# CHAPTER IV

# Material and Method

## I. Materials

### 1. Subjects, blood samples, and cell lines.

1) Experimental Rabbits.

Two New Zealand White rabbits (The national laboratory animal center, Mahidol University), three-month olds and approximately 2500 g. at the beginning of study. They were kept in cages covered with mosquito net to prevent exposure to mosquito bite.

2) Serum from highly exposure to mosquito bite.

Thirty sera samples of King Chulalongkorn Memorial Hospital' s guards were included in this study. They were present here as group of person with high risk to mosquito bite. Ten ml of whole blood was collected from each guard were centrifuged at 2500 rpm for 10 minutes and serum were collected and stored at $-20^{\circ}C$ for later analysis.

3) Patients with Dengue infection.

Thirty sera samples from patients admitted in King Chulalongkorn Memorial Hospital with dengue infection based on clinical and laboratory data were collected during 2001. The sera which are positive by RT-PCR confirmatory method, were used in this study. About 0.3-1 ml of serum was collected from each patients and stored at $-70^{\circ}C$ until required.

4) Cord blood.

A total of ten cord blood samples from the Obstetric-Gynecology Department of King Chulalongkorn Memorial Hospital, were included in this research project. About 0.5 ml of serum was collected from each sample and stored at $-20^{\circ}C$ until required.

5) C6/36 cell line

Continuous cultures of C6/36 cell line (epithelial cell from larva of *Aedes albopictus* mosquito, ATCC Number : CRL-1660) (C6/36 cell line from department of Medical Molecular Biology of Mahidol University) were cultured in 25 ml cell culture flasks (NUNCLON®, Denmark) containing L-15 medium (Gibco BRL®, USA) with 10% TPB (Sigma, USA), 10% FBS (Gibco BRL®, USA), 60 U/ml Penicillin G (Sigma, USA), and 60 µg/ml Streptomycin sulfate (Sigma, USA).

6) Mosquitoes

Two laboratory species of mosquitoes which are *Aedes aegypti* and *Aedes albopictus* were used in this study. The mosquitoes were obtained from Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University and maintained at 20-25°C. Until used in experiments, adult mosquitoes had access to 10% sucrose only. Mosquitoes were anesthetized by putting them into a test tube and chilled on ice for 20 minutes. Whole body extracts were made by grinding each mosquito in 100 µl loading buffer with a steriled plastic tissue grinder. The homogenate was briefly centrifuged at 1000 g., then the supernatant was collected and stored at −20°C until required.

## II. Methods

### 1. Immunization of Rabbits

Before start immunization programme, the preimmuned blood were collected for 15 ml from each of two experimental rabbits. Then first immunization with 100,000 C6/36 cells in 0.85% Normal saline by subcutaneous injection followed by three repeated boosting does on day 14, 42, and 70, respectively. About 10-20 ml of whole blood were collected from each rabbits on day 28, 52, 84, and 98, then centrifuged at 2500 rpm for 10 min to collect rabbit's serum. Serum were stored at

—20°C for later analysis.

**2. Viral RNA isolation**

Dengue viral RNA was extracted from infected patients serum, which dengue serotype have already been confirmed by RT-PCR, using a QIAamp Viral RNA Minikit (QIAGEN, USA) . Briefly, a 140 µl volume of serum was mixed with the 560 µl of buffer AVL-carrier RNA, mixed, incubated at room temperature for 10 min. Added 560 µl of absolute ethanol, mixed and loaded onto the spin column. This was followed by two time of washing with buffer AW1 and AW2, respectively. The final step, elution with the buffer AVE to a final volume of 60 µl, as recommended by the manufacturer.

**3. RT- nested PCR of Dengue viral RNA**

A one-step RT-PCR was performed in a 30 µl reaction volume containing 10 µl of extracted RNA solution, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 200 µM concentrations of each dNTP, 40 *p*mole of primer DENR, the dengue virus downstream consensus primer which homologous to the genomic RNA of the four serotypes, 100 U of M-MLV RT (Promega corporation, USA) and 20 U of RNasin Ribonuclease Inhibitor(Promega corporation, USA). The reactions was incubated for 30 min at 42°C and then inactivated for 5 min at 95°C. Subsequent Taq polymerase amplification, the first-round PCR amplification, was performed on the resulting cDNA with the upstream dengue virus consensus primer, DEN. Target cDNA was amplified in 50 µl volumes of the reaction mixture contained all the components of the amplified dengue RNA initial reaction with the following exceptions : 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton®X-100, 2 mM $MgCl_2$, 40 *p*mole of primer DEN and 2 U of Taq polymerase(Promega corporation, USA). The reactions was allowed to proceed in an Thermocycle programmed to incubate for 3 min at 94°C

initially then amplified for 35 cycles under the following conditions: 94°C for 30s, 55°C for 30s, 72°C for 45s, and a final extension at 72°C for 5 min. A second amplification reaction was initiated with 1 µl of initial amplification reaction. The reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton®X-100, 2 mM $MgCl_2$, 200 µM concentration of each dNTP, 20 *p*mole of primer DENG and primer DEN-1, 10 *p*mole of each primers DEN-2, DEN-3, and DEN-4 and 2U of Taq polymerase. The samples were incubated for 3 min at 94°C initially then amplified for 30 cycles under the following conditions : 94°C for 30s, 55°C for 30s, 72°C for 30s, and a final extension at 72°C for 5 min. A 10 µl portion of the reaction product were electrophoresis on a 2% composite agarose gel (Promega corporation, USA) in TAE buffer. The different PCR product were characterized by the position of priming with each of the dengue virus type-specific primers. All primer sequences are listed in Table 2.

