CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Animals

Rabbits were used for immunization with whole cells of leptospira. Eight rabbits of both sexes (5 males and 3 females) weighing form 2.2 - 2.5 kg, the age of which ranged from 6-8 months were used.

3.2 Bacterial Strains

Four leptospira strains were kindly supplied by the Bangkok Leptospirosis Research Centre namely *L.biflexa* serogroup Semaranga serovar patoc strain Patoc 1, *L.interrogans* serogroup Bataviae serovar bataviae strain Swart, *L.interrogans* serogroup Autumnalis serovar autumnalis strain Akiyami A and *L.interrogans* serogroup Icterohaemorrhagiae serovar icterohaemorrhagiae strain M20. The stock cultures were grown in Fletcher's semisolid medium.

3.3 Preparation of Sonic Extracted Leptospiral Antigens

Subcultures of 4 leptospiral strains were made by inoculating 1 volume of the actively growing 7 to 10 day-old leptospiral culture into 9 volumes of the cultural EMJH medium (7). The cultures were incubated aerobically in the dark at 30°c for 7 to 10 days. It was also possible to incubate the culture at room temperature (about 28-33°c). After incubation, the cultures were examined under a dark-field microscope and

subsequently grown on blood agar plates to check for growth density and contamination. The cultures were harvested by centrifugation at 11,300 g in a refrigerated high speed centrifuge at 4°c for 1 h. The pellets were suspended in 0.15 M phosphate-buffered saline (PBS) pH 7.2, washed twice, and resuspended in the same buffer to a concentration of 20% (w/v). The suspensions were kept in -70°c overnight. They were thawed and sonicated for 15 min. After that, the antigens were centrifuged at 12,000 g for 20 min, the supernatants collected and stored at -20°c.

3.4 Determination of Protein Concentration

Protein concentration of sonic extracted leptospiral antigen of *L. biflexa* serovar patoc, *L. interrogans* serovar bataviae, serovar autumnalis and serovar icterohaemorrhagiae were determined according to Lowry method (38), with bovine serum albumin as the standard. To 1.0 ml of sample was added 1 ml of solution D (see Appendix II), blended with a Vortex mixer and allowed to react for 15 min at room temperature. Three ml of Folin Ciocalteus phenol reagent (see Appendix II) was added and mixed. The samples were incubated for 30 min at room temperature and then read in a spectrophotometer at 540 nm. Lowry's protein assay revealed protein concentrations ranging from 1.95 - 4.29 mg/ml in each sonic extract (Table 3).

Table 3 : The protein concentration of sonic extracted leptospiral antigen (mg/ml) by Lowry method.

Sonic extracted antigen of	Protein concentration (mg/ml)
L. biflexa serovar patoc	3.12
L. interrogans serovar bataviae	4.29
L. interrogans serovar autumnalis	2.34
L. interrogans serovar icterohaemorrohagiae	1.95

3.5 Preparation of Rabbit Anti-Leptospiral Antisera

Subcultures of 4 leptospiral strains were made by inoculation of 1 volume of the actively growing 7 to 10 day-old leptospiral culture into about 9 volumes of the Fletcher's semisolid medium. The cultures were incubated aerobically in the dark at 30°c for 7 to 10 days. After incubation, the cutures were examined by dark-field microscopy for density and contamination. Two rabbits were immunized with each leptospira strain. They were injected intravenously at 7-day intervals with 0.5, 1.0, 2.0, 4.0 and 4.0 ml doses of a 7 day-old living culture of each leptospira strain in Fletcher's semisolid medium (18). The rabbits had no microscopic-agglutinating antibodies when tested before immunization, and their pre-unimmunisation sera were pooled and used as normal rabbit serum. The rabbits were bled by ear vein on the seventh day after the last injection. Two rabbit antisera to one strain of leptospira were pooled and

distributed into small aliquots and stored at -20 °c. The leptospiral antibody titer was tested in the microscopic agglutination test. (77).

