CHAPTER III

MATERIALS AND METHODS

1. Patients

Between June 2003 and December 2007, we enrolled ninety-five patients who had been diagnosed with dengue fever (DF) and dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) by the criteria of the World Health Organization (WHO) (45). The study has been approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University and written informed consents were obtained from all subjects.

2. Sample Size Calculation

Preliminary study showed the difference of dengue serotypes (P=0.1) the sample size was calculated by

$$N/group = Z2\alpha/2PQ/d2$$

When $P = 0.1$

$Q = 0.9$

$d = 0.05$

$Z\alpha/2 = 1.96$

$$N/group = 2(1.96)(0.1)(0.9)/(0.05)^2$$

$$= 139$$

Sample size calculation showing to samples number of patients were 139

3. Definition

DF and DHF/DSS are defined as the clinical diagnosis based on the WHO classification. Presence of fever or history of fever lasting 2-7 days, a hemorrhagic tendency shown by a positive tourniquet test or spontaneous bleeding (petechiae, ecchymosis, purpura, hematemesis, melena), thrombocytopenia (platelet count 100 x 10^9 /L or less), and evidence of plasma leakage shown either by hemoconcentration of packed-cell volume, or by the development of pleural effusion or as cites, or both.

4. Viral strain

The standard strains of dengue virus used in this study are DEN-1 (Hawaii), DEN-2 (16681), DEN-3 (H87), and DEN-4 (H241) for positive controls of nested RT-PCR and multiplex RT-PCR and 13 strains kindly provided by AFRIMS as positive controls for RSS-PCR (13 strains, Table 2) (85, 86). Viruses were propagated in C6/36 *Aedes albopictus* mosquito cells grew in MEM medium (Gibco BRL, Grand Island, NY) containing Earle's salts, L-glutamine, and nonessential amino acids, supplemented with 0.11% sodium bicarbonate, 100 unit/ml penicillin, and 10% fetal bovine serum. After incubation at 28°C for 7 days, the cellular component was harvested by centrifugation, supplemented with 20% FBS, and stored at -70° C until use.

Table 2. 13 strains of dengue virus as positive controls which match a previous study.

Study on	Dengue serotype	Strain	Location	Year
D80-101	DEN-1	101	Thailand	1980
D80-218	DEN-2	218	Thailand	1980
D80-244M	DEN-2	244	Thailand	1980
D80-215M	DEN-2	215	Thailand	1980
D80-603	DEN-2	603	Thailand	1980
D80-158	DEN-2	158	Thailand	1980
CH53489/73	DEN-3	CH53489D73-1(492)	Thailand	1973
D80-273M	DEN-3	D-80-273 (35)	Thailand	1980
D84-137	DEN-3	D-84-137(21)	Thailand	1984
D84-315	DEN-3	215	Thailand	1984
D84-734	DEN-3	734	Thailand	1984
D84-641	DEN-4	641	Thailand	1984
D84-013	DEN-2	013	Thailand	1984

5. Blood and Body fluid collections

Blood, urine, saliva and oral brush were collected from patients during a few days to 4 weeks after the onset of disease. The number of each blood and body fluid specimen ranged from one to three per patient. The first sample collected on the day of hospitalization and the second sample on the day of discharge. The third sample was occasionally collected after the discharge day. The blood sample centrifuged at 3,000 rpm for 10 minutes at 4°C to separate the red blood cells from the plasma. The plasma transferred to a new tube. Red blood cells diluted with Phosphate buffered saline (PBS) and overlaid on Ficoll Hypaque ™ (GE) to separate peripheral blood mononuclear cells (PBMC). Urine samples centrifuged at 1,500 rpm for 10 minutes to separate pellet from supernatant. The urine pellets resuspended with PBS buffer and transferred to a new tube. Saliva and oral brush samples centrifuged at 3,000 rpm for 15 minutes at 4°C. Pellets of saliva and oral brush resuspended with PBS buffer and collected in a new tube. All of specimens were stored at -80°C until RNA extraction.

