,400	0.382	0.329	0.053	0.662	1.115	-0.453
Grippoty	1	1:3,200	0.329	0.312	0.017	0.317	0.644	-0.327
	2	1:6,400	0.487	0.477	0.010	0.282	0.574	-0.292
	3	1:1,600	0.268	0.253	0.015	0.221	0.388	-0.167
	4	1:3,200	0.571	0.510	0.061	0.395	0.526	-0.131
Icterohae	1	1:6,400	2.238	0.512	1.726+	0.229	0.435	-0.206
	2	1:12,800	2.287	0.324	1.963+	0.201	0.235	-0.034
	3[c]	1:6,400	2.305	0.368	1.937+	0.226	0.362	-0.136
PatocI	1	1:102,400	0.779	0.718	0.061	0.658	0.73	-0.072
	2	1:102,400	0.835	0.380	0.455	0.359	0.65	-0.291
	3	1:102,400	0.388	0.342	0.046	0.326	0.364	-0.038
	4	1:204,800	0.319	0.274	0.045	0.244	0.34	-0.096

[a], Titre expressed as the reciprocal of the titre to the inoculated serovar.

[b], The reactivity rate of control was deduced for analysis of samples. [c], BALB/c mice were immunized with these serovars. +, Indicates a positive ELISA OD.

Table 11 (Continued)

Serovar	Serum	MAT titre[a]	ELISA (OD 650)					
			Lig	GST	Δ[b]	FlaB	His	Δ
Pomona	1	1:51,200	0.239	0.201	0.038	0.143	0.209	-0.066
	2	1:800	0.499	0.495	0.004	0.466	0.45	0.016
	3	1:6,400	0.293	0.265	0.028	0.184	0.428	-0.244
	4	1:200	0.188	0.147	0.041	0.525	0.234	0.291
Sejroe	1	1:6,400	0.289	0.219	0.070	0.149	0.195	-0.046
	2	1:6,400	0.440	0.321	0.119	0.296	0.575	-0.279
	3	1:6,400	0.320	0.624	-0.304	0.243	0.467	-0.224
Tarassovi	1	1:6,400	0.881	0.228	0.653	0.222	0.283	-0.061
	2	1:12,800	1.085	0.736	0.349	0.441	0.827	-0.386
	3	1:12,800	0.909	0.496	0.413	0.353	0.61	-0.257
	4	1:6,400	0.367	0.299	0.068	0.277	0.371	-0.094
Celledoni	1	1:3200	0.599	0.697	-0.098	0.829	0.712	0.117
	2	1:3200	0.817	0.655	0.162	0.767	0.72	0.047
Cynopteri	1	1:6400	0.379	0.472	-0.093	0.493	0.726	-0.233
Djasiman	1	1:3200	0.386	0.346	0.04	0.283	0.306	-0.023
Hebdomadis	1	1:12,800	0.644	0.490	0.154	0.594	0.405	0.189
Javanica	1	1:25,600	0.848	0.731	0.117	0.976	0.789	0.187
	2	1:51,200	0.911	0.827	0.084	0.966	0.768	0.198
Louisiana	1	1:400	0.712	0.613	0.099	0.592	0.585	0.007
	2	1:6,400	0.401	0.427	-0.026	0.482	0.489	-0.007
Mini	1	1:3,200	0.342	0.435	-0.093	0.553	0.358	0.195
	2	1:1,600	0.598	0.707	-0.109	0.610	0.858	-0.248
Panama	1	1:3,200	0.561	0.438	0.123	0.452	0.532	-0.080
Ranarum	1	1:3,200	0.320	0.463	-0.143	0.549	0.446	0.103
Sarmin	1	1:3,200	0.318	0.411	-0.093	0.302	0.303	-0.001
Shermani	1	1:3,200	0.236	0.260	-0.024	0.244	0.247	-0.003

[a], Titre expressed as the reciprocal of the titre to the inoculated serovar.

[b], The reactivity rate of control was deduced for analysis of samples. [c], BALB/c mice were immunized with these serovars. +, Indicates a positive ELISA OD.

Table 12 Frequency distribution of the optical densities at 650 nm (OD_{650}) values in an indirect ELISA using recombinant Lig antigen of positive (n = 101) and negative (n = 93) canine sera as examined by MAT[a]

OD_{650}	No. of sera	
	MAT positive	MAT negative
0-0.038	10	33
0.039-0.084	2	11
0.085-0.130	2	11
0.131-0.176	2	15
$\geq$ 0.177	87	21
Total (n)	103	91[b]
Mean OD $\pm$ SD		0.065 $\pm$ 0.028

[a]number of 77 samples of MAT negative were used for calculation of mead OD_{650} values, 14 samples showing high value of OD_{650} ($OD_{650} \geq 0.48$; mean OD of MAT positive samples) were excluded, [b]The cut-off values for the interpretation of positive and negative samples were determined as the mean plus 4 SD (Ribotta *et al*., 2002), of the amount MAT negative absorbance. The value was calculated to be 0.177.

Table 13 Comparison between MAT and ELISA. Relative sensitivity, specificity and accuracy values of the ELISA to detect leptospiral antibodies using MAT as a reference standard in canine samples. MAT was considered positive at 1:100 dilution of serum. ELISA was considered positive at 1:40 dilution of serum when the absorbance was above the cut-off value (0.177).

