CHAPTER IV

MATERIALS AND METHODS

1. Subjects, blood sample, and plasmid

1) Serum from patient with dengue infection

Sera from 100 patients (DEN-1 = 25, DEN-2 = 25, DEN-3 = 25, and DEN-4 = 25) admitted in King Chulalongkorn Memorial Hospital with dengue infection based on clinical and laboratory data were collected during 2002-2003. 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 patient and stored at −70°C until used.

2) Recombinant plasmid

Recombinant plasmid, pTT47, contains 4.7 kb of DEN-2 New Guinea C strain sequence that encodes all of structural proteins, and the nonstructural proteins NS1, NS2A, NS2B, and N-terminal portion of NS3. This 4.7 kb of DEN-2 sequence was cloned into the EcoRI site in the multiple cloning site of pGem7Zf (+) under the control of the T7 promoter. The recombinant plasmid was used for evaluated sensitivity of conventional PCR and Real-time PCR.

2. Viral RNA Extraction

Dengue viral RNA was extracted from infected patients' serum, using a QIAamp Viral RNA Mini Kit (QIAGEN, Germany). 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 minutes. Add 560 µl of absolute ethanol, mixed and loaded onto the spin column. This

was followed by two time of washing buffer AW1 and AW2, respectively. The final step, RNA in column was eluted with the buffer AVE to a final volume of 40 µl.

3. Primers and probes

RT-PCR was performed using seven oligonucleotide primers that are conserved among dengue viruses. Seven oligonucleotide primers are included in this assay: in the RT-step and first PCR amplification, the forward primer DEN that targets on the region of the capsid gene and the reverse primer DENR that conserved on the membrane glycoprotein precursor. In the nested PCR amplification, five primers are included in the PCR reaction: the forward primer DENG that targets on the capsid gene and four reverse primers, each of which is complementary to sequence unique to each genotype. The primers, DEN, DENR and DENG, are conserved in all four dengue virus genotype. All primer sequences are listed in Table 3. Primers and probes for nested PCR determination in the LightCycler consisted of the forward primer DENG, the reverse primer DENLC that conserved in all four dengue genotype, and two probe pair for determined the dengue virus genotypes. Each probe pair consists of two onligonucleotides, which are labeled with different fluorophores. One probe, called the detection probe, is labeled at the 5' end with a LightCycler-Red fluorophore (LC-Red 640 and LC-Red 705). The other probe, called the anchor probe, is labeled at the 3' end with fluoresein. First probe pair consisted of PDEN1, the anchor probe labeled with fluoresein and the detection probe labeled with LC-Red 640, PDEN2. Second probe pair consisted of PDEN3.1 and PDEN 3.2, the anchor probe labeled with fluoresein and PDEN4 the detection probe labeled with LC-Red 705. The first probe pair was designed to detect dengue virus genotype 1 and genotype 3 and the second probe pair was designed to detect dengue virus genotype 2 and genotype 4. Two pairs of hybridization probe labeled with different fluorophore were used

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PDEN 1          :  GCT CAC CGG CCA AGG ACC MAT G
Den 1           :  ... TT. A.. ... ... ... C.. .
Den1th          :  ... .T. A.. ... ... ... C.. .
Den 3           :  ... G.. ... ... ... ... A.. .
Den3th          :  ... G.A ... ... ... ... A.. .
Den 2           :  ... G.. G.. ... ... ... A.. A
Den2th          :  ... GCA G.. A.G ... ... AT. A
Den 4           :  TT. T.. ... ..A ... ... CT. A
Den4th          :  TT. TC. ... GA. ... ... CT. .

PDEN 2          :  AAC TGG TTA TGG CGT TCA TAG CTT TC
Den 1           :  ..T ... .G. ... .T. .T. ... .A. ..
Den1th          :  ... ... .G. ... .T. ... ... .A. ..
Den 3           :  ..T ... ... ... ... .T. ... ... ..
Den3th          :  ..T ... ... ... ... ... ... ... ..
Den 2           :  ..T ..T ... ... .CC .GG .G. .A. ..
Den2th          :  ... ..T .C. ... .CC .GG .G. ... ..
Den 4           :  GGA ... .GC .A. .A. ... .CA .G. .T
Den4th          :  GGA ... .GT ... .AT ... .CA .G. .T

PDEN 3.1        :  TT CGT TTC CTA ACA ATC CCA CC
Den 2           :  .. ... ... ... ... ... ... ..
Den2th          :  .. ... ... ... ... ... ... ..
Den 4           :  .G ..A G.. ..T T.C ... ... ..
Den4th          :  .G A.A G.. ..T T.C ... ... ..
Den 1           :  .A A.A ..T ... .C. ..A ..T ..
Den1th          :  .A A.A ..T ... GCC ..A ..C ..
Den 3           :  .C A.A ..T ... G.C ..T ... ..
Den3th          :  .C A.A ..T ... G.C ..T ... ..