6. Viral RNA Extraction

Total RNA extracted by Viral RNA mini kit (Qiagen, Chatworth, CA, USA) according to the instruction of manufacture. The kit combines the selective binding properties of a silica -based membrane with the speed of micro-spin technology. Briefly, 140 µl of blood (plasma, PBMC, serum) and body fluids (urine, saliva, oral brush) were first lyses with 560 µl of lysis buffer (AVL) in the presence of a highly denaturing chaotropic salt containing buffer and incubation at room temperature for 10 minutes, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol added to provide appropriate binding conditions, and the sample was then applied to spin column, where the total RNA binded to the membrane and contaminants were efficiently washed away and centrifuged. High-quality RNA was then eluted in 60 µl of RNase –free sterile distilled water by centrifugation at 8,000 rpm for 1 min and stored at -80°c until use for synthesis of cDNA.

7. Measurement of Viral RNA Concentration

To determine the quality of extracted RNA, each RNA sample aliquot at 5 ul for measuring of total RNA concentration. Aliquot RNA diluted 20 times (fill up with RNase-free water to 100 ul); total RNA measured by spectrophotometer at optical density 260 nm (OD_{260}) and 280 nm (OD_{280}). Calculation of total RNA was necessary for reverse transcription polymerase chain reaction (RT-PCR). One unit of optical density at 260 nm equal 40 ug of total RNA per ml. Pure RNA had an OD_{260}/OD_{280} ratio of 1.6-1.9.

8. Nested reverse transcription-Polymerase chain reaction (nested RT-PCR)

To screen dengue virus infected in various clinical specimens, synthesis of single-stranded cDNA carried out. RNA of 0.25 ug reverse-transcribed into cDNA by M-MULV Reverse Transcriptase Reagent (Biolab, New England). Fifteen microliters of reverse transcription master mix containing 2 µl of 10xRT buffer (75 mM KCl, 50mM Tris-HCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 1 µl of 10mM dNTPs, 0.75 µl of 20 µM consensus primer (Table 3.), 20 U of RNase inhibitor (Bio Basic, Canada) and 100 U of M-MuLV Reverse Transcriptase, 15 µl of master mix added into 0.25 µg (3.5 ul) RNA template and transcribe at 25°C for 5 minutes, 37°C for 60 minutes and 70°C for 15 minutes.

First round PCR carried out using cDNA from RT as a template for PCR by Hot Start Taq™ DNA Polymerase ((Qiagen, Chatworth, CA, USA). Seventeen point five microliters of PCR mastermix containing 2 µl of 10x buffer (contain 1.5 mM $MgCl_2$) , 1.6 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM dNTPs, 0.75 µl of 20uM outer consensus primer (table 1), 0.5 U of Hot Start Taq™ DNA Polymerase, 2.5 µl of cDNA added into 17.5 microliters of the mastermix. PCR conducted at 95 °C for 15 min, followed by 40 cycle amplification cycles of 95 °C for 1 minute, 50 °C for 1 minute, and 72 °C for 1 minute, with a final extension at 72 °C for 7 minutes

Second-round PCR carried out using first round PCR product as a template by Hot Start Taq™ DNA Polymerase (Qiagen, Chatworth, CA, USA). Nineteen

microliters of PCR mastermix containing 2 µl of 10x buffer (contain 1.5 mM $MgCl_2$) , 0.4 µl of 25 mM $MgCl_2$, 0.4 µl of 10mM dNTPs, 0.75 µl of 20 µM inner consensus primer (table 1), 0.5 U of Hot Start Taq™ DNA Polymerase, 1 of first PCR product added into 19 microliters of the mastermix. PCR conducted at 95 °C for 15 minutes, followed by 25 cycle amplification cycles of 95 °C for 1 minute, 58 °C for 1 minute, and 72 °C for 1 minute, with a final extension at 72 °C for 7 minutes. Ten microliters of the PCR product analyzed by electrophoresis on 1.5 % agarose gels in 1X TBE (89 mM Tris borate, 0.5 mM EDTA, pH 8) until the bromophenol blue dye had migrated two-thirds the length on the gel. A 100-bp ladder used as a size standard (Fermentas, Germany).

