CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

1.	-80°C Deep Freezer model MDF-U32V	Sanyo, Japan
2.	-20°C Freezer	Sanyo, Japan
3.	4°C Refrigerator	Sanyo, Japan
4.	4°C Refrigerator	Mirage,
5.	Autoclave model	Hirayama, Japan
6.	Balance	Ohaus, Switzerland
7.	Centrifuge	Beckman Coulter, USA
8.	Centrifuge	Sorvall, Germany
9.	Cuvette	Hellma, Germany
10.	DNA Thermal Cycle	Biometra, Germany
11.	Gel Document and Quantity One 4.4.1	Biorad, USA
12.	Gel Mate 2000 Electrophoresis	Toyobo, Japan
13.	Heat Block : Dri-Bath Type 17600	Thermolyne, USA
14.	Heat Block : Thermo Block TDB-120	Biosan, USA
15.	Hot Air Oven model	Shel Lab, USA
16.	Magnetic Stirrer	Clifton, USA
17.	Microcentrifuge : Minispin	Eppendorf, Germany
18.	Microcentrifuge Tube 0.5 ml and 1.5 ml	Axygen Scientific, USA
19.	Micropipette P2.5, P10, P100 and P1000	Biohit, Finland
20.	Microwave	Imarflex, Thailand
21.	PCR Tube 200 μl	Axygen Scientific, USA

22.	Power Supply	Hoffer Scientific Instrument, USA
23.	Rotor Gene RG-3000	Corbett Research, Australia
24.	Spectrophotometer	Biorad, USA
25.	Vortex Mixer	Gemmy Industrial, Taiwan

3.2 Chemicals and kits

1.	100 bp DNA Ladder	Fermentas, Canada
2.	100 mM dNTP Mix	Fermentas, Canada
3.	2X platinum qPCR supermix UDG	Invitrogen, USA
4.	6X Loading Dye Solution	Fermentas, Canada
5.	Absolute Ethanol	Bio Basic Inc., Canada
6.	Agarose Gel	Research Organics Inc., USA
7.	DEPC (diethypyrocarbonate)	Sigma Aldrich, USA
8.	DNAse I	Fermentas, Canada
9.	DNAzol Reagent	Invitrogen, USA
10.	EDTA (ethylenediaminetetraacetic acid)	Fermentas, Canada
11.	Ethidium Bromide	Sigma Aldrich, USA
12.	Glacial acetic acid	Merck, Germany
13.	Gel extraction kit	QIAGEN, Germany
14.	Isopropanol	Bio Basic Inc., Canada
15.	M-MuLV Reverse Trancriptase	Fermentas, Canada
16.	Magnesium Chloride ($MgCl_2$)	Fermentas, Canada
17.	Phase separation Reagent : BCP	MRC, UK
18.	Ramdom Hexamer Primer	Fermentas, Canada
19.	RNAlater : RNA stabilization Reagent	QIAGEN, Germany
20.	RibolockTM Ribonuclease Inhibitor	Fermentas, Canada
21.	Taq DNA Polymerase	Fermentas, Canada

22. Tris base Research Organics Inc., USA
23. Trizol Reagent Invitrogen, USA

3.3 Bacteria and animals

Golden Syrian Hamster (*Mesocrietus auratus*), age 4-6 weeks bought from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The hamsters were injected intraperitoneally with 10^8 *Leptospira interrogans* serovar Pyrogenes, an isolate previously shown to induce pathogenesis in hamsters (24). Infected hamsters were sacrificed at 3, 5, and 7 days post infection. Four hamsters were used for each timepoint. Four non-infected hamsters were used as a control group. Kidney and liver tissues were collected and kept in RNA later solution for the stability of tissues for further RNA extraction

(Hamster injection and tissue collection were done by the staff at Armed Forces Research Institute of Medical Science. The animal experiments were conducted under the approval of the Ethical Research Committee, Faculty of Medicine, Chulalongkorn University.)