3.6 Microscopic Agglutination Test (MA Test) for the Determination of Leptospiral Antibody (Droplet Method, Institute for Tropical Hygiene and Geographical Pathology, Amsterdam, Netherlands)

3.6.1 Preparation of Live Leptospiral Antigens

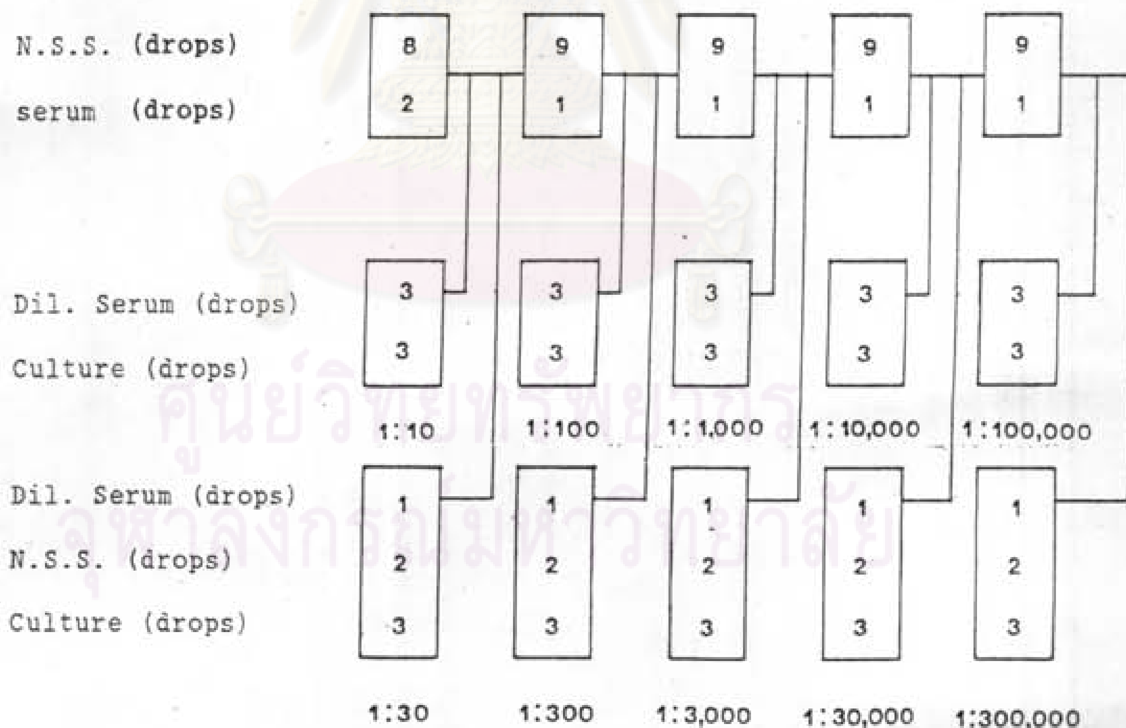
A battery of 12 viable *L. interrogans* serovars commonly associated with diseases in man and animals in Thailand were examined in the MA test. These serovars were australis, autumnalis, bataviae, canicola, grippotyphosa, hebdomadis, hyos, icterohaemorrhagiae, javanica, pomona, pyrogenes and wolffii. All serovars were subcultured and grown in Fletcher's medium broth (see Appendix II) for 4-6 days, checked for viability and non-autoagglutination under a dark-field microscope bofore use.

3.6.2 MA Test

Sera were first screened against all serovars at a 1:100 dilution in normal saline solution (N.S.S.). Sera showing positive reaction at 1:100 were than retested against the respective serovar(s) to determine the endpoint agglutination titer(s), or the highest dilution giving > 50% agglutination of leptospires. Thus rabbit hyperimmune sera or patient's sera were diluted from 1:5 to 1:150,000 in a dilution plate (Figure 1). To

each dilution was added an equal volume (3 droplets) of viable leptospira culture. The dilution plate was incubated at room temperature for 2 h, after which a loopful from each well was removed to examine for agglutination under a dark-field microscope. Positive controls and antigen controls were also included. Rabbit hyperimmune serum against *L. biflexa* serovar patoc, were tested by a modified tube method (15).