PDEN 3.2        :  TG AGA GTT CTT TCC ATC CCA CC
Den 2           :  .T C.T T.C ..A A.A ... ... ..
Den2th          :  .T C.T T.C ..A A.A ... ... ..
Den 4           :  .. C.. ..C ... ... ... ... ..
Den4th          :  .. ... ... ... ... ... ... ..
Den 1           :  .A ... T.. ..A G.. ..A ..T ..
Den1th          :  TA ... T.. ..A G.. ..A ... ..
Den 3           :  .C ... T.. ..A G.. ..T ... ..
Den3th          :  .C ... T.. ..A G.. ..T ... ..

PDEN 4          :  ACA GCA GGG ATT CTG AAG AGA TGG
Den 2           :  ... ... ... ..A T.A ..A ... ...
Den2th          :  ... ... ... ..A T.G ..A ... ...
Den 4           :  ... ... ... ... ... ... ... ...
Den4th          :  ... ... ... ... ..A ..A ... ...
Den 1           :  ... ... ..A ... T.. GCT ... ...
Den1TH          :  ... ... ..A ... T.. GCT ... ...
Den 3           :  ... ... ..A G.C T.G GCT ... ...
Den3th          :  ... ... ..A G.C T.G GCT ... ...
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Figure 2 The position and sequence of hybridization probe compared with region of dengue genotype 1-4 used for LightCycler typing. PDEN 1, PDEN3.1, and PDEN 3.2 labeled with Fluoresein. PDEN 2 and PDEN 4 labeled with LC-Red 640 and LC-Red 705, respectively. DEN 1-4 as a reference sequences from NCBI and DEN 1-4 TH as sequence from this study. M as base A and C. Anchor probe PDEN1 is designed for hybridize with both DEN-1 and DEN-3. PDEN3.1 and PDEN3.2 are perfectly matched with DEN-2 and DEN-4 is designed to detect DEN-1, 3 and DEN-2, 4, respectively.

Table.3 The sequences of oligonucleotide primers used for RT-PCR and Real-time PCR

Primer	Sequence	Length	PCR product	Nucleotides position
DENR	5' TTG CAC CAA CAG TCA ATG TCT TCA GGT TC 3'	29	511	genotype 1, 614-642; genotype 2, 616-644; genotype 3, 614-642; genotype 4, 618-646
DEN	5' TCA ATA TGC TGA AAC GCG CGA GAA ACC G 3'	28		genotype 1, 132-159; genotype 2, 134-161; genotype 3, 132-159; genotype 4, 136-163
DENG	5' GCG AGA GAA ACC G(C/T)G T(G/A)T C 3'	19		genotype 1, 147-165; genotype 2, 149-167; genotype 3, 147-165; genotype 4, 151-166
DEN1	5' CGT CTC AGT GAT CCG GGG G 3'	19	467	genotype 1, 595-613
DEN2	5' TTC CTG AAC CCT CTC AAA ACA 3'	18	204	genotype 2, 333-352
DEN3	5' TAA CAT CAT CAT GAG ACA GAG C 3'	19	275	genotype 3,400-421
DEN4	5' CTC TGT TGT CTT AAA CAA GAG A 3'	19	377	genotype 4, 506-527
DENLC	5' TTC AGG AAG GAG ATT GGA AGC AT 3'	23	220	genotype 1, 343-366; genotype 2, 354-368; genotype 3, 343-366; genotype 4, 347-370

in one reaction in order to typing the dengue virus genotypes. The position and sequences of the hybridization probes are shown in Figure 2. FRET probes were protected from light and high centrifugal forces to prevent degradation or precipitation of the fluorophores.

All primers were synthesized by Bio Basic Inc. (Canada) and fluorescence hybridization probes were synthesis by TIBMOL BioScince (Germany). The genome positions of each primer are given according to the published reference sequences of dengue genotype 1-4 from GenBank (URL:http:// www.ncbi.nlm.nih.gov): genotype 1, NC_001477; genotype 2, NC_001474; genotype 3, NC_001475; genotype 4, NC_002640.

