CHAPTER IV
MATERIALS AND METHODS

1. *Leptospira* spp. cultivation

Pathogenic and non-pathogenic leptospires used in this study were from the collections of the Department of Medical Sciences, Ministry of Public Health, Thailand.

Various *Leptospira* serovars **(Table 1)** were maintained individually in either liquid or semisolid EMJH medium containing Tween-80, albumin and pyruvate (Johnson and Harris, 1967; Johnson *et al.*, 1973; **Appendix A**). The culture medium (10 ml) was aliquoted in screw-capped glass tubes (size 16 mm × 150 mm). The bacteria were incubated aerobically in an incubator set at 28-30°C. Bacterial growth was checked by dark-field microscopy. Before subculturing, the bacteria were streaked onto TSA plate and the plate was incubated at 37°C for 48 h. Subcultures were performed at 3-4 week intervals (or more frequently if needed).

In cases of culture contamination, the *Leptospira* were rescued from the culture by filtering the culture fluid through a sterile 0.22 μm Millipore membrane. *Leptospira* that passed through the membrane were placed in a tube of fresh culture medium containing 200 μg/ml of 5-fluorouracil. At this concentration, the 5-fluorouracil does not inhibit the growth of the pathogenic *Leptospira* while it does to other contaminating bacteria.

2. Two dimensional difference gel electrophoresis (2D-DIGE) of *Leptospira* spp.

2.1 Preparation of *Leptospira* whole cell homogenates for 2D-DIGE

The leptospires were collected by centrifugation (Sorvall Legend MACH 1.6R, Kendro Laboratory Products, Osterode, Germany) at 12,000 × *g*, 4°C, 10 min and the bacteria were washed with standard cell washing buffer (10 mM Tris and 5 mM magnesium acetate) **(Section 1, Appendix B)** by centrifugation at 12,000 × *g*, 4°C, 10 min. Bacteria in the pellet were resuspended in standard cell lysis buffer (30 mM Tris, 2 M Thiourea, 7 M urea, 4% CHAPS) **(Section 2, Appendix B)** containing

Table 2 List of *Leptospira* spp. which their whole cell homogenates were used for protein extraction for 2DE

	Species	Serogroup	Serovar (strain)
1	*L. biflexa*	Andaman	Andamana (CH11)
2	*L. biflexa*	Semaranga	Patoc
3	*L. borgptersenii*	Javanica	Javanica
4	*L. borgptersenii*	Tarrassovi	Tarassovi
5	*L. interrogans*	Australis	Bangkok
6	*L. interrogans*	Autumnalis	Autumnalis (Akiyami A)
7	*L. interrogans*	Bataviae	Bataviae
8	*L. interrogans*	Canicola	Canicola
9	*L. interrogans*	Pomona	Pomona

protease inhibitors (Roche Diagnostics GmBH, Manheim, Germany). The whole cell homogenate was prepared by subjecting the preparation to sonication (Labsonic®P, Sartorious AG, Gottingen, Germany) at 30% amplitude, 2 sec pulse-on, 2.5 sec pulse-off, for a total of 5 min in an ice water bath. After sonication, the sample was centrifuged and the supernatant was transferred to a new tube and the pellet was discarded. The pH of the homogenates were adjusted to 8.5 by slow and careful addition of 50 mM sodium hydroxide. The homogenates were stored in aliquots at –70°C.

The *Leptospira* homogenates were treated with the 2D-Clean-up kit (PlusOne, Amersham Biosciences, San Francisco, CA) to eliminate detergents, salts, lipids, phenolics, and nucleic acids. After the treatment, the preparations were resuspended in the standard cell lysis buffer. The preparation were determined for their protein concentration using the 2D-Quant kit (PlusOne, Amersham Biosciences) before subjecting them to the protein labeling as described below.

2.2 Preparation of CyDye™ DIGE Fluor minimal dyes for protein labeling

The lyophilized CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3 and Cy5) (Amersham Biosciences) were taken from the –20°C freezer and left to warm at 25°C for 5 min. The stock dye solution (1 nmol/µl) was prepared and immediately diluted to the working dye solution (400 ρmol/µl) before useing in the labeling reaction.

Five microliters of dimethylformamide (DMF) (PlusOne, Amersham Biosciences) from its original container was transferred into a tube and containing dye in order to make 5 nM of the dye. The tube containing the dye was capped and mixed vigorously to dissolve the dye and centrifuged in a bench-top centrifuge at 12,000 × *g* for 30 sec. The stock dye solution was stored at –20°C until used.

2.3 Preparation of ™ DIGE Fluor minimal dye for labeling proteins of *Leptospira* spp. lysate

The vial containing the stock dye solution was briefly spun in a microcentrifuge. The working dye solution containing 400 ρmol/µl was made by pipetting 2 µl of reconstituted stock dye solution into a fresh microfuge tube containing 3 µl of DMF.

2.4 *Leptospira* protein labeling

2.4.1 Preparation of an internal standard

The pooled internal standard (50 µg/gel) was prepared from homogenates of various serovars of the *Leptospira* spp. **(Table 2)**. The pooled internal standard was prepared as shown in **Table 3**.

2.4.2 Protein labeling with the CyDye™ DIGE Fluor minimal dyes

A volume of *Leptospira* sample(s) containing approximately 50 µg of proteins was added to a microfuge tube. One microliter of working dye solution was added to individual tubes (*i.e.* 50 µg of proteins was labeled with 400 ρmol of dye in the labeling reaction) and the solution were mixed thoroughly by vortexing. The microfuge tubes were centrifuged briefly and left in an ice bath for 30 min in the dark. One microliter of 10 mM lysine was added to each sample tube to stop the reaction. The content in the tubes was mixed and the tubes were spun briefly in a microcentrifuge and left in an ice bath for 10 min in the dark. The labeled samples were processed for 2D-DIGE immediately.

2.4.3 Preparation of labeled protein samples for the first dimension separation

At this stage the *Leptospira* protein sample(s) was/were undergone labeling and the reactions were quenched by the addition of 10 mM lysine, as described in **Section 2.4.2.** The two differentially labelled *Leptospira* samples and one pooled internal standard were combined into a single microfuge tube and mixed. An equal volume of 2× sample buffer (2 M Thiourea, 7 M urea, 4% CHAPS) **(Section 2.1.1, Appendix B)** was added to the labelled protein samples and kept in an ice bath for at least 10 min.

2.5 Focusing using the Ettan IPGphor isoelectric focusing system

The combined *Leptospira* protein samples, labeled with the different CyDye™ DIGE Fluor minimal dyes, were mixed together as in **Section 2.4** so that they were focused on the same Immobiline DryStrip. This ensures that the *Leptospira* protein samples were subjected to exactly the same electrophoretic running conditions.

Table 3 The assignment for three-color analysis-comparison of protein abundance of nine different serovars of *Leptospira* spp.

Gel	Cy2 standard	Cy3	Cy5
1	50 μg (5.56 μg each of *Leptospira* spp. serovars 1–9)	50 μg serovar 1	50 μg serovar 2
2	50 μg (5.56 μg each of *Leptospira* spp. serovars 1–9)	50 μg serovar 3	50 μg serovar 4
3	50 μg (5.56 μg each of *Leptospira* spp. serovars 1–9)	50 μg serovar 5	50 μg serovar 6
4	50 μg (5.56 μg each of *Leptospira* spp. serovars 1–9)	50 μg serovar 7	50 μg serovar 8
5	50 μg (5.56 μg each of *Leptospira* spp. serovars 1–9)	50 μg serovar 9	50 μg serovar 1

2.5.1 Rehydrating IPG strips containing of protein samples

The total volume of the labeled proteins was made up to 350 µl for a 18 cm-IPG strip (Immobiline™ DryStrip, GE Healthcare Biosciences) using rehydration buffer (2 M Thiourea, 7 M urea, 4% CHAPS, 1% IPG buffer, 0.2% DTT) **(Section 2.1.2, Appendix B)**. The labeled protein solution was pipetted and slowly delivered to the center of a slot in the 18 cm strip holder of the IPGphor (GE Healthcare Biosciences). The protective covers of the 18 cm-IPG strips (Immobiline™ DryStrip, GE Healthcare Biosciences) were removed. The IPG strips were positioned with the gel side downward and the pointed (acidic) end of the strips against the end of the slot. The IPG strips were slided down onto the solution by gently lifting and lowering the strips along the surface of the solution. The PlusOne™ DryStrip Cover Fluid (PlusOne, Amesham Biosciences) was overlayed onto each IPG strips to prevent evaporation and urea crystallization. The IPG strips were rehydrated at the limited current of 50 µA/strip at 20°C for 12-15 h. After rehydration, the IPG strips were removed from the individual strip holder and transferred onto the manifold placed on the IPGphore 3 IEF Unit (GE Healthcare Biosciences) platform. The strips with anodic (+) end were placed on the strip resting with appropriately marked etch. The end of the gels was aligned with the etched mark. Distilled water (150 µl) was added to each pre-cut paper wick. The wicks were placed such that one end of the wick overlapped the end of the IPG strip. The electrode assembly was placed onto the wicks. The cover fluid was flooded on the channels of the manifold. The IPGphore lid was closed and programmed for the IEF separation was performed.

2.5.2 Isoelectric focusing parameters

The electrophoresis condition for the 18 cm IPG strips was set at 50 µA/IPG strip at 20°C. The electrophresis was performed initially at step and hold mode, 500 V, 0.5 kilovolt/hours (kVh) followed by gradient, at 1,000 V, at 0.8 kVh, gradient, 8,000 V, 13.5 kVh and step and hold mode, at 8,000 V and 12.2 kVh, respectively.