Table 3. The sequences of primers used to amplification dengue-1 to dengue- 4 by nested RT-PCR.

Primer	sequence	Tm (C°)
Den outer-1	5'-CCATGGAAGCTGTACGC	54
Den outer-2	5'-GARACAGCAGGATCTCTGGTCT	49
Den inner-1	5'-GGTTAGAGGAGACCCCTCCC	66
Den inner-2	5'-GGGGGTCTCCTMTAACCTCTAKTCCTT	60

9. Serological methods and antigens

All the specimens also tested for dengue virus-specific IgM and IgG by ELISA.

Preparation of biotin-linked anti-flavivirus IgG. Antiflavivirus IgG was covalently conjugated to biotin using 1 mg of IgG dissolved in 1 ml of 0.15 M phosphate- buffered saline (PBS), pH 7.2, and cleared by centrifugation at 300 3 *g* at

48C for 10 minute. It was then dialyzed against 0.1 M sodium bicarbonate, pH 8.2, at 48°C overnight, clarified by centrifugation again, and mixed with 1 mg/ml of freshly prepared N-hydroxy succinimidobiotin in dimethylsulfoxide. The mixture was rotated gently at room temperature for 4 hour. It was then extensively dialyzed against 0.15 M PBS, pH 7.2, and clarified by centrifugation. The supernatant mixed with an equal volume of glycerol and stored at 22°C.

Immunoglobulin M–captured biotin-streptavidin ELISA. The IgM-ELISA, which was used to determine dengue IgM in the serum samples. A 96-well microtiter plate (Nunc, Roskilde, Denmark) coated with goat anti-human IgM (m-chain specific) diluted 1:1,400 in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (100 ml/well) and incubated at 48°C overnight in a humid box. The plate washed six times with 0.15 M PBS, pH 7.2 (3 minute/wash). At the blocking step, a 150 ml of 2% bovine serum albumin (BSA) in PBS was added to each well to block the uncoated sites and the plate was incubated at 37°C for 1 hour. The plate was then washed with PBS containing 0.05% Tween-20 (PBS-T) and 50 ml of duplicate sera including eight positive and six negative control sera diluted 1:200 in PBS containing 1% BSA was added into each well, incubated at 37°C for 1 hour, and washed with PBS-T. Fifty microliters of tetravalent dengue antigens (a mixture of 32 hemagglutination units each of DEN 1-3 and 16 hemagglutination units of DEN-4) diluted 1:2 in PBS containing 20% acetone-extracted normal human serum was added into each well. The plate was incubated at 37°C for 1 hr and washed as above. A 25-ml sample of biotinylated anti-flavivirus IgG diluted 1:500 in PBS containing 20% acetone-extracted normal human serum and 0.05% BSA was applied to each well, incubated at 37°C for 1 hour, and washed as above. A 50-ml sample of streptavidin-peroxidase diluted 1:2,000 in PBS containing 1% BSA was distributed into each well, incubated at 37°C for 1 hour, and washed with PBS-T. A 100-ml sample of chromogenic substrate solution including 0.3 mg% *o*-phenylenediamine and 0.02% hydrogen peroxide in 0.1 M citrate phosphate buffer, pH 5.0, added to each well and incubated in the dark at room temperature for 30 min. The reaction stopped by adding 50 ml of 4 N sulfuric acid to each well. The result determined by reading the absorbance at 490 nm in an ELISA plate reader (Minireader II; Dynatech Laboratories, Inc., Chantilly, VA). The cut-off value calculated by the mean of negative controls plus

two standard deviations. The average OD of the serum above the cut-off value was considered reactive for dengue IgM. (AFRIMS)