Figure 1 : End point Determination of MAT on a dilution plate



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3.7 Study Design

The antigenicity and immunogenicity of sonic extracted from *L. biflexa* serovar patoc and pathogenic strains commonly found in Thailand namely *L. interrogans* serovar bataviae, autumnalis and icterohaemorrhagiae, were analyzed on SDS-polyacrylamide gel electrophoresis and immunoblotting. Anti-leptospiral antisera were from two sources-human sera from patients afflicted with proven strains of leptospires and rabbit antisera raised against live leptospires.

Immunoblotting was divided into 2 parts.

Part I : Immunoblotting of each leptospiral antigen (4 serovars) against homologous and heterologous rabbit anti-leptospiral antisera.

Part II : Immunoblotting of each leptospiral antigen (4 serovars) against homologous, heterologous human anti-leptospiral antisera and human positive-TPHA serum.

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3.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with the Discontinuous Buffer System of Laemmli (20).

3.8.1 Sample Preparation

After protein determination, each sonic extracted leptospiral antigen was diluted in the sample buffer (5X) at a ratio of 4:1, and kept at -20 °c until use. On electrophoresis, each sample was thawed and heated at 100°c in

boiling water for 5 min. Heating is important for the destruction of proteases in samples, as well as for protein solubilization and SDS-binding. The extract is then centrifuged at 12,000 g for 5 min to remove cell debris. The supernatant was collected and the protein concentration in each sample was calculated for optimal loading in SDS-polyacrylamide gel.

3.8.2 Preparation of Electrophoretic Gels

SDS-PAGE was performed in 1.0 mm thick, 10% slab separating gels and 3% stacking gels by the system of Laemmli (20) as follow.

After the construction and sealing of the gel cassettes, the separating gel (see Appendix II) was prepared by mixing 30% stock acrylamide gel, 1.5 M Tris-HCI pH 8.8, SDS-solution, 0.2 M EDTA and deionized D.W. The mixture was deaerated under vacuum (oil vacuum pump) 2-3 min in a swirling motion, 250 ml side-arm conical flask. TEMED and freshly prepared ammonium persulfate were added. The gel mixture was swirled and filled in the cassettes using a 20 ml syringe to set 12 cm gel height, care was taken so that no air bubble were trapped in the gel. A few drops of deionized distilled water were carefully layered on the top of gel mixture before the gel hardened. After gel polymerization was completed (1 h), the water was decanted, the top of the gel was rinsed and moulded with 3% stacking gel (see Appendix II) prepared as above. A comb was inserted to form wells for antigen application for 25 mm of

stacking gel height. The cassetted gel was left at room temperature for about 1-2 h to complete polymerization and then kept in the refrigerator overnight.

3.8.3 Electrophoresis

Before antigen loading, gels should be prerun at a constant current of 15 mA per 1.0 mm gel for 30 min in an electrophoretic chamber containing electrode buffer pH 8.3. Then 35 μg of sonic extracted leptospira and 2 μl of standard molecular weight were loaded in each well. Electrophoresis was performed at a constant current of 30 mA per 1.0 mm gel, which generally took about 3 h during which voltage usually rose to about 460 V. After electrophoresis, gels were removed from their cassettes, proteins were stained in Coomassie Brilliant Blue R-250 or transfered onto the nitrocellulose membrane.

3.9 Immunoblotting

3.9.1 Preparation for Blotting (19)

The transfer buffer (see Appendix II) was usually prepared and cooled to 4°c for improved heat dissipation. Following electrophoresis, the gel was equlibrated in transfer buffer for 30 min. The nitrocellulose membrane was cut to the same dimension of the gel, slowly placed into transfer buffer and allowed to soak for 30 min. Two pieces of filter paper and fiber pads cut to the dimension of the gel were needed for each gel/membrane sandwich (Figure 2). These and the buffer tank were, in turn, saturated and filled with transfer buffer.