2.6 Second dimension SDS-PAGE

For the second dimension gel electrophoresis, each electrofocused-IPG strip was equilibrated in 10 ml SDS-equilibration buffer **(Section 2.2, Appendix B)** containing 100 mg dithiotreitol (DTT) **(Section 2.3, Appendix B)** for 15 min. Subsequently, the

strip was placed in 10 ml of the equilibration buffer containing 250 mg iodoacetamide (IAA) **(Section 2.4, Appendix B)** for 15 min. The strip was washed by submerging in the measuring cylinder containing 1× electrode buffer **(Section 3.8, Appendix B)** and placed onto a 1 mm thick, 12.5% polyacrylamide gel **(Section 3.6, Appendix B)** that had been cast for Ettan DALTsix™ (Amesham Biosciences, Upsala, Sweden) system between low-fluorescence glass plates. After the warm molten agarose (0.5%) **(Section 3.9, Appendix B)** was placed onto the gel. Gel electrophoresis was performed at 15°C under 2.5 W/gel during the first 20 min and at 17.5 W/gel until the tracking dye reached the lower edge of the gel. After SDS-PAGE, the gel within the glass plates, fluorescently labeled proteins were visualized using the Typhoon™ 9400 series Variable Mode Imager (Amersham Biosciences) with the following wavelength settings: Cy2—488 nm excitation wavelength, 520 nm bandpass (BP) 40 nm (520 BP 40) emission filter; Cy3—532 nm excitation wavelength, 580 BP 30 emission filter; Cy5—633 nm excitation wavelength, 670 BP 30 emission filter. All gels were scanned at 100-µm resolution **(Table 4)**. The DeCyder Differential Analysis Software™ (Amersham Biosciences) was used to analyze the intra-gel co-detection of sample, internal standard protein spots and the inter-gel matching of internal standard samples across all gels within the experiment.

2.7 DeCyder 2D Differential Analysis Software for co-detection and matching using the internal standard

The protein spot volumes were compared across a range of experimental samples and gels. Two distinct steps are required as follows:-

2.7.1 Intra-gel co-detection

Up to three scans, Cy2, Cy3 and Cy5, were made for each gel. The scanned images of each sample and the internal standard were overlaid in DeCyder Differential Analysis Software (Amersham Biosciences). The algorithms within the software co-detected the spots present in each scan, and effectively identifying the position of each spot within the gel **(Figure 1)**. The spot boundaries were made identical for each image in the gel. This minimized variation from detection and background subtraction, with the added benefit that every protein in the sample is intrinsically linked to the corresponding protein spot in the internal standard sample.

Spot volume (*i.e*. the sum of the pixel values within a spot, minus background) for each experimental sample was compared directly to the internal standard by DeCyder 2D Differential Analysis Software™ version 5.0 (Amersham Biosciences). The protein abundance for each spot in each sample was expressed as a (normalized) ratio relative to the internal standard *e.g*., Cy3 sample 1:Cy2 standard.

2.7.2 Inter-gel matching

Experiments were designed to ensure that each gel contained the same internal standard. This enabled inter-gel comparisons of spot abundance. Before this could be done, it was important to ensure that the same protein spots were compararable between gels. DeCyder Differential Analysis Software™ (Amersham Biosciences) achieved this using the internal standard to match the position of each protein across all gels within the experiment. An internal standard image was assigned as the "Master". Following co-detection, each image had a spot boundary map. The spot boundary map for the internal standard assigned as the master, was used as a template to which all remaining spot boundary maps for the other internal standards (intrinsically linked to their co-detected sample images) were matched **(Figure 1b)**.

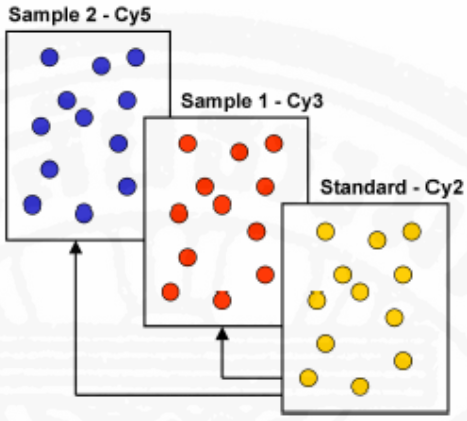
2.7.3 Image analysis

Gel analysis was performed using DeCyder™ Differential Analysis Software version 4.00, specifically designed for use with Ettan DIGE system. DeCyder Differential Analysis Software detects spots using a composite image formed by merging an image pair (Cy2 image with either Cy3 or Cy5 image) from the same gel. The fully automated co-detection algorithm ensures that all spots on co-detected gel images are represented identically. The estimated number of spots for each co-detection procedure was set to 1,200. Filtering was kept to a minimum to eliminate dust particles etc. while avoiding the exclusion of potentially valuable protein spots. The detection process incorporates background subtraction, quantitation, and normalization with respect to the standard. Gel-to-gel matching was performed using the Biological Variation Module (BVA) of DeCyder software™ (Amersham Biosciences). A series of manually set landmarks were entered for each of the standard Cy2 images with a master gel to aid the matching process. Once the

Table 4 Emission filters and laser combinations (From; Ettan DIGE™ system user manual, 2003)

Dye	Emission filter (nm)	Laser
Cy2	520 BP 40	Blue (488)
Cy3	580 BP 30	Green (532)
Cy5	670 BP 30	Red (633)

1a



1b

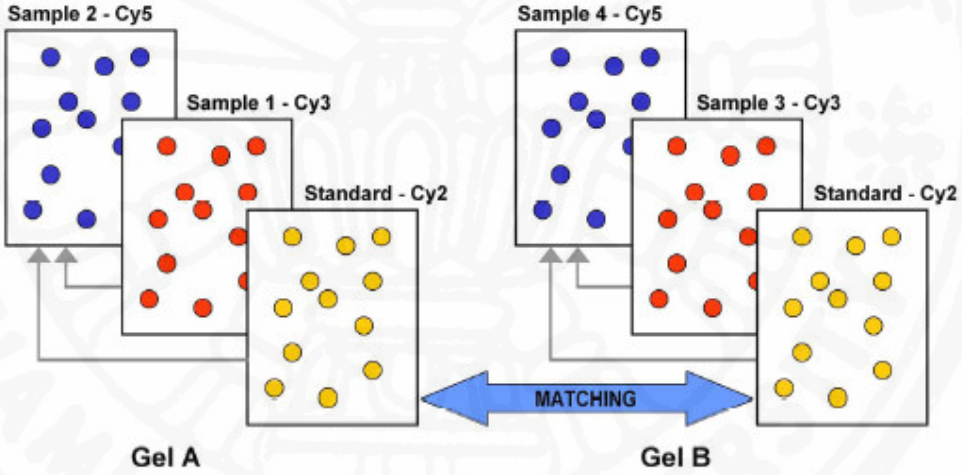


Figure 1 DeCyder 2D Differential Analysis Software for co-detection and matching using the internal standard

1a Intra-gel co-detection; all samples are co-detected with the internal standard.

1b Inter-gel matching; only the internal standards needed to be matched. These were derived from the same sample and therefore this aided matching (Cy5 sample 2:Cy2 standard). From this analysis, cross-sample comparisons could be made.

matching process was complete, statistical analysis of protein-abundance changes between samples was performed. Paired Student's t-test analysis was performed for every matched spot-set, by assigning each culture identity a different number.

Protein spots showing a significant ($p < 0.05$) quantitative difference between samples were manually assigned as "proteins of interest".

2.7.4 Colloidal Coomassie Brilliant Blue protein staining for spot picking

Leptospira protein samples were subjected to 2DE according to the established techniques described in **Section 2.6**.

Whole cell homogenates of a clinical isolate, *Leptospira interrogans,* serogroup Pomona, serovars *Pomona* were prepared as described in **Section 2.1**. The *Leptospira* homogenate (500 μg protein) was mixed with the rehydration solution containing 0.018 M DTT, 0.5% of pH 3-10 IPG buffer (Amersham Biosciences). The preparation was loaded into a slot of an 18 cm individual strip holder. Care was taken not to produce any bubbles. The IPG strip was placed (right side downward) into the strip holder containing the sample, then 2 ml of mineral oil or dry strip cover fluid (Amersham Biosciences) was added to the strip holder. The strip holder was placed into the Ettan IPG Phor 3 Electrofocusing System (Amersham Biosciences) and the IPG strip was allowed to rehydrate at 20°C with the current limited at 50 μA/stip for 12-15 h. After rehydration the strips were transferred onto the manifold which placed on the IPGphore 3 IEF Unit (GE Healthcare Biosciences) platform. The running condition was set at 50 μA/IPG strip at 20°C. The electrophresis was performed initially at step and hold mode, 500 V, 0.5 kVh followed by gradient, 1,000 V, 0.8 kVh and gradient and 8,000 V, 13.5 kVh and step and hold mode, at 8,000 V and 12.2 kVh, respectively.

For the second dimension gel electrophoresis, the electrofocused IPG strip was equilibrated in 10 ml SDS-equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% Bromphenol blue) containing 100 mg dithiothreitol for 15 min. It was placed in 10 ml of the equilibration buffer containing 250 mg iodoacetamide for 15 min. The strip was washed with electrode buffer and overlaid onto a 12.5% polyacrylamide gel **(Section 3.6, Appendix B)** casted in 24 cm-long slab-gel of Ettan DALTsix™ gel (Amesham Biosciences). The warm molten (0.5%) agarose **(Section 3, Appendix B)** was added on the gel. SDS-PAGE was

carried out at 15°C under 2.5 W/gel during the first 20 min and at 17.5 W/gel until the tracking dye reached the lower edge of the gel. After SDS-PAGE, the gel was stained with Coomassie Brilliant Blue G250 dye further 2D **(Section 4, Appendix B)**. The gel was fixed with fixing solution (1.3% *o*-phosphoric acid, 20% (v/v) methanol in ~20 fold excess of gel volume for a minimum of 1 h, at 25°C with gentle agitation. The fixation solution was poured-off and the staining solution containing 0.2% Coomassie Brilliant blue G-250, 1.6% *o*-phosphoric acid, 8% ammonium sulfate and 20% methanol **(Section 4.4, Appendix B)** was replaced in ~20 fold excess of gel volume with gentle agitation for 18 h. The gel was transferred into neutralization buffer (0.1 M Tris pH 6.5, **Section 4.5, Appendix B**) for 1-3 min and washed with 25% methanol for less than 1 min. Then the gel was transferred into stabilizing solution. The gel was imaged at this stage by Image Scanner Model UTA-1100 and ImageMaster™ Software (Amersham Biosciences).