		MAT		
		Positive	Negative	Total
ELISA	Positive	87 (a)	21 (c)	109
	Negative	16 (b)	70 (d)	85
	Total	103	91	194

Sensitivity = 84.5%, specificity 76.9%, accuracy = 80.9%; kappa value = 0.901

CONCLUSION

From the experimental results and discussion of this study, the conclusion can be drawn as follow:

1. The primers (*rrs, flaB* and *lig*) which were used in the PCR reaction are specific for *Leptospira*. Strains of all *Leptospira* species were amplified with specific primers of 16S rRNA gene (*rrs*). The primer sets of of *flaB* and *lig* gene can be used for differentiate between pathogenic and non-pathogenic leptospires.

2. *flaB* gene sequencing could preliminary screen *Leptospira* spp. The sequence similarities of *flaB* among species test varied between 50.6 % and 96.1%. The *flaB* gene was contained specific sequence information and was a recent addition in evolutionary terms to the leptospiral gene pool. Thus, this short sequence has become an accurate tool for discrimination and identification of leptospires.

3. The 35 kDa of FlaB protein and 96 kDa of conserver Lig protein were expressed successfully in *E.coli* as a fusion with 6X-histidine and glutathione *S*-transferase (GST) for FlaB and Lig protein, respectively. The fusion proteins were expressed upon IPTG induction and its molecular weight, deduced from mobility through a SDS-PAGE gel, were in agreement with that predicted from genetic data. The recombinant proteins had formed inclusion bodies within the cytoplasm of *E. coli*. The insoluble fractions (inclusion bodies) were purified by dissolved in 4M Urea and 1% sarkosyl. The purification protocols were successful for purification of recombinant proteins as a high purity concentration of interest with few contaminants of other cell debris of bacteria.

4. The 35kDa protein described here is a FlaB protein. The recombinant FlaB protein was purified by electroelution for preparation the antigens for immunization. The purified product represented by a strong single band at 35 kDa position was obtained. Rabbit was immunized with 100 µg/ml of rFlaB protein. After the immunization program was completed. Antiserum obtained from rabbit displayed

very strong immunoreactivities and specificities with Western blot immunoassay. The antibodies titre was very high as detected by dot blot analysis. To determine the specificity of polyclonal antibodies against FlaB recombinant proteins for detection of leptospires, the rabbit hyperimmune serum could recognize the band appeared within 35 kDa in all serovar of both pathogenic and non-pathogenic strain tested. The implication of the immunobloting data is that the flagellin is a highly cross reactive antigen within leptospires. By using canine leptospirosis sera with Western blot immunoassay, the sera could recognize a consistent pattern of immunodominant antigen from pathogenic leptospiral strains within 33-36 kDa. It is likely that the flagella protein of 33-36 kDa was found to be antigenically unique to *Leptospira.* The leptospiral flagella proteins have shown to be immunogenic and conserved antigens within this genus. From the preliminary study of application of polyclonal antibodies using IFA for detection of *Leptospira* spp., the polyclonal antibodies against rFlaB protein can be used for the detection of *Leptospiraceae* and had high specificity for discrimination between leptospires and other bacteria. The periplasmic flagellums of class B (FlaB) proteins are flagellin like protein of genus *Leptospira*. The leptospiral flagella proteins have shown to be immunogenic and conserved antigens within the genus *Leptospira*. The amino acid sequences of flagellin were likely to prove useful to differentiate and identify leptospires from other bacteria.

5. The rFlaB had poor potential to be used as an antigen to detect leptospiral antibodies, because it had no reaction with several MAT positive cases, can not distinguish MAT positive from normal cases.

6. The antibodies against recombinant Lig protein was found in mice sera induced with antigen obtained from in vitro cultured leptospires. This phenomenon may be due to these two serovars revealed the virulence mechanisms. They could be multiply into host animals which were recognized by host immune response.

7. The development and evaluation of recombinant Lig antigen-based ELISA for the detection of canine anti-leptospiral antibodies in this study is a simple, rapid, sensitive and specific test for screening canine sera. This assay used a nonhazardous

antigen, is easily standardized, is interpreted objectively, is repeatable and is already semi-automated. It is also safe than MAT since it eliminates the handling of live leptospires and do not have the facilities or expertise to perform. Lig antigen-based ELISA is suitable for large scale serological examination, routine diagnostics, epidemiology surveys and follow-up investigations for outbreaks. Due to, the diagnosis of leptospirosis based on a single serum sample, the ELISA described here would be particularly useful in canine with full consideration of clinical picture and vaccination history of animals.