Serotyping study

To determine dengue serotypes in clinical specimens. Synthesis of single-strand cDNA carried out, as used total RNA 0.25 ug for reverse-transcribed into cDNA by M-MULV Reverse Transcriptase Reagent (Biolab, New England). Fifteen microliters of reverse transcription mastermix containing 2 ul of 10xRT buffer (contain 3 mM MgCl2) 1 ul of 10mM dNTPs, 2.5 ul of 10pM consensus primer (Table 4.), 20 U of RNase inhibitor (Bio basic, Canada) and 100 U of M-MuLV Reverse Transcriptase, 15 ul of mastermix was added into 0.25 ug (2.5 ul) RNA template and transcribe at 25°C for 5 minutes, 37°C for 60 minutes and 70°C for 15 minutes.

First round PCR was carried out using cDNA from RT as a template for PCR by Hot Start Taq™ DNA Polymerase ((Qiagen, Chatworth, CA, USA). Eighteen microliters of PCR mastermix containing 2 ul of 10x buffer (contain 1.5 mM $MgCl_2$) , 0.8 ul of 25 mM $MgCl_2$, 0.4 ul of 10mM dNTPs, 0.5 ul of 10 pM consensus primer (Table 2.), 0.5 U of Hot Start Taq™ DNA Polymerase, 2 ul of cDNA added into 18 microliters mastermix. PCR conducted at 95 °C for 15 minutes, followed by 35 cycle amplification cycles of 95 °C for 30 second, 50 °C for 1 minute, and 72 °C for 2 minutes, with a final extension at 72 °C for 7 minutes

Second round PCR was carried out using first round PCR product as a template by Hot Start Taq™ DNA Polymerase (Qiagen, Chatworth, CA, USA). Eighteen microliters of PCR mastermix containing 2 ul of 10x buffer (contain 1.5 mM MgCl2), 0.4 ul of 25 mM $MgCl_2$, 0.4 ul of 10mM dNTPs, 0.5 µl of 10pM Types specific primer DEN-1 to DEN-4 (table 4.), 0.5 U of Hot Start Taq™ DNA Polymerase, 1 ul of first PCR product (diluted with distilled water 1:25) added into 18 microliters of the mastermix. PCR conducted at 95 °C for 15 minutes, followed by 25 cycle amplification cycles of 95 °C for 30 second, 50 °C for 1 minute, and 72 °C for 2 minutes, with a final extension at 72 °C for

7 minutes. Ten microliters of the PCR product was analyzing by electrophoresis on 1.5 % agarose gels in 1X TBE (89 mM Tris borate, 0.5 mM EDTA, pH 8) until the bromphenol blue dye migrated two-thirds the length on the gel. A 100-bp ladder was using as a size standard (Fermentas, Germany).

Table 4. The sequences of primers used to amplify serotypes of dengue-1 to dengue- 4 by semi-nested multiplex RT-PCR.

Primer	Sequence	Tm (C°)
DV1	5'-TGAATATGCTGAAACGCGCGAGAAACCG	69
DV2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC	64
TS1	5'-CGTCTCAGTGATCCGGGGG	64
TS2	5'-CGCCACAAGGGCCATGAACAG	62
TS3	5'-TAACATCATCATGAGACAGAGC	47
TS4	5'-CTCTGTTGTCTTAAACAAGAGA	43

10. Genotyping study

To determine dengue genotypes in various clinical specimens, RSS-PCR was carried out using RNA of 0.25 ug in one–step RT-PCR kit (Qiagen, Chatworth, CA, USA). Forty microliters of mastermix containing 10 ul of 5x one step RT-PCR buffer (contain 3 mM $MgCl_2$) 1 ul of 10mM dNTPs, 1.25 ul of 20μM restriction-site specific primers (table 5.), 10 U of RNase inhibitor (Bio Basic, Canada) 2 μM enzyme mix, 10 ul of RNA was added into mastermix. Reverse transcription conducted at 53°C for 60 minutes, followed by 35 amplification cycles of 95 °C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Ten microliters of the PCR product analyzed by electrophoresis on 1.5 % agarose gels in 1X TBE (89 mM

Tris borate, 0.5 mM EDTA, pH 8) until the bromphenol blue dye migrated two-thirds the length on the gel. A 100-bp ladder used as a size standard (Fermentas, Germany)

Table 5. The sequences of primers used to amplify genotypes of dengue-1 to dengue- 4 by Restriction site-specific – Polymerase Chain Reaction (RSS-PCR).