2.7.5 Spot picking, excision and spot handling work station

The 2D-DIGE gels were scanned and the selected spots corresponded to the proteins which were common to all pathogenic *Leptospira* spp. or found in different quantity to that in the pathogenic *Leptospira* and were not found in the non-pathogenic *Leptospira* spp., were carefully excised individually by hand from the respective Commassie Brilliant Blue-stained-gels. Tryptic digestion of the excised gel containing the protein spots was performed on Ettan™ Spot Handling Workstation (Amersham Biosciences). The steps were performed as the following: the gel plugs were destained using a washing solution containing 50% methanol and 5% acetic acid in UDW at 25°C for 18 h. Individual gel plugs were rinsed a few times in a period of 2-3 h with fresh aliquots of the washing solution. Proteins in the gel plugs were digested as described by Kinter and Sherman (2000).

2.7.6 MALDI-ToF and Mass spectrometry (MS) analysis

Dried droplet method was used for the sample preparation of MALDI/MS analysis onto MALDI-ToF–Target.

The gel plugs containing the proteins of interest were excised manually. Tryptic digestions were performed on Ettan™ Spot Handling Workstation (Amersham Biosciences). An equal volume of tryptic peptide digest was mixed with matrix

solution (α-Cyano-4-hydroxy-cinnamic acid, HCCA 10 mg/ml and acetonitrile/trifluor acetic acid (ATFA) solution **(Appendix F)**. One microliter of the mixture was spotted onto the target plate and allowed to dry at 25°C. The mass spectra were recorded on a reflector of MALDI-TOF mass spectrometer model Reflex V (Bruker Daltonik GmbH, Germany) delayed extraction MALDI-TOF mass spectrometer equibbed with a 2 GHz LeCroy digitizer and 337 nm N_2laser. Instrumental parameters were positive polarity; acceleration voltage 20 kV; IS/2 17 kV; focusing lens voltage 8.90 kV; extraction delay, 400 nsec. The detector was gated. Typically 100 shots were accumulated from three to five different positions within a sample spot. Protein identifications were obtained using MASCOT (MatrixScience) and by searching for matching peptide mass fingerprints in a protein database. The search criteria used were fix modification carboxamidomethylation of cysteine, variable modification methionine oxidation and considered the accuracy of the experimental to theoretical pI and molecular weight. Protein scores are significant when *p* value is smaller than 0.05.

2.7.7 LC/MS/MS

After MALDI-ToF/MS analysis was done the LC/MS/MS was performed after for confirmation. For peptide analysis, the LC/MS-MS model Finnigan LTQ Linear Ion Trap Mass Spectrometer (Thermo Electron Corp., CA, USA) was used. The HPLC system was a Finnigan Surveyor™ MS pump (Thermo Electron) with a flow splitter. A total of 2 µl of the digest mixture (1 µg/µl) from **Section 2.7.4** was loaded onto a reversed-phase of the C18 column (0.15 × 100 mm) (Thermo Electron) for LC/MS analysis. The flow rate was 200 µl/min. The two mobile phases were: A) water with 0.1% formic acid, and B) 100% acetonitrile with 0.1% formic acid. After washing to remove unbound peptides with A, the following gradients of B were applied to the column: 2-60% B for 20 min, 65-80% B for 5 min, and 80-2% B for 2 min. Peptides in the eluates were analyzed by mass spectrometry (Finnigan LTQ, Thermo Electron), which used NanoSpray, positive ion in ionization mode at a capillary temperature of 200°C with a 1.8 kV spray needle. The scanned sequence was a full-scan MS, zoom scan MS and MS-MS scan with a mass range of 400-1600

m/*z*. The acquisition modes were Normal, Data Dependent™ and Dynamic Exclusion™.

2.7.8 Database search

The ion spectra of the peptides generated by mass spectrometry were interpreted using the Turbo SEQUEST algorithm in the BioWorks™ 3.1SR1 software package (Thermo Electron) and nr.fasta database. Protein search parameters included a precursor peptide mass tolerance of ±1.25 amu, fragment mass tolerance of ±0.4 amu, methionine (M) oxidation, and threonine (T) or serine (S) phosphorylation. For the tryptic status requirement, at least one end of the peptide had to be a tryptic site. The identified peptides were further evaluated using charge state *versus* cross-correlation number (X_{corr}). The criteria for positive identification of peptides were X_{corr} >1.5 for singly charged ions, X_{corr} >2.0 for doubly charged ions, and X_{corr} > 2.5 for triply charged ions. A delta correlation of (ΔC_n) >0.8 was used as cut-off for peptide acceptance. The minimum number of one peptide per protein was specified by the software.

3. Antibodies and serum samples for the study of unique proteins of pathogenic *Leptospira* spp./*Leptospira* immunome/*in vivo* expressed antigens of *Leptospira* spp.

3.1 Antigens preparation for monoclonal antibody production

3.1.1 *Leptospira* spp. and their whole cell homogenates

Leptospira spp. were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium **(Appendix A)** (Difco, Detroit, MI, USA) at 30°C under aerobic condition. Whole cell homogenates were prepared from young cultures (7 days old). The bacterial culture was centrifuged at 12,000 × *g*, at 25°C for 30 min; the cell pellet was washed with of 0.15 M phosphate buffered saline, pH 7.4 (PBS) and finally resuspended in a small volume of ultrapure distilled water (UDW) containing protease inhibitors (Roche Diagnostics GmBH). The preparation was sonicated at 20 kHz ((Model VC750, Vibra Cell™ Sonics & Materials Inc, USA) in an ice bath at 30% amplitude, 2 sec pulse-on, 2.5 sec pulse-off, for a total of 5 min and the protein content was determined by the method of Bradford (1976).

3.1.2 Heterologous microorganisms

Panel of heterologous antigens other than the leptospires was kindly provided by Assistant Professor Dr. Patcharin Saengjaruk, Faculty of Medicine, Srinkharinwirot University. These included 18 strains of pathogenic *Leptospira* spp. (serovars Autumnalis, Bangkok, Bataviae, Bratislava, Bullum, Canicola, Copenhageni, Cynopteri (3522C), Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona (2 strains), Pyrogenes, Saigon, and Wolfi, strain CH11 of serovar Andamana, 3 strains of saprophytic *L. biflexa* serovar Patoc (strains Patoc 1, P136 and P138), and other organisms, *i.e.* 17 strains of *E. coli*, 2 strains of *Shigella* spp., 8 strains of *Salmonella* spp., 1 strain each of *Klebsiella pneumoniae*, *Proteus vulgaris*, *Staphylococcus aureus* (coagulase positive), *S. aureus* (coagulase negative), *Streptococcus pneumoniae*, group A *Streptococcus* spp., group B *Streptococcus* spp., *Burkholderia pseudomallei*, *Pseudomonas aeruginosa, Orientia tsutsugamuchi*, *Candida albicans*, Cytomegalovirus, Japanese encephalitis virus, Dengue virus types 1 to 4.

Rubella virus, strain H1N1 Influenza virus type A, strain H3N2 Influenza virus type A and one isolate of Influenza virus type B were kindly provide by Associate Professor Taweesak Songserm, Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University.

The whole cell lysates (Ly), sonicates or extracts were prepared from the organisms and they were used for testing the cross reactivities of the monoclonal antibodies secreted by hybridomas produced in this study (Saengjaruk *et al* 2002).

3.2 Murine hybridomas and murine monoclonal antibodies

The culture fluid of hybridoma clones secreting monoclonal antibodies to pathogenic *Leptospira* antigens were obtained and used for 2DE-immunoblotting in the identification of the specific epitopes of pathogenic *Leptospira* spp. proteins.

3.2.1 Immunization of BALB/c mouse

A male BALB/c mouse (six weeks old) was immunized intraperitoneally (i.p.) with 50 μg (100 μl) whole cell homogenate of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae mixed with an equal volume of complete Freund's adjuvant (CFA). Two booster doses were given at two-week intervals *via* the same route using the same dose of immunogen mixed with

incomplete Freund's adjuvant (IFA). Seven days after the last booster dose the mouse was bled and the serum was tested by indirect ELISA against the homologous antigen (Saengjaruk et al., 2002). When a satisfactory level of serum antibody was reached, the mouse was given an intravenous injection with ~1 × 10^6 cells of heat killed *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae. Three days after the intravenous booster the mouse was bled and the immune serum was collected.

3.2.2 Preparation of myeloma cells

The stock P3x-63-Ag8.653 myeloma cells were recovered from cryopreservation in a liquid nitrogen tank at the Molecular Immunlogy Laboratory, Faculty of Allied Health Sciences, Thammasat University Rangsit Center, Pathum-thani. The cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Australia) in a loosen capped culture flask (Costar, Corning Incorporated, New York, USA) at 37°C in a CO_2 incubator until the cells were healthy and actively proliferated. The cells that had been cultured in the medium containing 0.02 mM azaguanine for two weeks were used in the cell fusion. One day prior to the cell fusion, the healthy cells were fed with medium containing 20% FBS **(Section 2, Appendix C)** instead of the previous 10% FBS supplemented medium **(Section 2, Appendix C)**. On the day of the cell fusion, the myeloma cells were harvested and washed two times by centrifugation at 200 × *g* for 15 min with plain RPMI-1640 medium and resuspended in the same RPMI-1640 medium **(Section 1, Appendix C)**. The cells were counted in a hemocytometer and checked for viability using 0.02% trypan blue dye exclusion method. The myeloma cell preparation having the viability more than 95% were used in the cell fusion.