**Table. 2** The sequences of oligonucleotide primer used for PCR amplification, DNA sequencing and typing of dengue gene.

| **Primer of amplified** | **sequence*** | **size, in bp, DNA product** |
|---|---|---|
| DENR | 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3' | 511 |
| DEN | 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' | 511 |
| DENG | 5'-GCGAGAGAAACCG[C/T]GT[G/A]TC-3' | depended on viral type |
| DEN-1 | 5'-CGTCTCAGTGATCCGGGGG-3' | 467 |
| DEN-2 | 5'-TTCCTGAACCCTCTCAAAACA-3' | 204 |
| DEN-3 | 5'-TAACATCATCATGAGACAGAGC-3' | 275 |
| DEN-4 | 5'-CTCTGTTGTCTTAAACAAGAGA-3' | 377 |

* The annealing positions of each primers were as follows:

DENR : serotype 1, 614-642; serotype2, 616-644; serotype3, 614-642; serotype4, 618-646.

DEN : serotype 1, 132-159; serotype2, 134-161; serotype3, 132-159; serotype4, 136-163.

DENG : serotype 1, 147-165; serotype2, 149-167; serotype3, 147-165; serotype4, 151-166.

DEN-1 : serotype 1, 595-613.

DEN-2 : serotype 2, 333-352.

DEN-3 : serotype 3, 400-421.

DEN-4 : serotype 4, 506-527.

The genome position of each primer are given according to the published reference sequences of dengue serotypes 1-4 from GenBank (www.ncbi.nlm.nih.gov): serotype1, NC_001477; serotype2, NC_001474; serotype3, NC_001475; serotype4, NC_001475.

**4. Detection and typing of PCR products**

The 2% agarose gel was prepared by completely dissolving agarose powder upon heating in 1X TAE buffer then allowed to cool to 50°C before pouring into an electrophoresis chamber set, with comb inserted. A 10 µl DNA sample was mixed with 2 µl of 6X loading dye and loaded into gel slots in a submarine condition with 2.5 µg/ml ethidium bromide solution. Electrophresis was performed at 100 volts for 30 min. DNA patterns were visualized and photographed under UV light exposure with UV transilluminator (Ultra-lum, USA).

**5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The C6/36 cell protein were separated by SDS-PAGE in a discontinuous

system according to Laemmli (113) using a Biorad MINI-PROTEAN II apparatus.

1) Polyacrylamide gel preparation

Polyacrylamide gel was prepared by diluted of the stock solution, 30% acrylamide (Pharmacia Biotech, Sweden) to 15% with 1.5 M Tris buffer. To prepare a 15% separating gel, 2.5 ml of 30% acrylamide mix, 1.3 ml of 1.5 M Tris buffer (pH8.8), 50 µl of 10% SDS and 1.1 ml of distilled water were mixed to make a final volume of 5 ml gel solution. The gel solution was added with 50 µl of 10% APS, 2 µl of TEMED (Sigma, USA) and immediately poured the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel, used a pasture pipette to carefully overlayer the acrylamide solution with distilled water. The gel was let to polymerized at room temperature for 30 min. After complete polymerization, poured off the overlay and wash, then prepare 5% stacking gel, 0.17 ml of 30% acrylamide mix, 0.13 ml of 1.0 M Tris buffer (pH6.8), 10 µl of 10% SDS and 0.68 ml of distilled water were mixed to make a final volume of 1 ml gel solution. The gel solution was added with 10 µl of 10% APS, 1 µl of TEMED and immediately poured between the glass plates until the glass plate sandwich was full and then the comb was placed into the assembled glass sandwich. The gel was iet to polymerized at room temperature for 30 min, after that removed the comb carefully. Used a squirt bottle to washed the wells immediately with distilled water to remove any unpolymerized acrylamide.

2). Electrophoresis

The gel was mounted in the electrophoresis apparatus, added Tris-glycine electrophoresis buffer to the top and bottom reservoirs. The bubbles trapped at the bottom of the gel between the glass plates were removed. The extract of $10^5$ cells of C6/36 cell was added with 15 µl of loading buffer and heat them to 100°C for 3 min. The 10 µl of each of the sample were loaded into the bottom of the wells. Then

attached the electrophoresis apparatus to an electric power supply. Apply constantly voltage about 130 volts to the gel. After the dye front has moved into the separating gel, decrease the voltage to 100 volts and run the gel until the dye front reach the bottom of the separating gel. Then turn off the power supply. Removed the glass plates from the electrophoresis apparatus, carefully pry the plates apart and removed the gel into buffer until used.