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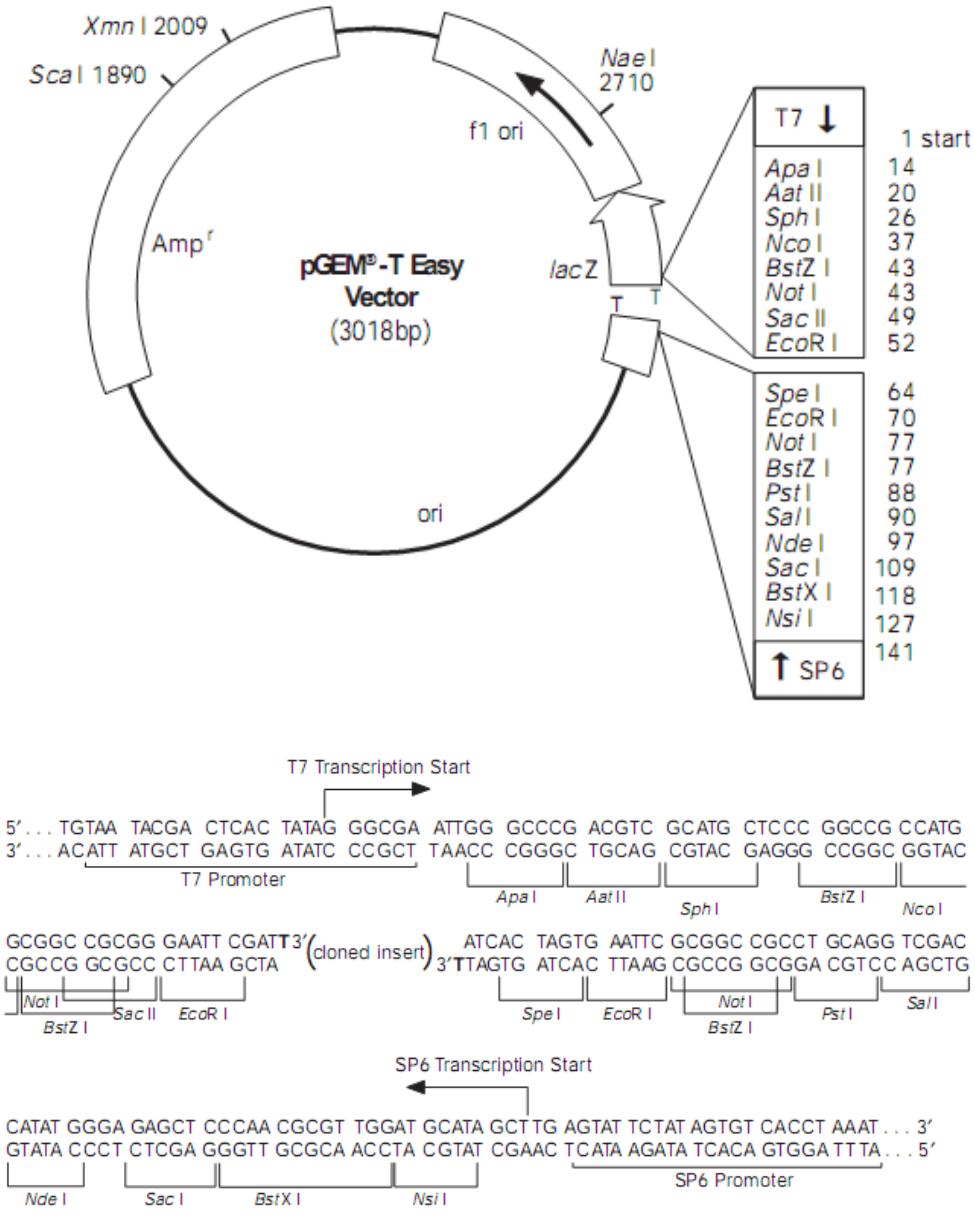
APPENDICES