Primer	Sequence	Genome position	Strand	Specificity
RSS-9	5'-CTG TTC TAG TGC AGG TTA	1897-1914	+	DEN-1
RSS-10	5'-CAT TTT CCC TAT ACT GCT TCC	2124-2144	-	DEN-1
RSS-11	5'-GTC ACA AAC CCT GCC GTC CT	1089-1108	+	DEN-1
RSS-12	5'-CGC AGC TTC CAT GCTCCA AT	1013-1032	-	DEN-1
RSS-1	5'-GGA TCC CAA GAA GGG GCC AT	1096-1715	+	DEN-2
RSS-2	5'-GGC AGC TCC ATA GAT TGCT	2277-2259	-	DEN-2
RSS-3	5'-GGT GTT GCT GCA GAT GGA A	1524-1542	+	DEN-2
RSS-4	5'-GTG TCA CAG ACA GTG AGG T	2371-2353	-	DEN-2
RSS-5	5'-CCA ACA TAA CAA CTG ACT C	1131-1149	+	DEN-3
RSS-6	5'-GGC AAG GGA AGC (C/T)TG GTA	1259-1276	+	DEN-3
RSS-7	5'-CTA CAT TTT AAG TGC CCC G	1785-1767	-	DEN-3
RSS-8	5'-GAC AGG CTC CTC CTT CTT G	2023-2005	-	DEN-3
RSS-21	5'-GGA C/TCA ACA GTA CAT TTG CCG GA	1196-1218	+	DEN-4
RSS-22	5'-GTT TTC ATG CTC GGG GAA GAT	1292-1313	+	DEN-4
RSS-23	5'-CTT CTG ATG TGT CTG CTC CT	1604-1623	-	DEN-4
RSS-24	5'-GAG AAC TTT CCT GAA/G CAC ATC GT	1863-1858	-	DEN-4

11. Interpretation of RSS-PCR patterns

To test the reproducibility of the RSS-PCR technique, each strain amplified more than once to ensure that the same pattern was obtained. A control strain with a known pattern was amplified along with test strains. Only when the control strain generated the expected patterns were the patterns of the test strains included in the analysis.

12. PCR products purification

To ensure the accuracy of dengue serotypes and genotypes, the region of the 3' UTR was amplified by nested RT-PCR with consensus primers and purified with the PCR product purification kit (Qiagen, Chatworth, CA, USA). Amplified PCR products purified by QIAquick PCR Purification Kit (Qiagen, Chatworth, CA, USA). 250 µl of Buffer PB added to 50 µl of PCR product in QIAquick spin column attached to the (provided) 2 ml collection tube. The column centrifuged at 13,000 g for 60 seconds, Flow-though was discarded. The sample washed by adding 0.75 ml of Buffer PE and centrifuged at 13,000 g for 60 seconds, the flow-though was discarded and centrifuged once at 13,000 g for 60 seconds. Then, the column was placed in a clean 1.5 ml microcentrifuge tube purified DNA eluted by added 50 µl of EB Buffer (10 mM Tris·Cl, pH 8.5), let the column stand for 1 minute for increased DNA concentration and centrifuged at 13,000 g for 1 minute. The purified DNA was kept at -20 ^{o}C.

13 Sequencing of the 3' UTR or Casid/prM gene

Amplified PCR products were purified by precipitation with QIAquick PCR Purification Kit, (Qiagen) Kit. The pellet was diluted in 20 µl of EB buffer with 5 pmole of primer (sense and/or reverse), and sent to sequencing by Macrogen, Korea. Identification of all sequences was performed by submitting the sequences to BLAST program from GENBANK database.