3.2.3 Preparation of the immune spleen cells

On the day of cell fusion, the immunized mouse was bled *via* retro-orbital plexus to collect immune serum (IS); then the mouse was killed humanly by cervical dislocation. The whole mouse body was soaked in 70% ethanol to disinfect the hair and the skin. The spleen was removed aseptically, washed several times in serum-free RPMI-1640 medium (Gibco Co., USA) **(Section 1, Appendix C)** and placed in a glass petridish which contained the serum-free RPMI-1640 medium. The spleen was crushed gently with a constant force by the frosted end of glass slides until the spleen

looked pale in color. The cell suspension was filtered through a 70 μm cell strainer (Becton Dickinson, New Jersey, USA) into a sterile 50 ml conical tissue-culture tube (Costar), and washed once with the serum-free RPMI-1640 medium. Viability of the cells was checked by 0.02% trypan blue dye exclusion method. The spleen cells with more than 95% viability were fused with P3x-63-Ag8.653 myeloma cells.

3.2.4 Preparation of the feeder cells

Spleen cells of a normal BALB/c mouse were used as the feeder cells in nurturing the hybrids in the cell cloning process (monocloning). These feeder cells were prepared as the following:

A BALB/c mouse was killed, the spleen was removed aseptically, washed three times with serum-free RPMI-1640 and the spleen cells were prepared in the H-medium supplemented with 20% FBS as for the preparation of the immune splenocytes **(Section 3.2.3 above)**. The spleen cell concentration was adjusted to 10^6 cells/ml and 100 μl aliquots were plated out into wells of the 96-well tissue culture plates. The feeder cell layer was prepared one day before cell cloning.

3.2.5 Cell fusion

The myeloma cells from Section 3.2.2 and the immune spleen cells from Section 3.2.3 were fused by the method of Galfre *et al*., 1977 and which modified by Chaicumpa *et al.* (1998). Details of the procedure are as the following:

Approximately 10^7 myeloma cells were added to a tube containing about 10^8 spleen cells (the ratio of one myeloma cell to ten spleen cells). The cells were gently mixed and centrifuged at 200 × *g* at 25°C for 10 min and the supernatant was removed. One ml of 50% (w/v) PEG-4,000 (Sigma Chemical Co., St Louis, Minnesota, USA) in NSS, pre-warmed at 37°C, was added drop by drop to the cell pellet with regular agitation to stir up the cells in the pellet. The tube was immediately transferred to a 37°C water bath with constant shaking for 90 seconds. The mixture was then gradually added with about 20 ml of the serum-free RPMI-1640 medium with gentle agitation over a period of 10-15 min and the volume was brought up to 40 ml. The cell preparation was centrifuged at 200 × *g* at 25°C for 10 min and the supernatant was discarded. Warm hypoxanthine-azaserine selective medium **(Section 5, Appendix C)** was added to the cell pellet to give the cell concentration of

approximately 5×10^5 cells/ml. The cell preparation was distributed in 200 µl aliquots into wells of the 96-well tissue culture plates (Costar). The plates were incubated at 37°C in a humidified 5% CO_2 incubator and inspected daily for cell growth or contamination using an inverted microscope (CK2, Olympus, Japan). Contents of the well(s) showing rapid color change and turbidity of the medium which indicated contamination was (were) discarded.

3.2.6 Nurturing of the newly growing hybrids

Approximately 7 days after the cell fusion, some of the growing hybrid cells could be seen under the inverted microscope. The culture medium in the wells was changed every three days by removing 100 µl of the uuper layer of the medium from each well and then replaced with 100 µl of the fresh HA medium containing 20% FBS **(Section 2, Appendix C)**. When the hybrids had grown sufficiently, 100 µl of the spent culture fluids from individual wells were collected, and checked for the presence of specific antibodies to the homologous antigen by an indirect ELISA. The growing hybrids from wells which were positive for the antibodies were transferred from the 96 well plates to the 24 well plates (Costar) containing 1.5 ml of RPMI-1640 medium supplemented with 20% FBS in the individual wells. The culture fluids from the growing hybrid cells were re-screened for antibodies against the homologous antigen by indirect ELISA and Western blotting. Cells in culture wells containing the antibodies with interesting Western blot patterns were individually cloned by the limiting dilution method.

3.2.7 Cloning of the hybridomas

The cells from the antibody-positive wells that showed appropriate bands by SDS-PAGE and Western blot analysis against various pathogenic *Leptospira* antigens WB were identified as polyclones or polyhybrids. They were cloned immediately in order to get rid of the non-antibody producing hybrids. Limiting dilution was the method used for cloning of the polyclones to monoclones which produced the monoclonal antibodies. The details of limiting dilution method are as follows:

The splenocytes of the normal isologous BALB/c mouse were used as the feeder cells for nurturing the hybrids in the cell cloning process. The culture plate of the feeder cells was prepared one day before the cloning **(Section 3.2.4)**. The feeder

cells were diluted to a concentration of 1×10^6 cells/ml and plated 1×10^5 cells (100 µl) into each well of the 96 well tissue culture plate(s) (Costar). The plate(s) was (were) incubated overnight at 37°C in 5% CO_2 incubator for contamination checking before use as the feeders.

The polyhybrid cells from antibody-positive wells were harvested, counted and tested for their viability. A portion of these polyclones were cryopreserved, while cells of another portion were diluted with the serum-supplemented RPMI-1640 medium to the desired concentration. Aliquots of 0.1 ml were allocated into wells of the 96-well plates containing feeder cell layer and incubated as previously mentioned. After five days of cloning, the medium in each well was changed to the fresh serum-supplemented RPMI-1640 medium. The clones were examined daily for cell growth. Only wells containing single clones were fed, propagated and their spent media tested for antibodies by an indirect ELISA and Western blot analysis. The antibody-positive wells containing single clones and the spent culture fluids that gave a specific reaction to homogenates of various *Leptospira* serovars by Western blot analysis were expanded in the 75 mm^3 tissue culture flasks (Costar). The spent medium of each clone was collected and further tested for (1) cross-reactivity to other heterologous bacterial homogenates by the indirect ELISA, (2) determining antibody titers against the homologous antigen by the indirect ELISA, and (3) immunoglobulin isotyping.

3.2.8 Cell cryopreservation

Polyclones, hybridomas and myeloma cells lines can be kept for a long period of time under liquid nitrogen. This frozen storage will protect the cell lines from change by genetic drift and from risk of contamination. In this study, the cells to be frozen were checked for healthy growth and appearance, no contamination, and the presence of the required characteristics for subsequent use. All cells were frozen slowly to avoid damage by forming of intracellular ice crystals. They were also best protected by medium, which has a reduced freezing point. The best freezing medium for hybridomas or myelomas is 90% FBS which contained 10% dimethylsulphoxide (DMSO) (Campbell, 1991). Cells were frozen in a logarithmic growth with high percentage of viability (> 95%).

Cells were grown up to the late logarithmic phase and harvested by centrifugation at 200 × *g* for 10 min. The pellet was resuspended by dropwise adding

of the freezing medium **(Appendix C)** with gentle agitation to make the cell concentration at approximately 1×10^6 to 1×10^7 cells/ml. The cell suspension was aliquoted into the pre-labeled cryovials (0.5 ml/vial; Costar). After the vials were sealed tightly, they were put in a freezing container (Nalgene, Greenville, South Carolina, USA) and placed in a -70°C freezer overnight. The vials were finally transferred to a liquid nitrogen tank for prolonged storage.

3.2.9 Cell thawing from the cryovials

The cryovial of cells was removed from the liquid nitrogen tank and the bottom part was immediately immersed in a 37°C water bath. Care was taken to completely thaw the frozen samples quickly and the vial was not left in the water bath for too long. As soon as liquefied, the cell vial was removed from the water bath. The cells were pipetted carefully with a Pasteur pipette and resuspended in a tube containing 10 ml of the serum-free RPMI-1640 medium. After the cells were washed once with the medium and pelleted by centrifugation at $200 \times g$ for 10 min at 25°C, they were gently resuspended in the serum-supplemented RPMI-1640 medium, transferred to a 75 mm^3 tissue culture flask and incubated at 37°C in the humidified, CO_2 incubator. Growing cells were subcultured every two to three days.

3.3 Characterization of monoclonal antibodies

3.3.1 Indirect enzyme linked immunosorbent assay (ELISA)

The method was used for detecting the specific antibodies and determining the antibody titers and also for cross-reactivity checking in the immune mouse sera and culture supernatants of the polyclones and monoclones. The indirect ELISA **(Appendix D)** was carried out by the following procedure:

Wells of the microtiter plates (Costar) were coated with 0.1 ml of appropriate antigen at the concentration of 10 μg protein/ml in coating buffer (carbonate-bicarbonate buffer, pH 9.6). These optimal concentrations had been previously titrated by using the pools of positive (PS) and negative (NS) control sera and 1:3,000 of the goat anti-mouse immunoglobulins-horseradish peroxidase conjugate (Southern Biotechnology). The antigen was allowed to attach to the solid surface of the plates by incubating at 37°C overnight. The unbound antigen was extensively washed away with phosphate buffered saline, pH 7.4 (PBS), containing 0.05% Tween-20 (PBST).

The unoccupied sites on the surface of the wells were blocked with the blocking solution, *i.e*. the PBS containing 1% bovine serum albumin (BSA) (200 µl per well) at 37°C in a humidified chamber for one hour and washed again with three changes of the PBST. After washing, appropriate antibody preparations and dilutions were added (100 µl per well) to appropriate wells. The positive and negative control sera were also included in the test. The PBS was included in some wells as blanks. The plates were incubated as for the blocking step; after the incubation they were washed as above and incubated with the rabbit anti-mouse immunoglobulin-horseradish peroxidase (HRP) conjugate, diluted 1:1,000 in the diluent for one hour. The excess conjugate was washed away, 100 µl of *p*-phenylene-diamine dihydrochloride (PPD) (Sigma Chemical Co.) substrate solution which was freshly prepared was added to each well and the plates were kept at room temperature (25°C) in the dark for 30 min. The enzyme-substrate reaction was stopped by adding 50 µl of 1 N NaOH solution. The optical density (OD) of the content in each well was determined against the blank (wells to which PBS was added instead of the antibody preparation) at 492 nm using an ELISA reader (Multiscan *EX,* Labsystems, Helsinki, Finland). The OD at 0.05 or more was taken as positive reaction. The smallest amount of the antibody preparation which still gave positive indirect ELISA was one ELISA unit (one EU). The antibody titer of any preparation was the highest dilution which still gave positive indirect ELISA.