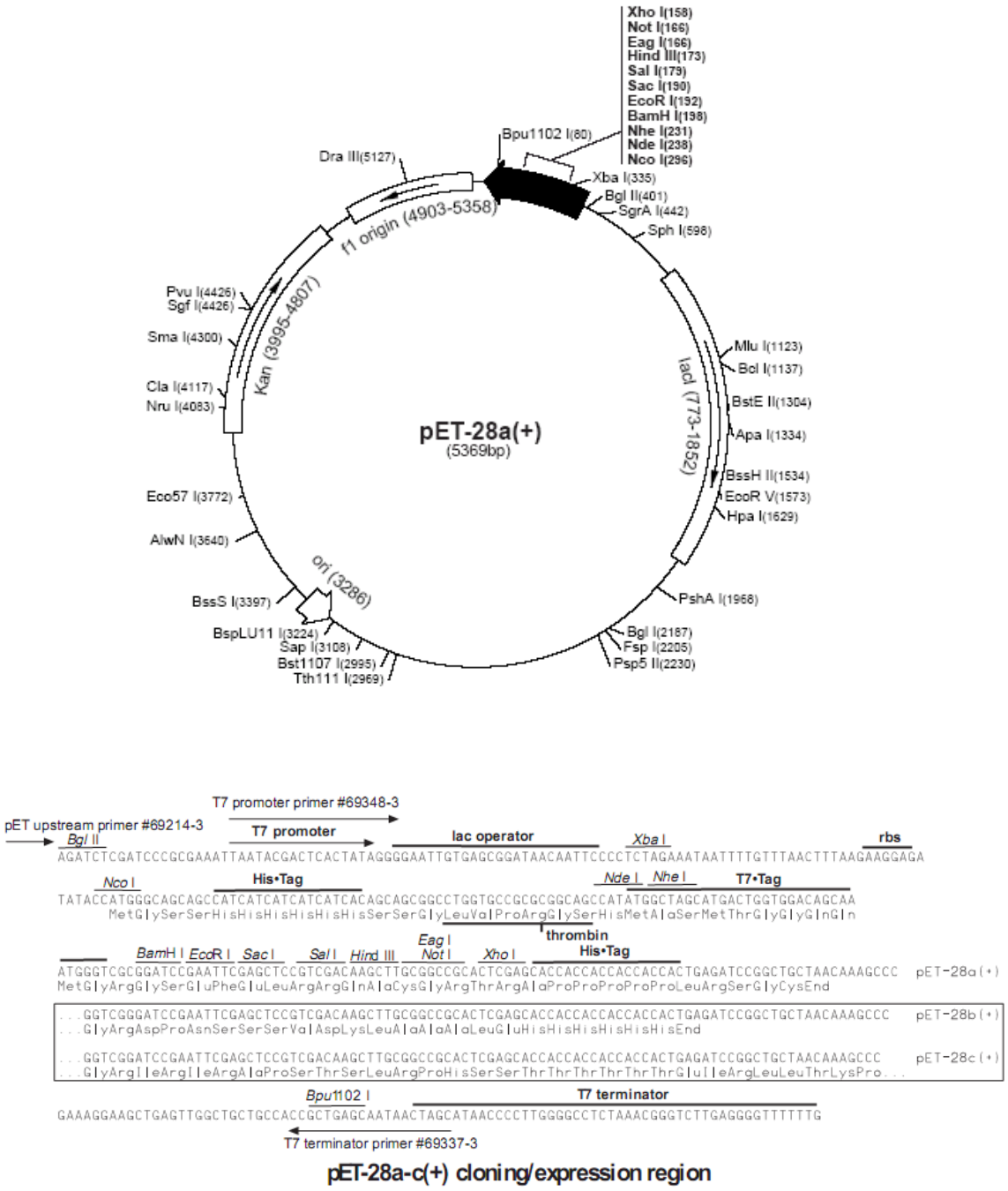
Appendix A

Physical map of plasmids



Appendix Figure A1 Physical map of pGEM-T® easy vector system

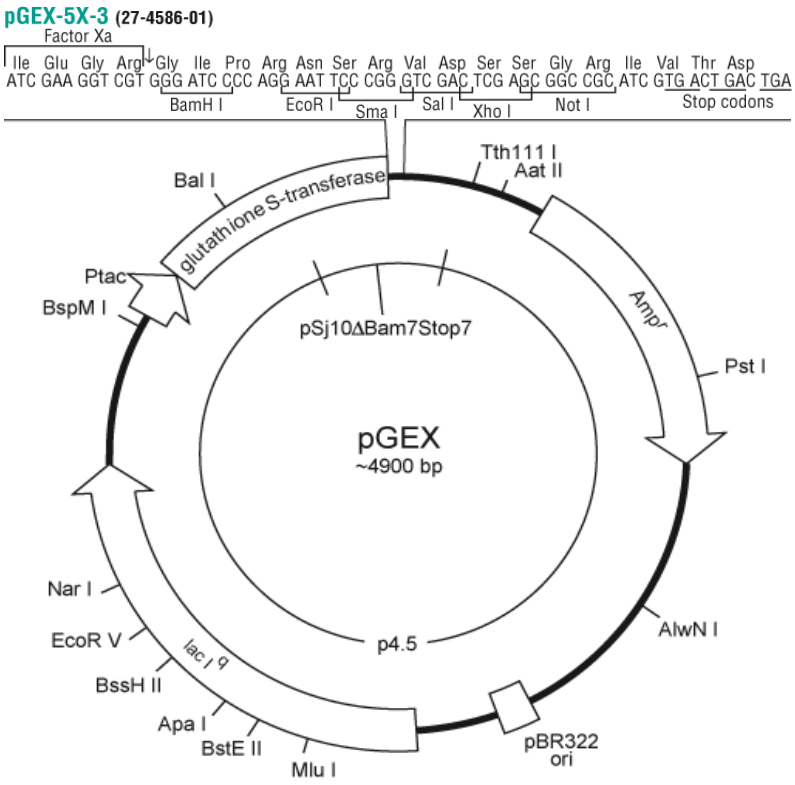
Source : Promega, 1998



pET-28a-c(+) cloning/expression region

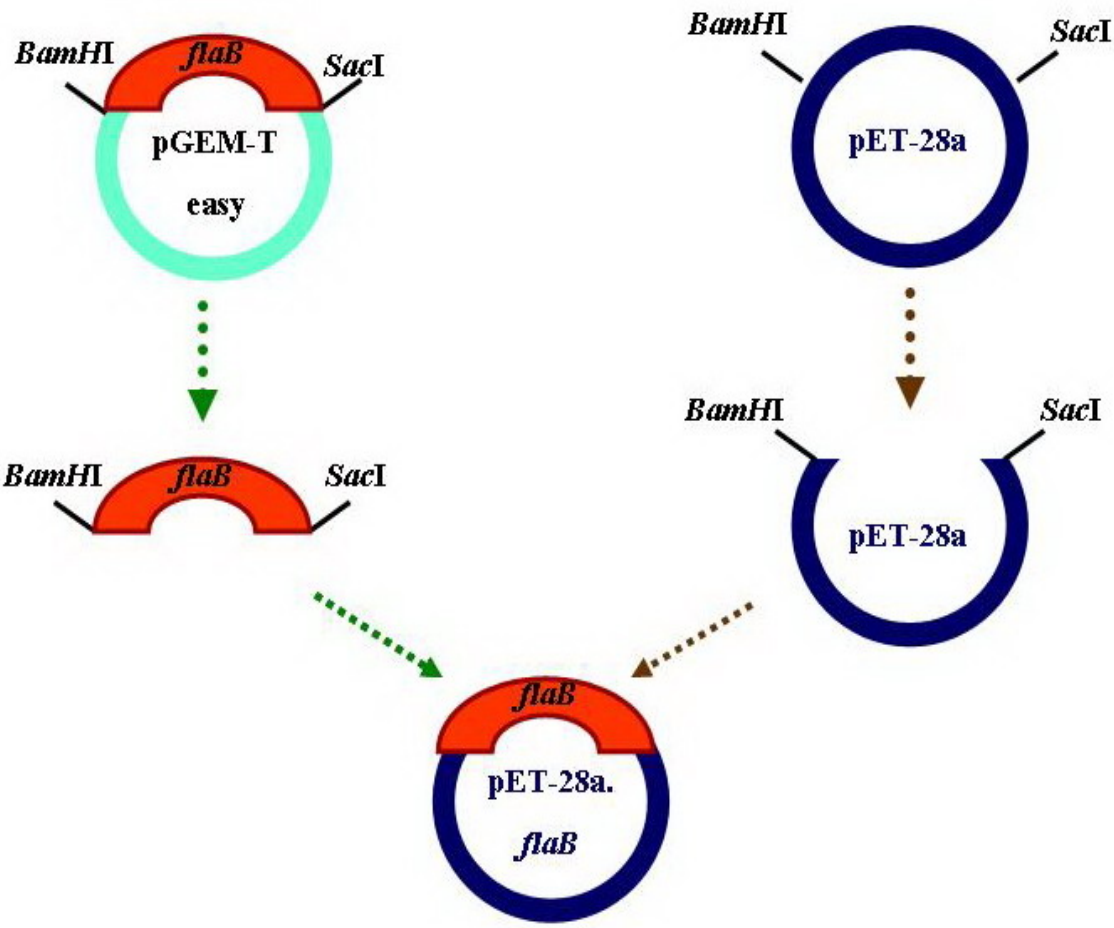
Appendix Figure A2 Physical map of pET.28a cloning vector

Source : Novagen, 2000

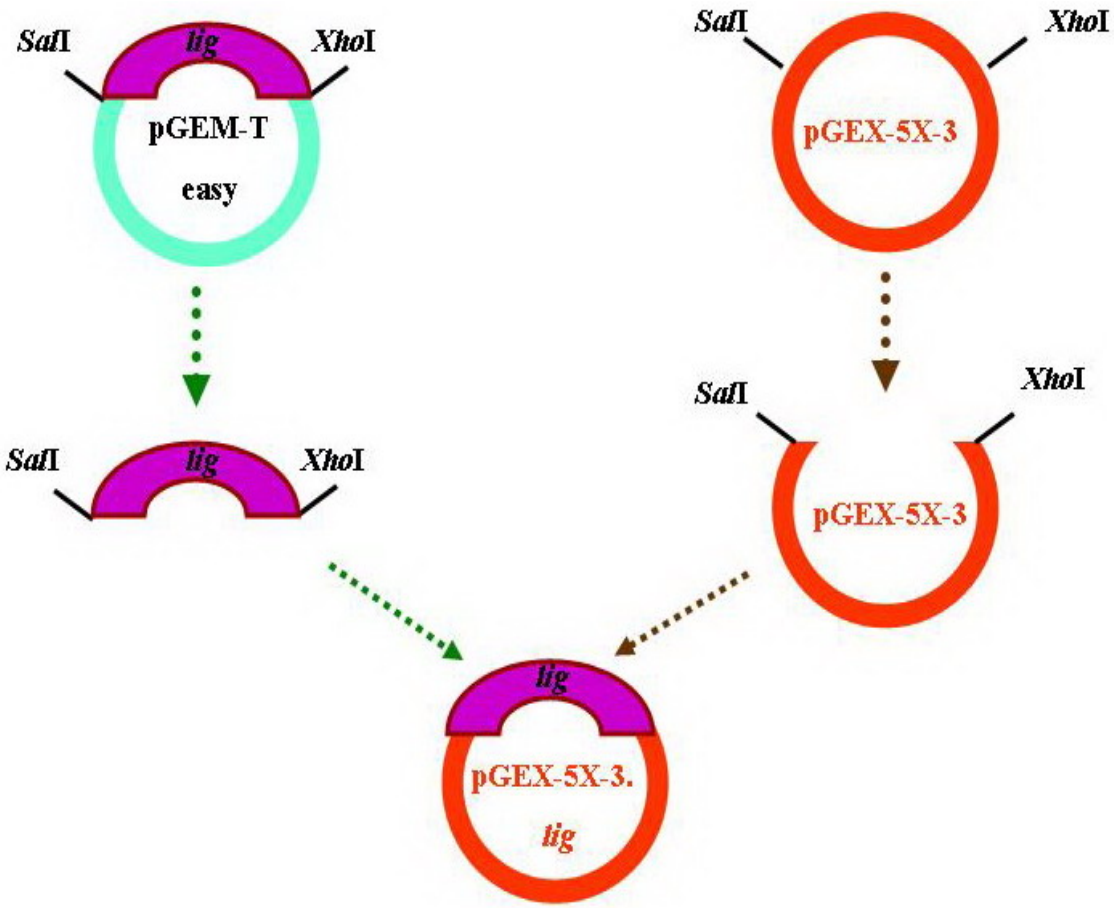


Appendix Figure A4 Physical map of pGEX-5X-3 cloning vector

Source : Amersham phamacia biotech, 2000



Appendix Figure A5 Plasmid construction of pET-28a.*flaB*



Appendix Figure A6 Plasmid construction of pGEX-5X-3.*lig*

Appendix B

Bacterial media and stock solution,

General buffers and reagents

Bacterial media and stock solution

1. Bacterial media

1.1 EMJH medium for leptospiral culture

1.1.1 Stock solution for albumin fatty acid supplement (per 100 ml distilled water)

$CaCl_2.2H_2O+MgCl_2.6H_2O$	1.0 g (each)
$ZnSO_4.7H_2O$	0.4 g
$CuSO_4.5H_2O$	0.3 g
Vitamin B12	0.02 g
Tween 80	10.0 g (approximate 9.3 ml)

All solution were sterilized by filtration and stored at 4^oC.