3.9.2 Electrophoretic Blotting

The physical assembly for protein transfer is shown diagrammatically in Figure 2. The gel holder was opened by sliding and lifting the latch. The clear panel represents the anode, and the gray panel the cathode. The opened gel holder was placed in a shallow tray, with the grey panel lying flat on the bottom. The clear panel should then rest at an angle against the wall of the tray. A pre-wetted fiber pad was placed on the grey panel of the holder, followed in order by a piece of saturated filter paper, the pre-equilibrated gel and a pre-wetted nitrocellulose membrane. Protein transfer was best done by holding the nitrocellulose membrane at opposite ends so that the center portion is sagging, and allowing the center protion to contact the gel first. The transfer membrane was then carefully rubbed from side to side to push out all air bubbles and displaced excess liquid between gel and membrane. Then a second piece of saturated filter paper was placed on top of the membrane, again removing air bubbles by rolling a glass tube over the filter paper. A second saturated fiber pad was added on the top of the filter paper. The gel holder was closed, holded firmly and placed in the Trans-Blot tank such that the gray panel of the holder was on the cathode side of the tank. The buffer tank was filled with transfer buffer and a stir bar added to the cell. The lid was put in place and the unit plugged into the power supply at 250 mA for 3 h, after which the current was decreased to 50 mA for 19 h. Normal transfer polarity takes place from the cathod to the anode.

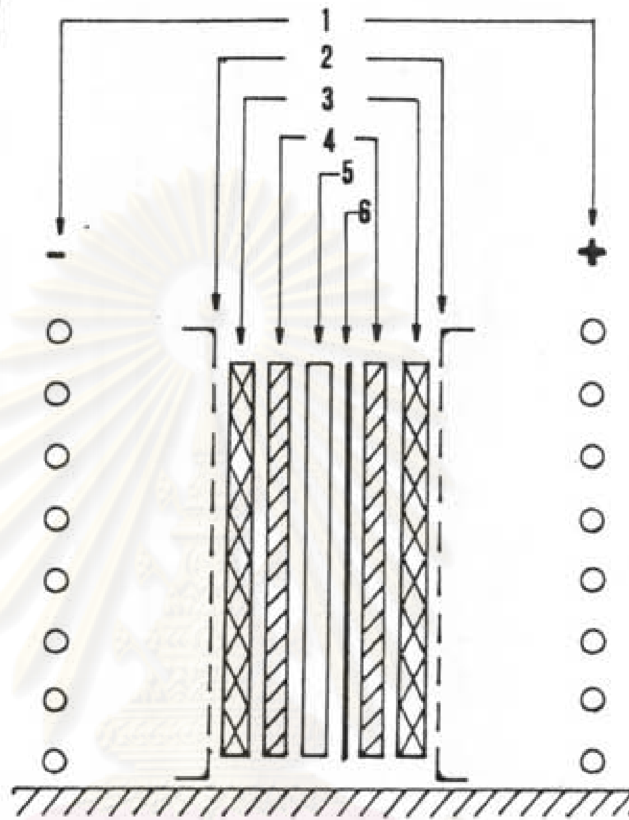

Figure 2 : Assembly for electrophoretic blotting procedure 1, Electrode; 2, gel holders; 3, fiber pads (Scotch-Brite pads); 4, filter papers; 5, polyacrylamide gel; 6, nitrocellulose membrane. Assembly parts are shown separated for visualization only.

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3.9.3 Immunoblotting

After protein transfer, the antigenic component were detected by immunostaining with leptospiral antisera. Various conditions used for immunoblotting were optimized. In short, blots were rinsed with PBS pH 7.4 containing 0.1% Tween 20 (washing buffer), dried and soaked in 5% nonfat dry milk (116) in washing buffer for 3 h at 37 c in order to saturate unused protein-binding sites and reduce non-specific reaction with the exception of transblotted molecular weight markers to be stained with Pilikan-fount india drawing ink (117). The blots were then rinsed in washing buffer, dried and kept in the refrigerator until immunostained. The gel after transfer was stained for remaining protein with Coomassie brilliant blue R-250.