**6. Western blot analysis**

1) Electro-blotting

Wet one sheet of nitrocellulose membrane (Biorad, USA) (in similar size to the gel) in Transfer buffer. Used gloves to handle the membranes. Cut two pieces of Whatmann paper (Whatman®, UK) in a bit large than the size of the gel, then placed them and fiber gel pads in container with enough transfer buffer. Equilibrate them for 5 min at room temperature, then placed the wet nitrocellulose membrane on top of the gel. The operation should be done under the buffer. Rub the membrane from one end to the other to eliminate bubbles. Placed the gel on top of a piece of wet Whatmann paper and place another wet paper on top of the nitrocellulose membrane. Placed the "sandwich" on top of the fiber gel pad and place another fiber gel cover the "sandwich". Opened the gel holder, put them on the black holder of the holder then lose it and placed it in the Transfer-Blot tank containing about 1000 ml of transfer buffer. The black side of the holder should face the cathode side. Connect the electrodes, and run for 3 hrs at constant 100 volts.

2) Western blot analysis

Place the nitrocellulose membrane in a container with blocking buffer, incubate with shaking for 2 hrs at room temperature. Wash three times for 10 min in washing buffer. Cut the appropriate area of nitrocellulose membrane to incubate with diluted serum (1:20) in blocking buffer for 3 hrs with shaking at room temperature.

Wash three times for 10 min with washing buffer. Add 1:1000 dilution of peroxidase-conjugated anti-rabbit immunoglobulins (KPL®, UK) or peroxidase-conjugated anti-human immunoglobulins (DAKO, Denmark) in blocking buffer. Shake for 2 hrs at room temperature. Wash three times for 10 min in washing buffer. Incubate the NC with HRP color development solution, fresh prepared, and shake at room temperature for 15 min. Stop reaction by incubate NC with distilled water.

**7. Immunofluorescence assay**

Preparation of C6/36 cell 's slide for immunofluorescence assay. Dilute susceptible C6/36 cells to 20,000 cells per ml in PBS then load 100 µl of cell suspension in each slide cytospin casetle after that spin at 3000 rpm for 3 min and then place on room temperature until slide dry. After slide preparation, the cells are fixed with cold acetone (-10°C) for 30 min. Allow the antiserum to react for 30 min with the fixed cells. Then wash with PBS three times for 15 min and react for 30 min with an anti-immunoglobulin conjugate at optimal dilution, human antiserum; 1:200, rabbit antiserum; 1:50 then wash with PBS three times for 15 min. Finally, counterstaining with 0.003% Evan's blue for 12 seconds and then wash with PBS.

**8. Enzyme-linked immunosorbent assay (ELISA)**

The increased rabbit anti-mosquito cell antibody level was measured by a modification of the enzyme-linked immunosorbent assay (ELISA) protocol as described by Engvall (114). Microtiter plates (Corning, New York) were coated with 10 µg of mosquito cell antigen, C6/36 cell and *Aedes aegypti* mosquito extract, in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 37°C. The antigen-coated wells were then washed 3 times with 0.85% NaCl containing 0.05% Tween-20. After washing, the plates were blocked with 5% BSA in PBS/Tween 20 (100 µl/well) for 1 hr at 37°C. The plates were then incubated sequentially with 50 µl/well of the

appropriately diluted antiserum in PBS/Tween 20 containing 1% BSA, goat anti-rabbit antibody conjugated to peroxidase, and the substrate orthophenylenediamine (Sigma, USA) in citrate buffer (pH 4.5). Plates were washed between incubations with 0.05% Tween 20/0.85% NaCl. The final enzyme reaction was stopped with 2 N $H_2SO_4$ , and the color change was measured at 492 nm on a plate reader (Organon, Belgium).

**9. Automated DNA sequencing**

Automated DNA sequencing was performed by using ABI 310 DNA sequencer (PE Applied Biosystem, USA) to characterize the dengue sequence. Cycle sequencing reaction was performed by using Big Dye Terminator kit according to the protocols as recommended by the manufacturer. The sequencing reaction was performed in 10 µl of total volume containing 4 µl of termination mix, 4 *p*mole of sequencing primer and 50-100 ng of purified PCR product. The reaction was performed in 25 cycles with the following temperatures and time lengths: 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. After cycle sequencing, 10 µl of sterile distilled water were added, followed by 0.1 volume of 3 M sodium acetate pH 4.8 and 2.5 volumes of 95% ethanol. The reaction tube was left at room temperature for at least 15 min then centrifuged. After centrifugation at 14,000 rpm for 20 min, the pellet was washed with 70% ethanol. The pellet was briefly dried before resuspended with 15 µl of Template suppressor reagent. The reaction mixture was heated at 95°C for 2 min then immediately cooled on ice before loading into the DNA sequencer.