1.1.2 Stock solution for basal medium (per 100 ml distilled water)

NH_4Cl	25.0 g
Thiamine	0.5 g
Na-pyruvate	10.0 g
Glycerol	10.0 g (approximate 8 ml)

All solution were sterilized by filtration and stored at 4^oC.

1.1.3 Albumin fatty acid supplement solution

This solution was prepared by dissolving 10 g of bovine serum albumin fraction V in 50 ml of distilled water. And added these stock solution as described

$CaCl_2.2H_2O+MgCl_2.6H_2O$	1.5 ml
$ZnSO_4.7H_2O$	1.0 ml
$CuSO_4.5H_2O$	0.1 ml
$FeSO_4.7H_2O$	0.05 g
Vitamin B12	1.0 ml
Tween 80	12.5 ml

This solution was adjusted the pH to 7.4 with 1N NaOH and added the distilled water to 100 ml. The solution was sterilized by filtration.

1.1.4 Basal medium

The solution was prepared by dissolving 1.0 g Na_2HPO_4, 0.3 g KH_2PO_4 and 1.0 g NaCl in 900 ml of distilled water and added these stock solution as described

NH_4Cl	1.0 ml
Thiamine	1.0 ml
Na-pyruvate	1.0 ml
Glycerol	1.0 ml

This solution was adjusted the pH to 7.4 with 1N HCl and added the distilled water to 1,000 ml. The solution was sterilized by autoclaving at 121^{o}C, 15 lb/inch^2 for 15 minutes.

1.1.5 Solution for preparig liquid EMJH medium

Liquid EMJH medium was prepared by mixing 9 volume of basal medium supplemented with 1 volume of albumin fatty acid under sterilization. This EMJH medium was incubated at 28^{o}C for over night to check the sterility.

1.2. Luria-Bertani (LB) medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Add the distilled water to 1,000 ml. The solution was sterilized by autoclaving at 121^{o}C, 15 lb/inch^{2} for 15 minutes.

LB agar and LB agar with ampicillin or kanamycin

Add 15 g agar to 1 liter of LB medium. Allow the medium to cool to 55^{o}C before adding ampicillin (100 µg/ ml final concentration) or kanamycin (25 µg/ ml final concentration).

1. 4. SOC medium (1M MgCl2.6H2O, 1M MgSO4. 7H2O, filter sterilized)

Tryptone	2 g
Yeast extract	0.5 g
1M NaCl	1 ml
1M KCl	0.25 ml
2M glucose, filter sterilized	1 ml
Mg^{2+} stock	1 ml

Add H_2O to final volume of 100 ml, the solution was sterilized by autoclaving at 121^{o}C, 15 lb/inch^{2} for 15 minutes.

2. Stock solution

2.1 Ampicillin

A stock solution of 100 mg/ ml of sodium salt was prepared in distilled water, sterilized by filtration, dispensed into aliquots and stored at -20^{o}C.

2.2. Kanamycin

A stock solution of 25 mg/ ml was prepared in distilled water, sterilized by filtration, dispensed into aliquots and stored at -20^{o}C.

2.3. IPTG (1M)

Dissolve 238 mg/ ml solid IPTG in distilled water, sterilized filter, dispensed into aliquots and stored at -20^{o}C.

General buffers and reagents

1. D-solution (4M guanidine thiocyanate, 25 mM sodium citrate, pH7.0, 0.5% N-laurylsarcosine)

2. Phosphate buffer saline (PBS pH 7.2)

The buffer was prepared by dissolving 8.0 g NaCl, 0.2 g KH_2PO_4, 2.17 g Na_2HPO_4. $7H_2O$ and 0.2 g KCl in 800 ml distilled water. The pH of the buffer was adjusted to 7.2 with 1N HCl and the volume was brought up to 1,000 ml.

3. TE buffer (10M Tris-HCl, pH 7.4 and 1mM EDTA pH 8.0)

4. Alkaline lysis buffer for mini preparation of plasmid DNA

Alkaline lysis solution I
50 mM Glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)
Solution I can be prepared in batches of approximately 100 ml, autoclave for 15 minutes at 10 Ib/sq. in. on liquid cycle, and stored at 4^oC.

Alkaline lysis solution II
0.2 N NaOH (freshy diluted from a 10 N stock)
1% SDS

Alkaline lysis solution III

5 M potassium acetate	60	ml
Glacial acetic acid	11.5	ml
DW	28.5	ml

5. Buffer for agarose gel electrophoresis

50 x Tris-acetate buffer (TAE)

Tris (ultrapure)	242	g
Glacial acetic	57.1	g
0.5 M EDTA	100	ml

Adjust the volume to 1,00 ml with DW, sterile by autoclaving

10X loading buffer/ dye

20% glycerol, 0.01% bromphenol blue, add TE to final volume

5mg/ml ethidium bromide (EtBr)

EtBr	100	mg

Add distilled water to 100 ml

1% agarose gel

Dissolve 1 g of agarose in 1 x TAE buffer then make up volume to 100 ml and melt by microwave.