3.4 RNA extraction

RNA was extracted by TRIzol reagent according to the manufacturer's instruction as follows: 20 mg of tissue was homogenized in one ml of TRIzol reagent using a pastel and incubated for 5 minutes at room temperature. Then, 100 µl of phase separation reagent was added and incubated with the homogenized tissue for 3 minutes before centrifugation at 12,000g for 15 minutes at 4°C. After that, the aqueous phase was transferred to a new tube. The RNA was precipitated by mixing with 0.5 ml of isopropyl alcohol. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed twice with 1 ml of 75% ethanol. Finally, 20 µl of DPEC water was added to dissolve the RNA pellet.

RNA was stored at -80°C.

RNA concentration was determined by diluting to 100-fold dilution in RNase-free water. The absorbance of diluted RNA was measured at 260 and 280 nm using spectrophotometer. An OD_{260} of 1.0 corresponds to a concentration of 40 μg/ml single stranded RNA. Then, the concentration of RNA was calculated by using the following equation.

$$RNA\ (\mu g/ml) = OD_{260} \times 40 \times \text{dilution factor}$$

The purification of RNA was evaluated from a ratio of OD_{260} / OD_{280}. The ratio of appropriately purified RNA was in the range of 1.8-2.0.

3.5 RT-PCR for *Leptospira* and hamster gene expression

3.5.1 cDNA synthesis

Before the cDNA synthesis, residual DNA removal by treating with RNase-free DNase I was done according to the manufacturers as followed: 1 μg of total RNA was incubated with 1u of RNase-free DNase I in 1X DNase buffer at 37°C for 1 hour. Then, DNase I was inactivated by incubating with 2.5 mM EDTA at 65°C for 10 minutes.

cDNA was synthesized by mixing 1 u. of DNase I-treated RNA with 0.2 μg of random hexamer. The mixture was incubated at 70°C for 5 minutes and chilled on ice. Then, 1X RT buffer, 1 mM dNTP and 20u of Ribonuclease inhibitor were added into the mixture and incubated at room temperature for 5 minutes. Finally, 200 units of M-MuLV Reverse transcriptase were added and the mixture was incubated at 25°C for 10 minutes and 42°C for 1 hour for cDNA synthesis. The reaction was stopped by heating at 70°C for 10 minutes. cDNA was used for PCR later.

3.5.2 PCR

Amplification of cDNA transcribed from tissue RNA was carried out in sterile 0.2 ml PCR tubes containing the mixture as follow; 1X PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1u Taq DNA polymerase, and 500 nM of each primer in 25 μl reaction. The primer sequences and PCR product sizes were shown in the Table 2.

3.5.2.1 PCR for *Leptospira* 16S rRNA expression

PCR for *Leptospira* 16S rRNA was performed using the following conditions: heat denaturation at 94°C for 3 minutes, and then 30 cycles of heat denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and DNA extension at 72°C for 1 minute. After the last cycle, elongation step was extended for 5 minutes.

3.5.2.2 PCR for LipL32 expression

Since the expression of LipL32 mRNA could not be demonstrated by regular RT-PCR, nested-PCR was performed for LipL32 gene expression detection. The external amplification was performed using the following conditions: heat denaturation at 94°C for 3 minutes, and then 30 cycles of heat denaturation at 94°C for 1 minute, primer annealing at 59°C for 1 minute, and DNA extension at 72°C for 1 minute. After the last cycle, elongation step was extended for 5 minutes. The internal amplification was performed using the following conditions: heat denaturation at 94°C for 3 minutes, and then 30 cycles of heat denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and DNA extension at 72°C for 1 minute. After the last cycle, elongation step was extended for 5 minutes.

3.5.2.3 PCR for HPRT expression

The HPRT cDNA amplification was performed using the following conditions: heat

I24594593

denaturation at 94°C for 2 minutes, and then 40 cycles of heat denaturation at 94°C for 40 seconds, primer annealing at 62°C for 1 minute, and DNA extension at 68°C for 1 minute. After the last cycle, elongation step was extended for 1 minute.