3.9.3.1 Immunostaining with Rabbit Anti-Leptospiral Antisera.

Each transblotted leptospiral antigen was incubated for 3 h at $37^{o}c$ with five different sera namely, pooled normal rabbit sera (1:30), rabbit antisera against *L. biflexa* serovar patoc (1:30), *L.interrogans* serovar bataviae (1:100),serovar autumnalis (1:40) and serovar icterohaemorrhagiae (1:50). Excess antisera were removed by five 10-min washes in washing buffer, and the membranes were subsequently incubated for 1 h with peroxidase-conjugated swine anti-rabbit immunoglobulins diluted 1:50 in washing buffer. The membranes were finally washed 3 times, to remove the excess peroxidase conjugate, and developed in 4-chloro-1-naphthol solution (substrate buffer, see Appendix II) for 30 min, then rinsed with deionized distilled water.

3.9.3.2 Immunostaining with Human Antisera

Each transblotted leptospiral antigen (4 serovars) was incubated for 3 h at 37°c with pooled normal human sera; human antisera against *L. interrogans* serovar bataviae, serovar autumnalis, serovar icterohaemorrhagiae; and human antiserum which obtained positive result by TPHA diluted 1:100 in washing buffer containing 5% non fat dry milk (116). The excess serum was removed by five 10-min washes in washing buffer, and the membranes were subsequently incubated for 1 h with peroxidase-conjugated rabbit anti-human immunoglobulin G diluted 1:50 in 5% non fat dried milk in PBS-Tween. The membranes were finally washed 3 times, to remove the excess peroxidase conjugate and developed in 4-chloro-1-naphthol solution for 30 min, then rinsed with deionized distilled water. Optimal conditions are summarised in Table 4.

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Table 4 : Summary of optimal conditions for immunostaining.

The optimal conditions for immunostaining
1. The blots were incubated in rabbit or human anti-leptospiral antibody at 37°c for 3 h.*
2. The blots were washed with five 10-min changes of washing buffer.
3. The blots were incubated in peroxidase conjugated at 37°c for 1 h.*
4. The blots were washed with three 10-min changes of washing buffer.
5. The antigen-antibody complexes were developed in substrate buffer at room temperature for 30 min.
6. The reaction was stopped by rinsing with deionized distilled water.

3.10 Coomassie Brilliant Blue R-250 Staining

The electrophoresed gel was stained in 0.25% Coomassie brilliant blue R-250 for at least 2 h, destained until the background was clear and soaked in a fixing solution for 2 h before drying.

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*Washing buffer was used as the diluent for immunostaining with rabbit antisera but blocking buffer as the diluent for immunostaining with human antisera.

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3.11 India Ink Staining (117)

The transblotted nitrocellulose membrane containing the high molecular weight (HMW) protein standards was washed 4 times each for 10 min in 50 ml of PBS pH 7.4 with 0.3% Tween 20 at room temperature in a shaker, stained in a solution of india ink (see Appendix II) with agitation for 2-3 h, rinsed with deionized D. W. for 5 min and dried.

3.12 Estimation of Molecular Weight by Relative Standard Molecular Weight Curve. (Modified from the methods of Weber and Osborn (99) and Davies and Stark (115).

The molecular weight of unknown protein can be determined by comparing its electrophoretic mobility with standard protein markers. A linear relationship is obtained by plotting the molecular weight of the standard polypeptide chains logarithimically against their respective electrophoretic relative mobilities (Rf). (Appendix III)

After electrophoresis or immunoblotting, the dried gel and immunostained nitrocellulose membrane were photographed. The migration distance of each protein was measured from the top of the gel and the relative mobility was determined.

$$Rf = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$