6. Reagents for SDS-PAGE

Polyacrylamide gel, acrylamide/Bis (30%)

Acrylamide	29.2	g
Bis	0.8	g
DW to	100	ml

1.5 M Tris-HCl, pH 8.8

Tris base	27.23	g
DW	80	ml

Adjust to pH 8.8 with 6N HCl, Bring total volume to 150 ml with deionized water.

0.5 M Tris-HCl, pH 6.8

Tris base	6	g
DW	60	ml

Adjust to pH 8.8 with 6N HCl, Bring total volume to 100 ml with deionized water.

10% Ammonium persulfat (APS) (fresh daily)

Ammonium persulfate	100	mg

Dissolve in 1 ml of deionized water

5x SDS-PAGE sample buffer

Tris	0.3784	g
SDS	0.5	g
Glycerol	5.0	ml
2-mercaptoethanol	2.5	ml
Bromphenol blue	0.0005	%
Deionized distilled water	10	ml

Adjust pH to 6.8

Runninng buffer

Tris	3.0	g
Glycine	14.4	g
SDS	1.0	g
Dieonized distilled water to	1,000	ml

Coomasie Brilliant Blue stain

Coomassie brilliant blue	0.25	g
Methanol	40	ml
Glacial acetic acid	10	ml
Deionized distilled water	454	ml

Dissolve the dye in methanol: deionized water (1:1 v/v), then add glacial acetic 10 ml. Remove insoluble material by filtration through filter paper.

Destain solution

Methanol	250	ml
Glacial acetic acid	100	ml
Deionized distilled water	650	ml

7. Reagent for immunobloting

Transfer buffer

Tris base	3.03	g
Glycine	14.4	g
Methanol	800	ml
Add deionized distilled water to	1,000	ml

PBS buffer, pH 7.4 (1 liter)

NaCl	8.0	g
$K_2H_2PO_4$	0.2	g
KCl	0.2	g
Na_2HPO_4	1.15	g
Add deionized distilled water to	1,000	ml

PBS-Tween buffer (PBS-T)

Add to final concentration of 0.05% Tween 20

Blocking buffer

Non fat dry milk	10	g
PBS-T	100	ml

Tris buffer saline (TBS)

Tris base	2.4	g
NaCl	29.2	g
DW	800	ml

Adjust pH to 7.5 using HCl and bring the solution to 1 litre by DW

DAB substrate solution

TBS	10	ml
3% H_2O_2	100	µl

Dissolved solid DAB (3,3'-diaminobenzidine) at final concentration 6 mM

8. Reagent for indirect-ELISA

Coating buffer (5x bicarbonate buffer, pH 9.6)

Na_2CO_3	1.12	g
$NaHCO_3$	2.94	g
NaN_3	0.20	g

Adjust to pH 9.6 with NaOH. Bring total volume to 200 ml with DW.

Stop solution

NaOH	12	g
DW to	100	ml

Appendix C

The standard protocols

The standard protocols

1. Preparation of genomic DNA

Preparation of small-scale genomic DNA from bacteria was prepared by a modified protocol from the procedure of Trochimchuk *et al*., 2002.

1. Bacterial pelleted cells were resusprnded in 200 µl of D-solution and incubated at room temperature for 5 minutes.

2. 150 µl of phenol and 150 µl of chloroform were added , vortexed and phases separated by centrifugation at 13,000 rpm for 5 minute.

3. The aqueous phase was transferred to a clean tube, about 2.5 volume of 95% ethanol was added and DNA was precipitated overnight at -20^{o}C.

4. The precipitated sample was then centrifuged at 13,000 rpm for 10 minutes and the pellet was washed twice with 0.6 volume of 75% ethanol.

5. The pellet was dried, then redissolved in 25 µl of steriled TE buffer and store at -20^{o}C until used.

2. Preparation of plasmid DNA

Small-scale plasmid DNA from *E.coli* strains was prepared by a modified protocol from the method of Sambrook and Russell, 2001.

1. Pour 1.5 ml of an overnight culture into a microcentrifuge tube. Centrifuge at 13,000 rpm for 60 seconds.

2. The cell pellet was resuspended in 200 µl of solution I containing 2 µl of RNase (10 mg/ ml)

3. Add 200 µl of solution II and inverting the tube five to ten time to mix the content.

4. Add 200 µl of solution III and inverting the tube five to ten time to mix the content.

5. Add 10 µl of chloroform and centrifuge at 13,000 rpm for 5 minutes

6. The aqueous phase (500 µl) was transferred to a clean tube , about 1-1.5 volume of isopropanol was added and DNA was precipitated overnight at -20^{o}C.

7. The precipitated sample was then centrifuged at 13,000 rpm for 10 minutes and the pellet was washed twice with 0.6 volume of 75% ethanol.

8. The pellet was dried, then redissolved in 25 µl of steriled TE buffer and store at -20^{o}C until used.

3. Ligatin of DNA fragment

The concentration of DNA in 20 µl ligation reaction volume was adjusted so that the insert:vector molar ratio was approximately 3:1. DNA fragments were incubated with the T4 DNA ligase in 1 x ligation buffer at 16^{o}C for overnight.