3.5.2.4 PCR for TNF-α expression

PCR for TNF-α expression was performed using the following conditions: heat denaturation at 94°C for 2 minutes, and then 40 cycles of heat denaturation at 94°C for 40 seconds, primer annealing at 62°C for 1 minute, and DNA extension at 68°C for 1 minute. After the last cycle, elongation step was extended for 1 minute.

3.5.2.5 PCR for TGF-β expression

The amplification of TGF-β cDNA was performed using the following conditions: heat denaturation at 94°C for 2 minutes, and then 30 cycles of heat denaturation at 94°C for 40 seconds, primer annealing at 60°C for 1 minute, and DNA extension at 68°C for 1 minute. After the last cycle, elongation step was extended for 1 minute.

3.5.2.6 PCR for IL-10 expression

The amplification of IL-10 cDNA was performed using the following conditions: heat denaturation at 94°C for 3 minutes, and then 35 cycles of heat denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and DNA extension at 72°C for 1 minute. After the last cycle, elongation step was extended for 5 minutes.

3.5.2.7 PCR amplification of IP-10

PCR for IP-10 expression was performed using the following conditions: heat denaturation at 94°C for 3 minutes, and then 35 cycles of heat denaturation at 94°C for 1 minute,

primer annealing at 55°C for 1 minute, and DNA extension at 72°C for 1 minute. After the last cycle, elongation step was extended for 5 minutes.

3.5.3 Agarose gel electrophoresis

PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. 1.5% agarose gel was prepared by mixing 0.6 grams of agarose powder with 40 ml of 1X TAE buffer. The mixture was heated in a microwave oven until completely dissolved. After cooling the solution to about 60°C, the agarose was poured into a casting tray containing a sample comb and allowed to solidify at room temperature about 40 minutes. After the gel was solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing 10 µl of DNA mixed with 1X loading buffer were then pipetted into the sample wells, the lid and power leads were placed on the apparatus, and a current was applied. When adequate migration occurred, the gel was stained in ethidium bromide and the images were captured by Gel Document.

The band density was measured by selected the target band and used the Quantity One program for calculated the concentration and area by compared with the reference band (band of DNA ladder). Then, the program was measured the density of selected band.

3.5.4 DNA purification and sequencing

PCR product was confirmed by sequencing. The bands obtained by PCR and agarose electrophoresis were cut from agarose gel and purified by QIAquick Gel Extraction kit according to the manufacturer's instruction as follows. DNA fragment was excised from the agarose gel with a clean sharp scalpel and the gel slice was weighed in a sterile tube. 300 µl of buffer QG was added into the tube which containing 100 mg of gel and incubated at 50°C for 10 minutes for agarose gel dissolving. Then, 100 µl of isopropanol was added into the sample and mixed for increasing the yield of DNA fragments. After that, the samples were loaded into the QIAquick column and centrifuged at

13,400 rpm for 1 minute. The flow-though was discarded and 0.5 ml of buffer QG was added into QIAquick column. The column was centrifuged for 1 minute again to remove all traces of agarose. DNA fragment was washed by adding 0.75 ml of buffer PE and centrifugation for 1 minute. Lastly, QIAquick column was placed into a clean 1.5 ml sterile tube and DNA was eluted with 30 μl of buffer EB and centrifuged for 1 minute. Purified DNA was used for sequencing later.

3.6 Real-time PCR for quantitation of LipL32 and IP-10 mRNA levels in hamster kidneys

cDNA amplification was carried out in a 25 μl reaction volume containing 2.5 μl of cDNA template, 12.5 μl of 2X platinum qPCR supermix UDG, 400 nM of each primer and nuclease-free water to a final volume of 25 μl. The primer sequences for real-time PCR were shown in the Table 2. Data acquisition and analysis of the real-time PCR assay were performed using delta-delta Ct method. This method was used to calculate the relative expression levels of a target relative to calibrator (RNA from non-infected kidneys). The amount of target gene in kidneys of hamsters infected by pathogenic *Leptospira*, normalized to an endogenous housekeeping gene (reference gene) and relative to the normalized calibrator, is then given by $2^{-\Delta\Delta_{Ct}}$, where

$$\Delta Ct\ (\text{sample}) = Ct\ (\text{target gene of sample}) - Ct\ (\text{reference gene of sample})$$

$$\Delta Ct\ (\text{calibrator}) = Ct\ (\text{target gene of calibrator}) - Ct\ (\text{reference gene of calibrator})$$