3.3.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate (SDS-PAGE) was used to analyze the complexity of protein profiles and resolution of the antigens prior to electroblotting onto nitrocellulose paper (NC) for WB analysis or staining for direct visualization of the separated components. The techniques described by Laemmli (1970) were followed with some modifications. The 8.0 × 9.5 cm vertical slab gel was prepared by using the casting apparatus (SE260 Mini Vertical, Amersham Biosciences) while electrophoresis was done in an electrophoretic chamber with an electric power supply (model EPS 301, Amersham

Bioscience). A 4% acrylamide stacking gel and 12% acrylamide separating gel were used in this process **(Appendix E)**.

3.3.3 Preparation of samples for loading into the slab gel

Samples were denatured by diluting with four volumes of sample buffer **(Section 1, Appendix E)** and heating at 100°C for 4 min before carefully loaded into the slots in the stacking gel. The desired amount of the sample was carefully applied into each well. Prestained SDS-PAGE broad range molecular weight standard (Bio-Rad, Hercules, California, USA) were included in one slot of each gel slab. Care was taken not to contaminate the adjacent wells with the samples.

3.3.4 Running the gel

Electrophoresis was carried out in an electrophoretic chamber at 10 mAmp/gel during the first 15 min and at 20 mAmp/gel until the tracking dye reached the lower edge of the gel.

After electrophoresis, the gel was removed from the glass. It was then either stained and destained for direct visualization of the separated components or proceeded to the electro-transblotting onto the nitrocellulose membrane for Western blot analysis.

3.3.5 Staining and destaining of protein bands

Protein bands were revealed by staining the gel in Colloidal Coomassie Brilliant Blue staining solution **(Section 4, Appendix B)** as follows:

The slab gel from SDS-PAGE containing separated proteins was fixed in fixing solution **(Section 4.1, Appendix B)** for one hour on an orbital shaker or platform rocker then the gel was transferred into the Colloidal Coomassie Brilliant Blue staining solution (PlusOne, Amesham Biosciences) at 25°C for 18 h **(Section 4.4, Appendix B)**. After that, the staining solution was removed and the gel was soaked in neutralization solution **(Section 4.5, Appendix B)** for 10 min and transferred to the washing solution **(Section 4.6, Appendix B)** for 1 min or until the background color was adequately reduced. When destaining was completed, the gel was store in stabilizing solution **(Section 4.7, Appendix B)** for one hour or for over weekend. The gel was imaged at this stage by Image Scanner Model UTA-1100 and ImageMaster™ Software (Amersham Biosciences).

3.3.6 Silver staining

Silver stain detects a wider variety of macromolecules including nucleic acids, glycoproteins and lipoproteins. In this study, Amersham Biosciences's Silver staining kit was used **(Section 5.10, Appendix E)**. First, the gel was placed in the fixing solution with gentle agitation for 30 min, washed 3 times with 20 ml deionized distilled water (UDW) for 5 min with gentle agitation. After washing with distilled water the gel was placed into the sensitizing reagent with gentle agitation for 30 min, washed with 20 ml UDW for 5 min for 3 times with gentle agitation. The gel was placed into the sensitizing reagent with gentle agitation for 20 min, washed 2 times with 20 ml UDW for 1 min with gentle agitation. The gel was placed into the developing solution and left shaking for 2-5 min or until desired staining intensity was reached. The gel was stained until the bands turned brown or black, then it was placed in stop solution to stop the reaction for 10 min. The stop solution was removed and the gel was finally washed in UDW three times for 5 min each and stored in 1% glycerol (v/v) in UDW prior to drying.

3.3.7 Western blot analysis

The homogenate of *Leptospira* spp. resolved in the polyacrylamide gel after electrophoresis were electroblotted onto a sheet of nitrocellulose membrane (NC) (Hybond ECL, Amersham Biosciences, Upsala, Sweden). Western blot analysis as described by Towbin *et al.* (1973) was followed **(Appendix G)**. After blotting, the unbound sites on the NC were blocked by soaking in a blocking solution (3% BSA, in TBS, pH 7.4) **(Section 5, Appendix G)** at 25°C with gentle rocking for 1 hour. The NC was then washed to remove the excess BSA with three changes of washing buffer **(Section 4, Appendix G)** (over a period of 15 min). It was then reacted with the antibody preparation (monoclonal antibodies, polyclonal antibodies or immune mouse serum; IS) for an hour at 25°C on a rocking platform. The paper was washed three times with the washing buffer. The membrane was then put in a solution containing anti-mouse immunoglobulin-alkaline phosphatase conjugate (Southern Biotech, AL, USA) (1:3,000 in 0.2% BSA in TBS, pH 7.4) for an hour at 25°C with continuous shaking. After excessive washing (5 times for 5 min each) with the washing buffer, the NC was rinsed with Tris buffer (0.15 M Tris-HCl, pH 9.6) and placed in a freshly prepared substrate solution (The BCIP/NBT phosphatase substrate [Kirkegaard &

Perry Laboratory, USA] **(Section 8, Appendix G)** until the protein bands appeared. Then the NC was washed with DW until the background was cleared.

3.3.8 Isotyping of the monoclonal antibodies

Isotypes of MAbs were determined by Mouse typer kit (Bio-Rad) which could subtype the mouse immunoglobulins into IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, kappa (κ) and lambda (λ) chains by ELISA. One hundred microliters of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Icterohaemorrhagiae homogenate in coating buffer (10 μg/ml) was added into each well of ELISA plate. The plate was covered and incubated at 37°C for overnight. Unbound Ag was removed by washing three times with PBST. MAb preparation (100 μl) was added to appropriate wells, using the suggested format outlined in **Figure 2**. The plates were incubated for 1 hour at 37°C, then washed with PBST three times. Rabbit anti-mouse Ig panel reagents were added to the wells using the format in **Figure 2**. Rows A to H were filled with the respective panel reagents, 100 μl/well. The plates were incubated for 1 hour at 37°C and washed three times with PBST. The wells were then added (100 μl/well) with diluted (1:3,000) goat anti- rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) and incubated for 1 hour at 37°C. After washing four times with PBST, 100 μl of freshly prepared peroxidase substrate solution was added to each well. Positive reaction was assessed after 30 min at 25°C. Color development was stopped by adding 100 μl/well of 2% oxalic acid. The results were read at 405 nm with ELISA reader (Multiskan, EX Labsystem, Helsinki, Finland).

3.4 Mouse immune serum

The immune serum of the splenocyte donor mouse for the production of hybridomas secreting monoclonal antibodies to pathogenic *Leptospira* spp. antigens was used for 2DE-immunoblotting of the antigenic components and immunomes of pathogenic *Leptospira* spp. proteins.

3.5 Acute phase and convalescence phase sera of leptospirosis patients

Serum samples were collected from patients admitted to Khon Kaen Provincial Hospital. The patients were clinically diagnosed of having leptospirosis according to WHO criteria and whose sera were positive for antibodies to *Leptospira* spp. by IgM Dipstick and MAT. The first serum samples were collected from the patients on the

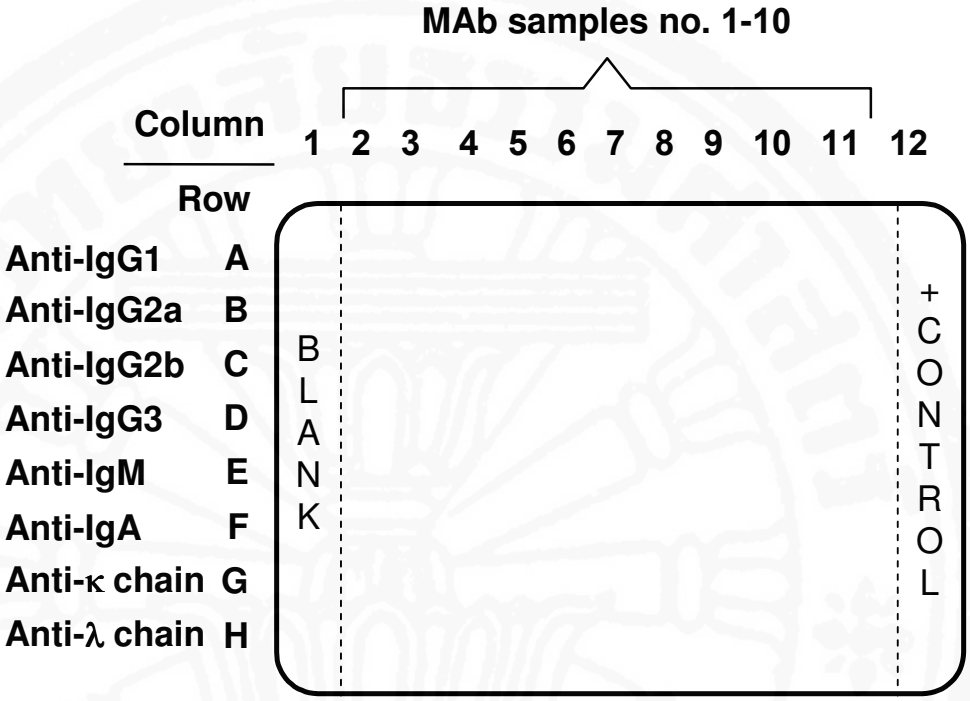


Figure 2 Work chart for isotyping and sub-isotyping of ten MAb samples (column 2-11) on one microtitration plate

first day of their hospitalization and the follow-up samples were collected during day 3rd, day 7th, day 14th of the convalescence phase.

Sera from patient number 1 were collected on the first (D1) and third day (D3) of the hospital arrival which were the fourth and sixth day of fever onset. These sera were positive by IgM dipstick assay (Organon, Belgium), Immunofluorescence assay and MAT (Bratislava 1:100).