4. RT-Nested PCR of Dengue viral RNA

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 concentration of each dNTP, 40 *p*mole of primer DENR, the dengue virus downstream consensus primer with homologous to the dengue genome RNA of the four genotypes, 100 U of MMLV 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 50 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 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 Thermocycler programmed to incubate for 5 min at 94°C initially then amplified for 35 cycles under the following conditions: 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a

final extension at 72°C for 5 min. After completing PCR, 2 µl was diluted in 198 µl of distilled water, and 2 µl of this dilution was used for real-time genotyping.

A second amplification reaction was initiated with 2 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 consensus primer DENG and genotype specific primers (DEN-1, DEN-2, DEN-3, and DEN-4), and 2 U 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 30 s, 55°C for 30 s, 72°C for 30 s, 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 (BioWhittaker Molecular Applications, USA) in TAE buffer. The different PCR product was characterized by the position of priming with each of the dengue virus type-specific primers.

5. Real-time PCR with LightCycler-FastStart ENA Master Hybridization Probe Kit

Nested, hot start PCR reaction were performed in a final volume of 20 µl, using LightCycler-FastStart DNA Master Hybridization Probes Reaction Kit (Roche Diagnotic, Germany). The reaction combined 1 µl of diluted first round PCR product with 19 µl of master mixture in glass capillaries, and nested PCR was performed in the Roche LightCycler. Each 20 µl nested PCR reaction contained 3 mM $MgCl_2$, 0.5 µM of each primers, DENG and DENLC, 0.15 µM of each probes for genotype detection, and 1x LightCycler-FastStart DNA Hybridization probe mix. Only after hybridization do the two probes come in close proximity, resulting in fluorescence resonance energy transfer between the two fluorophore, is excited by the light source of the LightCycler instrument and part of the excitation energy is transferred to LightCycler-Red, the acceptor fluorophore. The emitted fluorescence to the acceptor fluorophore is measured.

After a preincubation step at 95°C for 10 min to activate the FastStart polymerase, PCR amplification was performed in 45 cycles of denaturation at 93°C for 5 s and the temperature transition rate of 20°C/s, annealing at 50°C for 10 s with a temperature transition rate of 20°C/s, a single fluorescence measurement taken at the end of the annealing step, and extension at 72°C for 15 s with a temperature transition rate 0.5°C / s. After amplification, melting curve analysis was performed by heating 95°C for 5 s with temperature transition rate of 20°C / s, cooling to 30°C for 30 s, and then heating the sample at 0.05°C / S to 70°C. The fluorescence signals of the LC-Red 640-labeled probes were first measured in channel 2 (F2), and afterwards the LC-Red 705-labeled probes were measured using F3. The four genotype of dengue virus were discriminated by the different melting temperature (T_m) of the amplicons.

6. Real-time PCR with in-house DNA hybridization probes reaction

The LightCycler – FastStart DNA Master Hybridization probe kit was replaced by in-house DNA hybridization probe reaction. The 19 µl reaction contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100, 3 mM $MgCl_2$, 200 µM concentration of each dNTP, 0.5 µM of each primer, DENG and DENLC, 0.15 µM of each probe, and 0.5 U of *Taq* polymerase.

7. Sensitivity of the LightCycler assay

The molecular sensitivity of LightCycler assay was compared to that the conventional assay using tenfold dilutions plasmid, pTT47. Starting from 625 pg of plasmid which is equal to $7.5x10^7$ molecules was serially diluted with 10 mM TE to 0.75 molecules. Ten microliter of each dilution was use as template in both PCR reactions.

8. Detection of PCR products

The 2% agarose gel was prepared by completely dissolving agarose powder upon heating in 1x TAE buffer, contain Tris base, glacial acetic acid, and 0.5 M EDTA (pH 8.0) then allowed cooling 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 (Promega Corporation, USA) and loaded into gel slots in a submarine condition with 2.5 µg/ml ethidium bromide solution. Electrophoresis was performed at 100 volts for 30 min. DNA patterns were visualized and photographed under UV light exposure with UV transilluminator.

9. Nucleotide sequencing

Nucleotide sequencing was performed by using ABI 310 DNA Sequencer (PE applied Biosystem, USA) 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 pmole 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 s, 50°C for 5 s, and 60°C for 4 min. After cycle sequencing, 10 µl of sterile distilled water were added, followed by 0.1 volumes 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 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. The nucleotide sequences from forward primer and reverse primer were aligned and compared with Sequence Navigator program. The

sequence was then retrieved in the Fasta format and aligned with dengue reference sequence using clustalX program. Dengue virus genotypes were compared with published reference sequences as previously described.