Ct was the point at which the fluorescence crosses the threshold. Then, $\Delta\Delta Ct$ and Ratio (folds of difference) of sample: calibrator was calculated by using the following equation.

$$\Delta\Delta Ct = \Delta Ct\ (\text{sample}) - \Delta Ct\ (\text{calibrator})$$

$$\text{Ratio (folds of difference) of sample: calibrator} = 2^{-\Delta\Delta_{Ct}}$$

3.6.1 Real-time PCR for quantitation of LipL32 mRNA levels

Real-time PCR assays were done to determine LipL32 and 16S rRNA mRNA levels. The thermal profile was as followed: an initial denaturation step of 2 minutes at 95°C followed by 45 cycles of 15s at 95°C, 30s at 55°C and 30s at 72°C. Data acquisition and analysis of the real-time PCR assay were performed using delta-delta Ct method which was used to calculate the relative quantitation of LipL32 normalized with 16S rRNA level in each individual sample and then compared between in the infected and uninfected hamsters.

3.6.2 Real-time PCR for quantitation of IP-10 mRNA levels

The thermal profiles for quantitation of IP-10 and HPRT expression levels were as followed; for HPRT detection: an initial denaturation step of 3 minutes at 95°C, followed by 40 cycles of 15 s at 95°C, 50 s at 60°C and 35 s at 72°C; for IP-10 detection: an initial denaturation step of 2 minutes at 95°C followed by 45 cycles of 15s at 95°C, 30s at 55°C and 30s at 72°C. Data acquisition and analysis of the real-time PCR assay were performed using delta-delta Ct method which was used to calculate the relative quantitation of IP-10 normalized with HPRT level in each individual sample and then compared between in the infected and uninfected hamsters.

Table 2 Primer sequences and PCR product sizes

Primer name	Sequences	Product size (bp)
16S-C	5'-CAAgTCAAgCggAgTAgCAA-3'	290 (145)
16S-D	5'-CTTAACCTgCTgCCTCCCgTA-3'	
HPRT-F	5'-CTgAAgAgCTACTgTAAYgAT-3'	206
HPRT-R	5'-TTTCACCARCAAgYTTgCAA-3'	
LipL32-F	5'-TTACCgCTCgAggTgCTTTCggTggTCTgC-3'	782
LipL32-R	5'-TgTTAACCCgggTTACTTAgTCgCgTCAgA-3'	(external round)
LipL32-FF	5'-TggATCTgTgATCAACTATTACg-3'	506
LipL32-RR	5'-CACTTCACCTggTTTgTAggTA-3'	(internal round)
LipL32-F (for real-time PCR)	5'-TggATCTgTgATCAACTATTACg-3'	286
LipL32-R (for real-time PCR)	5'-gAACTCCCATTTCAgCgATTAC-3'	
TNF-α-F	5'-CCCAAAgggAAgAgAAgTTC-3'	126
TNF-α-R	5'-CCACTTggTggTTTgCTACA-3'	
TGF-β-F	5'-CAACTATTgCTTCAgCTCCAC-3'	186
TGF- β-R	5'-gTASAgggCMAggACCTTRCT-3'	
IL-10-F	5'-TgCAggACTTTAAgggTTAC-3'	163
IL-10-R	5'-gggAgAAATCgATgACAgC-3'	
IP-10-F	5'-gAATCCCTCTTTCAAggACAgT-3'	486
IP-10-R	5'-TAgTAgAgTTggggACTCTTgT-3'	
IP-10-F (for real-time PCR)	5'-TTTATACgTCggCCTATggC-3'	186
IP-10-R (for real-time PCR)	5'-TAgTAgAgTTggggACTCTTgT-3'	