Sera from patient number 2 were collected on the first (D1) and fourteenth day (D14) of the hospital arrival which were the fifth and eighteenth day of fever onset. These sera were positive by IgM dipstick assay (Organon, Belgium), Immunofluorescence assay and MAT (Pyrogenes 1:100, Saigon 1:400).

Sera from patient number 3 were collected on the first (D1) and fourteenth day (D14) of the hospital arrival which were the sixth and nineteenth day of fever onset. These sera were positive by IgM dipstick assay (Organon, Belgium), Immunofluorescence assay and MAT (Saigon 1:100).

Sera from patient number 4 were collected on the first (D1) and fourteenth day (D7) of the hospital arrival which were the fourth and tenth day of fever onset. These sera were positive by IgM dipstick assay (Organon, Belgium), Immunofluorescence assay and MAT (Bratislava 1:100, Wolffi 1:100).

3.6 Sera of other febrile illnesses

Serum samples were collected from non-leptospirosis patients admitted to Khon Kaen Provincial Hospital. They were clinically diagnosed of having other febrile illness (patient control) *e.g.* melioidosis and dengue hemorrhagic fever (DHF).

3.7 Sera of normal individuals residing in leptospirosis endemic area

The serum samples of normal healthy individuals who lived in Khon Kaen Province were colleted and used as negative controls.

Written informed consent was obtained from each subject or the legal representative before serum sample collection.

4. Two-dimensional gel electrophoresis (2DE)

4.1 Preparation of *Leptospira* whole cell homogenates for 2DE

Leptospira interrogans, serogroup Icterohaemorrhagiae, serovars Copenhageni and Icterohaemorrhagiae and *L. borgpetersenii*, serogroup Tarassovi, serovar Tarassovi at log phase of growth in liquid EMJH medium were harvested individually

by centrifuging the culture at 12,000 × *g* at 4°C for 30 min (Sorvall). After the supernatant was discarded, the pellet was washed three times with standard cell wash buffer by centrifugation as above but only for 5 min each time. Then the standard cell wash buffer **(Section 1, Appendix B)** was removed. The bacterial pellet was resuspended in standard cell lysis buffer (30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS, 2% IPG buffer pH 3-10) **(Section 1, Appendix F)** containing protease inhibitors (Roche Diagnostics GmBH) and was left on an ice bath for 10 min. The bacterial suspension was subjected to sonication for 5 min in the ice bath at 30% amplitude, 2 sec pulse-on, 2.5 sec pulse-off, for a total of 5 min. After sonication, the samples were centrifuged. The supernatant in each tube was transferred to a new tube. This homogenates were stored in aliquots at −70°C.

Each *Leptospira* homogenate was cleaned with the 2D-Clean-up kit (PlusOne, Amersham Biosciences) to eliminate detergents, salts, lipids, phenolics, and nucleic acids. After cleaning, the preparation was resuspended in the rehydration buffer (2 M thiourea, 7 M urea, 4% CHAPS) **(Section 3, Appendix F)**. The protein concentration of each sample was determined using the 2D-Quant kit (PlusOne, Amersham Biosciences) before subjecting it to the first dimension electrophoresis.

4.2 Rehydrating IPG strips in the presence of protein samples

The *Leptospira* homogenate (30-60 µg protein) was added to the rehydration solution containing 0.18 M DTT or 1.2% Destreak reagent™ (Amersham Biosciences) and 0.5% IPG buffer (pH 3-10NL, IPG strip 3-10NL; pH 3.5-5.0, IPG strip 3-5.6NL; pH5.5-6.7, IPG strip 5.3-6.5; pH6-11, IPG strip 6.2-7.5; pH 7-11NL for IPG strip 7-11NL, respectively) (Amersham Biosciences). A portion (125 µl) of each homogenate was pipetted and delivered slowly into each slot of the reswelling tray. Care was taken not to produce any bubbles. The IPG strip of each range was placed (right side down) into the strip holder containing the sample corresponded to the pH range of their IPG buffer, then 1 ml of dry strip cover fluid was added to the strip holder. The IPG strip was allowed to rehydrate at 25°C for 12-15 h.

4.3 Isoelectric focusing parameters for 7 cm IPG strip

After complete rehydration, the IPG strips were transferred with gel faced up onto the IPGphor manifold, which placed on the IPGphore platform. IEF was

performed at 20°C, 50 μA/IPG strip, using the parameters provided by the instruction manual of Immobiline™ DryStrip of each pH range **(Table 5)**.

4.4 Second dimension SDS-PAGE

The second dimension gel electrophoresis was performed after IEF steps were completed, the electrofocused IPG strip was equilibrated in 10 ml SDS-equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% Bromphenol blue) containing 100 mg dithiothreitol for 15 min **(Section 4.1, Appendix F)**. Subsequently, the strip was placed in 10 ml of the equilibration buffer containing 250 mg iodoacetamide for 15 min. The strip was washed with electrode buffer and overlaid onto a 12.5% polyacrylamide gel (8 × 9.5 cm) cast in SE260 apparatus (Mini Vertical, Amersham Biosciences). SDS-PAGE was carried out at 10 mAmp/gel during the first 15 min and at 20 mAmp/gel until the tracking dye reached the lower edge of the gel. After SDS-PAGE, the gel was either stained with colloidal Commassie Brilliant Blue G-250 dye or silver staining for the *Leptospira* spp. proteomics or the separated components in the gel were electro-transblotted onto a PVDF membrane for further 2DE-immunoblotting with either monoclonal antibodies (MAb), acute phase sera and convalescence phase sera of leptospirosis patients, sera of patients with other febrile illnesses or sera of normal individuals.

4.5 2DE-immunoblotting

The NC or PVDF membrane blotted with the 2DE-gel separated-*Leptospira* components was placed in a blocking solution (3% bovine serum albumin in Tris buffered saline, pH 7.4 [TBS]) **(Section 2 and 5 Appendix G)** at 25°C for 1 hour. After washing with the washing buffer (TBS containing 0.05 Tween-20 [TBS-T]) **(Section 4, Appendix G)**, the blot was incubated with specific mouse monoclonal antibodies to pathogenic *Leptospira* spp. (MAbLPF1 and MAbLPF2), mouse immune serum, acute and convalescing leptospirosis patients' sera, sera of patients with other febrile illnesses and sera of normal healthy individuals from leptospirosis endemic area at 25°C for 2 h or at 4°C for 18 h. The membrane was washed thoroughly with the washing buffer; it was placed in a goat anti-mouse immunoglobulins-alkaline phosphatase (AP) conjugate (Southern Biotechnology, Bermingham, Alabama, USA; diluted 1:2,000) or goat anti-human-immunoglobulin G (IgG)-alkaline phosphatase

Table 5 Guidelines for running 7-cm Immobiline DryStrip™ gels on Ettan IPGphor II Isoelectric Focusing Unit. Running condition**:** temperature at 20°C; current at 50 μA *per* strip

pH intervals	Voltage mode	Voltage (V)	Time (h:min)	kVh
3–11NL	1 Step and Hold	300	0:30	0.2
3–10	2 Gradient	1000	0:30	0.3
6–11	3 Gradient	5000	1:20	4.0
	4 Step and hold	5000	0:06–0:25	0.5–2.0
	Total		2:26–2:45	5.0–6.5
3–10NL	1 Step and Hold	300	0:30	0.2
4–7	2 Gradient	1000	0:30	0.3
3–5.6NL	3 Gradient	5000	1:30	4.5
	4 Step and hold	5000	0:12–0:36	1.0–3.0
	Total		2:42–2:45	6.0–8.0
7–11	1 Step and Hold	300	0:30	0.2
	2 Gradient	1000	1:00	0.7
	3 Gradient	5000	1:30	4.5
	4 Step and hold	5000	0:20–0:55	1.6–4.6
	Total		3:20–3:55	7.0–10.0
5.3–6.5	1 Step and Hold	300	1:00	0.2
6.2–7.5	2 Gradient	1000	1:00	0.7
	3 Gradient	5000	2:30	7.5
	4 Step and hold	5000	0:45–1:30	3.6–7.6
	Total		5:15–6:00	12.0–16.0

Source: Görg A. In: 2-D electrophoresis: Principles and Methods. Buckinghamshire, UK. GE Healthcare Limited, 2005.

(AP) conjugate (Southern Biotechnology; diluted 1:2,000) in a diluent at 25°C for 1 h. After washing with the washing buffer and placing it in 0.15 M Tris-HCl, pH 9.6 for 10 min, the membrane was incubated with BCIP/NBT substrate (KPL, Gaithersburg, Maryland, USA) in the dark for 10 min. The enzyme-substrate reaction was stopped by rinsing the membrane with deionized water.

4.6 Liquid chromatography/mass spectrometry (LC/MS)

The proteins in the gel plugs revealed by the Colloidal Coomassie Brilliant Blue stain (Neuhoff *et al*., 1985) corresponded to the proteins reactive to the leptospirosis patients' convalescing sera but not react or react only weakly to sera of leptospirosis patient's acute phase sera and did not react to sera of patients with other febrile illnesses and sera of normal individuals in the 2DE-WB, were subjected to mass spectrometry. The peptide mass fingerprints were identified by comparing with those in the data bank.

The post stained-gel corresponded to the proteins reactive to murine monoclonal antibodies (MAb) specific to pathogenic *Leptospira* spp. (epitopes unique to pathogenic *Leptospira* spp.) and mouse immune serum (*Leptospira* immunome) were similarly studied.

From this experiment, the *in vivo* expressed proteins of pathogenic *Leptospira* spp. were revealed.