4. Preparation competent cells

1. Grow cell overnight in 1 ml LB broth at 37^{o}C with vigorous shaking (~250 rpm)

2. On the follow day, incolate 1 ml of the overnight culture into 50 ml of fresh LB medium. Grow at at 37^{o}C with vigorous shaking (~250 rpm) for 3 hour.

3. Centrifuge the cell at 4,000 rpm for 10 minutes, discard the supernatant and resuspended the pellet with 17.5 ml of ice-cold of 100 mM $CaCl_2$,, incubate on ice for 10 minutes.

4. Centrifuge the cell at 4,000 rpm for 7 minutes, discard the supernatant and resuspended the pellet with 4 ml of ice-cold of 100 mM $CaCl_2$, then add 7% DMSO (250 µl), mix gently and incubate on ice for 10 minutes.

5. Aliquot the cell to micro tube each 200 µl and store at -70^{o}C until use.

5. Transformation (Calcium chloride)

1. Thaw the competent cell on ice and add ligation mix or plasmid DNA, then leave the cell on ice for 30 minutes.

2. Heat shock the cells at 42oC for 90 seconds and returned to ice immediately for 2 minutes.

3. Add 900 µl of SOC medium and incubate with shaking at 37^{o}C for 1 hour.

4. Spin the cells in the centrifuge for 1 minute and pour off all the media except the last drop. Resuspend the pellet in the last drop and plate on a selective media. Incubate the cells overnight at 37^{o}C.

5. The recovery clone was determined by PCR assay, restriction endonuclease and sequencing

6. Restriction endonuclease digestion

DNA was digested using restriction enzymes obtained from Fermentas with the appropriate buffer supplied and according to the manufacturers' instruction. Restriction endonuclease fragments were analyzed by agarose gel electrophoresis.

7. DNA fragment isolation

DNA fragments were excised from gel and purified using a NucleoSpin® kit (MACHEREY-NAGEL GmbH) according to the manufacturers' instruction.

8. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used in the analysis of the recombinant protein. The procedure was applied from Sambrook and Russell (2001) and mini-PROTEAN® instruction manual (BioRAD). Prepare the gel by the recipe as follow 2 gels.

- Separating gel (12%)

H_2O	2.8 ml
4x Tris HCI/SDS pH 8.8.	2.0 ml
30% Acry/0.8%bis-Acrylamide	3.2 ml
10% APS	26.6 µl
TEMED	5.3 µl

- Stacking gel

H_2O	3.05 ml
4x Tris-HCl/SDS pH 6.8	1.25 ml
30% Acryl/0.8%bis-Acryl	0.65 ml
10% APS	25 μl
TEMED	5 μl

If not already in electrophoresis sample buffer, add an equal volume of 2X sample buffer to all samples and boil for 5 minutes. Apply 20-25 µl (1 - 10 µg total protein) of cell lysate to each well of a 0.75-1.0 mm thick gel. Run electrophoresis (100 volt, 90 minutes, constant ampere)

9. Copper staining

    1. Wash gel with distilled water
    2. Place the gel in glass or plastic tray
    3. Add at least 5 volumes of staining solution
    4. Incubate at room temperature with agitation for 5 minutes
    5. Wash the gel with destaining solution
    6. Observe against a dark background

10. Western blot analysis

    1. Separate protein sample using SDS-PAGE

    2. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Rinse gel with cool transfer.

    3. Set the transfer apparatus and then transfer proteins from gel to nitrocellulose membrane at constant 400 mAmp for 3 hours in transfer buffer. The transfer set should be placed in ice box to control the temperature in avoiding of loss of buffer quality from heat

    4. Remove the blot from transfer apparatus immediately place into blocking buffetr (5% skim milk in PBS-T) and incubate at 37^{o}C for 1 hour.

    5. Dilute the primary antibody with protein absorb (Canine sera were adsorbed at 4^{o}C overnight using lysate from induced *E. coli* BL21 containing plasmid pGEX-5X-3 and rabbit hyperimmune serum anti-recombinant FlaB protein was absorb with lysate from induced *E. coli* BL21 containing plasmid pET-28a). Decant the blocking buffer and add the primary antibody to membrane. Incubate with agitation at 37^{o}C for 90 minutes.

    6. Wash 3 times with PBS-T for 5 minutes each.

7. Dilute the secondary with 1% skim milk in PBS-T and incubate with agitation at 37^{o}C for 30 minutes.

8. Decant the secondary antibody and wash 3 times with PBS-T for 5 minutes each.

9. Add DAB substrate and incubate for 5-10 minutes

10. To stop enzyme-substrate reaction, place the blot in distilled water.

Appendix D

Nomenclature of 20 standard amino acids

IUPAC Nucleotide ambiguity codes

Appendix Table D1 Nomenclature of the 20 standard amino acids

Amino Acid	Three-Letter Symbol	One-Letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Th reonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix Table D2 IUPAC Nucletide ambiguity codes

Nucleotide ambiguity symbol	Nucleotide
A	A
T	T
C	C
G	G
M	A/C
R	A/G
W	A/T
S	C/G
Y	C/T
K	G/T
V	A/C/G
H	A/C/T
D	A/G/T
B	C/G/T
X/N	A/T/C/G