Protein spots corresponding to the criteria as mentioned above were cut from the 2DE- Colloidal Coomassie Brilliant Blue post stained-gel (previously described in **Section 2.8.4**). They were subjected to in-gel reduction, alkylation and trypsin digestion (previously described in **Section 2.7.5**). For LC/MS analysis, a nano-LC system (Finnigen) was coupled to a Finnigen LTQ (Thermo Electron Corporation). Peptide separation was done using a C18 reversed phase column (BioBasic-18; column dimensions 100 × 0.15 mm, 5 μm particle size) and a gradient of 0 to 60% B in A (A = 0.1% formic acid in water; B = 0.1% formic acid in acetonitrile) for 22 min at a constant flow rate of 200 μl/min. Each digest was analyzed in LC/MS mode for identification. Fragmentation of the peptides was performed in data-dependent mode and mass spectra were acquired in continuum mode.

5. Construction of DNA sequences for DNA prototype vaccine (s)

5.1 Selection of *Leptospira* genes candidate

The representative candidate gene for leptospirosis DNA vaccine was selected from the data obtained from **Section 2** and **Section 4**.

5.2 Preparation of *Leptospira* genomic DNA for DNA templates

The genomic DNA of pathogenic *Leptospira* was extracted from *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni by phenol-chloroform extraction by the step as follows;

Leptospira interrogans serogroup Icterohaemorrhagiae serovar Copenhageni was grown in modified Tween 80-albumin EMJH medium **(Section 1, Appendix A)**. Two milliters of the fully-grown leptospires was collected by centrifugation (Sorvall) at 12,000 × *g* at 4°C for 10 min and the pellet was washed twice with PBS pH 7.4 by centrifugation as above. The pellet was resuspended in 567 µl of TE buffer **(Section 1, Appendix J)** and mixed by vortexing. Then 30 µl of lysis buffer containing 10% SDS, 3 µl of 20 mg/ml proteinase-K, 5 µl of 20 mg/ml RNase A, were added, mixed and incubated at 37°C for 1 h. Then 100 µl of 5 M sodium chloride (NaCl) and 8 µl of Cetyltrimethylammonium bromide (CTAP) were added. The mixtures were centrifuged at 12,000 × *g* at 25°C for 5 min. The top phase was transferred to a new tube and 0.6 volume of isopropanol was added and mixed. The DNA pellet was collected by centrifugation at 12,000 × *g* at 25°C for 10 min and washed with 70% ethanol then allowed to be air. The pellet was dissolved by adding 50 µl of TE buffer. The quality of the genomic DNA template was determined by amplification of 16S RNA gene using two primers (forward primer, 5′-GGCGGCGCGTCTAAACATG-3′ and reverse primer, 5′-TTCCCCCCATTGAGCAAG-3′) generated from 16SrDNA of *L. interrogans* serovar Canicola strain Moulton (Suwimonteerabutr *et al,* 2005). The mixture of PCR reaction for the amplification of the 16S RNA gene contained, UDW (13.25 µl), 10 × buffer (2.5 µl), 100 mM dNTPs mix (2 µl), 0.5 µm forward primer (2.5 µl), reverse primer (2.5 µl), template (genomic DNA) (2 µl), and Taq polymerase (2 units/µl) (0.25 µl). The PCR condition was; 94°C, 5 min; 94°C, 1 min; 63°C, 30 sec; 72°C, 2 min; 30 cycles and extension at 72°C for 10 min. The DNA amplicons were examined by 1% agarose gel electrophoresis stained with ethidium bromide and

visualized under a UV transluminator (Biodoc-It ™ Imaging system, UVP Transluminator, Cambridge, UK).

5.3 Primer designs for amplification of DNA vaccine candidate genes of *Leptospira* spp.

The primers for amplification of DNA vaccine candidate gene (ID LIC 10973) encoded the outer membrane protein (OmpL1) of *L. interogans* serogroup Icterohaemorrhagiae serovar Copenhageni were designed using *Leptospira* whole genome sequences that were available in Genbank database.

Forward primer:

5′-GGCTCGAGCCATGGCATTATCTTCGGCTGCA-3′ (*Xho*I)

Reverse primer:

5′-GCTCTAGACTGTAGATTTGCCCACCGA-3′ (*Xba*I)

The amplified gene segment products were cloned into an appropriate vector.

5.4 Amplification of DNA gene segments

The *ompLI* of *Leptospira* was amplified by PCR using the appropriate DNA primers. The gradient PCR reactions were performed for determining the optimum annealing temperatures.

The PCR mixtures for the amplification of the gene contained; UDW (17.25 µl), 10 × buffer (2.5 µl), 100 mM dNTPs mix (2 µl), 0.5 µm forward primer (1.0 µl), reverse primer (1.0 µl), template (genomic DNA) 2 µl, and *Taq* polymerase (2 units/µl) (0.25 µl). The gradient annealing temperature of the PCR reactions were between 52°C to 61°C. The DNA amplicons were examined by 1% agarose gel electrophoresis and stained with ethidium bromide and visualized under a UV transluminator

5.5 Cloning of DNA segments into pGEM-T Easy vector

5.5.1 Purification of DNA amplicons

The PCR amplicons of *ompL1* from section 5.4 were purified by the ethanol precipitation method. One volume of the DNA amplicons was transferred to a new tube and 1/10 volume of 3 M sodium acetate and two volumes of absolute ethanol were added to the tube. The reaction mixture was placed on ice bath for 2 h. The

DNA pellet was collected by centrifugation at 12,000 × *g* for 15 min and washed with 70% ethanol then air dried. The pellet was dissolved with a small volume of UDW.

5.5.2 Ligation of DNA amplicon into pGEM-T Easy vector

The purified PCR amplicon was ligated into pGEM®-T Easy vector (Promega, Wisconcin, USA) *via* the overhang T of the vector and A of the DNA amplicon. The following components were added in order to the ligation tube in the following order; 5 µl of 2x rapid ligation buffer, 1 µl of pGEM®-T Easy vector (50 ng), 3 µl of eluted *ompL1* gene fragment (~150 ng), 1 µl of T4 DNA ligase (3 Weiss units/µl) and sterile UDW to a final volume of 10 µl. The mixture was incubated overnight at 4°C for the maximum number of transformants.

5.5.3 Transformation of recombinant plasmid into competent *E. coli*

5.5.3.1 Preparation of competent cells

A single colony of *E. coli* JM109 strain was inoculated into 5 ml of LB broth **(Section 1, Appendix I)** and incubated for 18 h at 37°C in a shaking incubator. One hundred microliters of the overnight cultured was inoculated into 10 ml of LB broth and incubated at 37°C with shaking until it reached the log phase (OD_{600} = 0.5). The cells were then spun at 4,000 × *g* for 10 min at 4°C and the supernatant was carefully discarded. The cell pellet was gently resuspended in 1 ml of ice-cold TSS **(Section 3.1, Appendix J)** and placed on ice. Cells must be used for transformations within 2-3 h.

5.5.3.2 DNA transformation

The ligation product was used to transform into the competent *E. coli* cells by chemical transformation protocol (Hanahan, 1983). Three microliters of the ligation product was added into 100 µl of competent *E. coli* strain JM109 cells. After gently mixing, the cells were incubated on ice for 20 min to allow attachment of the vector to the competent cells. Subsequently, the mixture was heat shocked at 42°C in a water bath for 1 min and immediately placed on ice for 2 min. Then, 900 µl of warm SOC medium **(Section 3.2.2, Appendix J)** was added to the mixture and incubated at 37°C in a shaking incubator for 1 hour. Two-hundred microliters of the cultured was spreaded on each LB-ampicillin (100 µg/ml)/100 mM IPTG/5% X-Gal agar plate and incubated for 18 h at 37°C. After which transformant *E. coli* colonies

were randomly picked and screened for the recombinant clones carrying the recombinant plasmids using PCR amplification.

5.5.3.3 Screening for positive clones

Positive colonies (white colonies) were selected and subcultured into LB-ampicillin broth. The bacterial suspension was incubated at 37°C for 18 h in a shaking incubator. The cell suspension was harvested by centrifugation at 4,000 × *g* for 5 min. Plasmid extraction of these clones was performed using the standard alkali lysis protocol.

The *E. coli* cell pellet was resuspended with 200 µl of solution I (50 mM Glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) **(Section 4.1, Appendix J)** containing ribonuclease A (RNase A, 20 µg/ml) and mixed by tube inverting. Four hundred microliters of solution II (0.1 N NaOH, 1% SDS) **(Section 4.2, Appendix J)** was then added and the preparation was incubated on ice for 5 min. The mixture was added with 300 µl of solution III (3 M potassium acetate, pH 5.2) **(Section 4.3, Appendix J)**, incubated on ice for 5 min and centrifuged at 12,000 × *g* for 5 min. The supernatant was collected from the top layer of the mixture. The plasmid was precipitated from the top phase of the mixture by adding 0.6 volumes of isopropanol and centrifugation at 12,000 × *g* for 5 min at 25°C. The plasmid pellet was air-dried at 25°C before dissolving in small amount of UDW.

The integrity of the plasmids was analyzed by 1% agarose gel electrophoresis, ethidium bromide staining and visualiztion under a UV transluminator. The positive clones which had higher sizes when compared with the original pGEM®-T Easy vector were chosen for double digestion with *Xho*I and *Xba*I restriction endonuclease enzymes for the presence of the DNA inserts.

5.6 Cloning of *Leptospira* vaccine candidate genes into pcDNA 3.1(+) vector

The positive recombinant plasmid containing the *ompL1* in the pGEM-T Easy vector **(Figure 3)** was cut with the appropriate restriction endonucleases at 5′ and 3′ ends. The cut plasmid was purified by 1% agarose gel electrophoresis. The DNA fragments were cut from the gel and purified with GENECLEAN II kit (Bio101, La Jolla, California, USA). The gel slices (0.1 g, equal approximately 100 µl) were mixed with 3 volumes of 6 M NaI in a microcentrifuge tube and melted at 55°C for

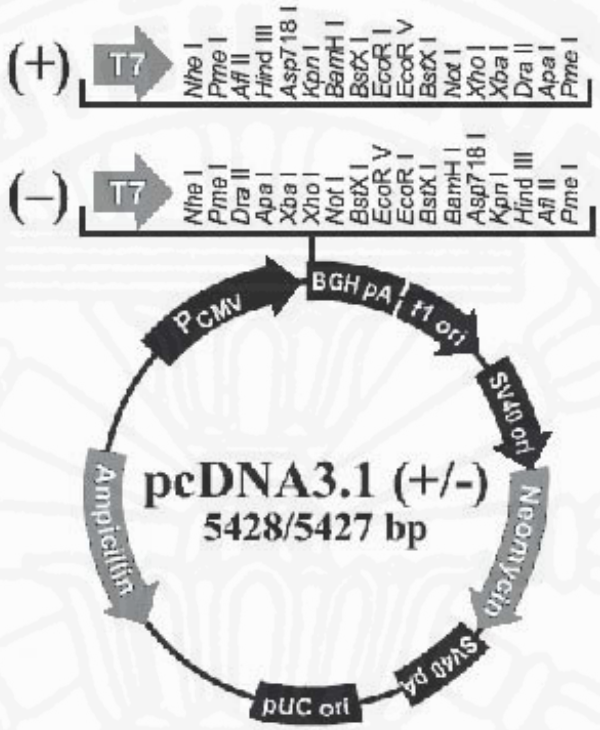


Figure 3 Map of pcDNA3.1[+]

5-10 min until all gel were completely dissolved. Then 5 µl of glass milk suspension was added and incubated at 25°C for at least 10 min with mixing every 1-2 min to allow binding of the DNA to the silica matrix. The contents were then spun down at 12,000 × *g* for approximately 10 sec and the supernatant was discarded. The glass milk pellet was washed with 500 µl of washing buffer and spun at 12,000 ×*g* for 5 sec. After 3-time washing, the pellet was air-dried by leaving the microcentrifuge cap open for 5-10 min. The pellet was then dissolved in 20 µl of sterile UDW and spun at 12,000 × *g* for 30 sec. The supernatant containing the eluted DNA was carefully removed to a new tube.

The purified *ompL1* PCR amplicon was ligated into the cut expression vector pcDNA 3.1(+) between the appropriate restriction endonuclease sites. The following components were added to the ligation tube in the following order; 5 µl of 2× rapid ligation buffer, 1 µl of pcDNA 3.1(+) (50 ng), 3 µl of eluted *ompL1* gene fragment (≈ 150 ng), 1 µl of T4 DNA ligase (3 Weiss units/µl) and sterile UDW to a final volume of 10 µl. The mixture was incubated at 4°C for 18 h. Then the recombinant plasmid was transformed into the competent *E. coli* strain Top10 prepared by the methods mentioned above. The transformants were spreaded on LB-ampicillin (100 µg/ml) agar plate.

5.6.1 Examination of the positive *E. coli* clones

The positive clones harboring the plasmid with the *Leptospira* genes were picked and cultured in LB-ampicillin (100 µg/ml) broth and incubated at 37°C in a shaking incubator (250 rpm) for 18 h. The bacterial cell suspension was collected by centrifugation at 7,000 × *g*, at 25°C for 5 min. The plasmid was isolated and examined by cutting with the appropriate restriction endonuclease enzymes. The size of *Leptospira* DNA inserts were examined and estimated by comparison with the 1 kb DNA ladder after agarose gel electrophoresis.

5.6.2 Preparation of recombinant expression vector

The recombinant plasmid in an *E. coli* transformant carrying *ompL1* of *Leptospira* was propagated in LB-ampicillin broth. The bacterial suspension was incubated at 37°C for 18 h in a shaking incubator (250 rpm). The *E. coli* culture was centrifuged at 7,000 × *g*, 25°C then the plasmid DNA containing the *ompL1* was

extracted and examined for the correct size of the *Leptospira* DNA insert. The stock cultures of *E. coli* clones harboring plasmid DNA with the o*mpL1* were kept in 80% (v/v) glycerol at –70°C.

5.6.3 Preparation of recombinant pc DNA 3.1(+) plasmid

The pcDNA 3.1(+) plasmid containing o*mpL1* insert of *Leptospira* for DNA vaccine immunization in experimental animal was prepared by the following steps.

5.6.3.1 Preparation of *E. coli* with recombinant plasmids

Both *E. coli* strain Top10 containing original pcDNA and those with pcDNA containing the *ompL1* insert of *Leptospira* were isolated on LB agar plate (100 μg/ml ampicillin) **(Appendix I)**. The isolated colonies were subcultured in 500 ml of LB broth (100 μg/ml ampicillin) **(Appendix I)** in 2,000-ml erlenmeyer flasks. The flasks were incubated in a shaking incubator (200 rpm) at 37°C for 18 h. The bacterial cells were harvested by centrifugation at 4,200 × *g* at 4°C for 30 min. The pellet in each flask was washed with sterile normal saline (NSS) by centrifugation as mention above.

5.6.3.2 Extraction of pcDNA plasmid

The plasmid extraction was performed on the bacterial pellets obtained from section 5.5.3.1 was extracted by mixing with 16 ml of solution I **(Section 4.1, Appendix J)** and RNase A was added to yield the final concentration at 20 μg/ml. Then 24 ml of solution II **(Section 4.2, Appendix J)** was added, kept on ice bath for 5-10 min then 24 ml of solution III **(Section 4.3, Appendix J)** was added. The mixture was mixed and centrifuged at 12,000 ×*g*, 4°C for 30 min. The supernatant was transferred to a new tube then 38.4 ml (1 volume) of isopropanol was added, mixed and centrifuged at 12,000 × *g*, 4°C for 15 min. Then the plasmid pellet was washed with 70% ethanol and air-dried at 25°C. The pellet was resuspended with 1 ml of UDW. The crude plasmid was purified by adding 1 ml of phenol-chloroform-isoamyl alcohol (PlusOne, Amersham Biosciences), mixed and centrifuged at 12,000 × *g*, 4°C for 10 min. The supernatant was transferred to new tube then 0.6 ml of isopropanol was added. The pellet obtained after centrifugation of the mixture was washed with 70% ethanol. The quantity of the purified plasmid was measured by UV

spectrometry (at 260/280 nm) and the quality was examined by agarose gel electrophresis.

5.7 Study of the immunogenicity and protective efficacy of the DNA vaccine against *Leptospira* infection

The purified plasmids from **Section 5.6.3.2** were used as an *ompL1* DNA vaccine for the immunogenicity and protective efficacy against *Leptospira* infection in experimental animal model (Golden Syrian hamsters).

Animal experiments were performed following the guideline of the National Research Council of Thailand and were approved by the Ethical Committee of the Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand. Twenty-four female Golden Syrian hamsters (*Mesocricetus auratus*) at four week-old were obtained from the National Laboratory Animal Center, Mahidol University Salaya Campus, Nakhon Pathom province, Thailand. They were allowed to adapt to the domesticated condition at the animal facility at the Faculty of Alled Health Sciences, Thammasat University for one week before commencing the experiment.

Six hamsters (group 1) were immunized intramuscularly with plasmid-*Leptospira* o*mpL1* gene DNA, 100 µg in 100 µl PBS per dose at fore and hide limbs; Six hamsters in group 2 were immunized with the same dose of empty plasmid DNA Six hamsters of group 3 (placebo) and group 4 (negative control) were injected with PBS three time at two-week intervals. The blood of individual hamsters in each group was collected on days 0, 7, 14, 28 and 42 after the first immunization. The sera were examined for the specific anti-*Leptospira* antibodies using an indirect ELISA.

The protective efficacy against heterologous *Leptospira* infection was performed by challenging all hamsters in each group except group 4 (negative control) with heterologous human clinical primary isolate, *Leptospira interrogans* serogroup Pomona serovar Pomona. One thousand leptospires (10 LD50) in 100 µl of PBS was injected intraperitoneally to each hamster at day 49. The animals in all groups were daily observed and any dead animals were recorded until day 21 post-challenging.

Brain, lung, heart, liver, spleen and kidneys of all dead and survived hamsters on day 21 in all groups were collected and processed for further examination.

An Indirect ELISA

Indirect ELISA was used for detecting and determining the specific antibodies and antibody titers in the immune sera of *ompL1* vaccine immunized hamsters. The indirect ELISA **(Appendix D)** was carried out by the following procedure:

Wells of the microtiter plates (Costar) were coated with 0.1 ml of appropriate antigen at the concentration of 10 μg protein/ml in coating buffer (carbonate-bicarbonate buffer, pH 9.6). These optimal concentrations had been previously titrated by using the pools of positive (PS) and negative (NS) control sera and 1:3,000 of the goat anti-hamster immunoglobulins-horseradish peroxidase (HRP) conjugate (Southern Biotechnology). The antigen was allowed to attach to the solid surface of the plates by incubating at 37°C for 18 h. The unbound antigen was extensively washed away with phosphate buffered saline, pH 7.4 (PBS), containing 0.05% Tween-20 (PBST). The unoccupied sites on the surface of the wells were blocked with the blocking solution, *i.e*. PBS containing 1% bovine serum albumin (BSA) (200 μl per well) at 37°C in a humidified chamber for 1 h and washed again with three changes of the PBST. After washing, the diluted serum (1:100) of each sample was added to the first row, two-fold dilution of the serum in diluent (0.2% BSA and 0.2% galatin in PBS pH 7.4) was performed until the last well (1:6,400). The positive and negative control sera were also included in the test. The PBS was included in some wells as blanks. The plates were incubated as for the blocking step then transferred to 4°C, 18 h. After the incubation they were washed as above and incubated with the goat anti-hamster immunoglobulin-horseradish peroxidase (HRP) conjugate, diluted 1:3,000 in the diluent for 1 h. The excess conjugate was washed away, 100 μl of *p*-phenylene-diamine dihydrochloride (PPD) (Sigma Chemical Co.) substrate solution which was freshly prepared was added to each well and the plates were kept at 25°C in the dark for 30 min. The enzyme-substrate reaction was stopped by adding 50 μl of 1 N NaOH solution. The optical density (OD) of the content in each well was determined against the blank (wells to which PBS was added instead of the antibody preparation) at 492 nm using an ELISA reader (Multiscan *EX,* Labsystems). The OD at 0.05 or more against blank was taken as positive reaction.