CHAPTER III

MATERIALS AND METHODS

Patient and specimen recruitments

Plasma, PBMCs, saliva and urine of both DENV and non-DENV-infected patients collected at least 2 time points during febrile, early and late convalescent periods were taken from our previous project "Survival of dengue virus in blood, urine, saliva and buccal mucosa in complete-recovery dengue patients" [30]. The inclusion criteria for this study were confirmed adult dengue-infected patients (Age ≥ 15 years old) and had been admitted to King Chulalongkorn Memorial Hospital during April 2007 to October 2008. Adult non-dengue-infected patients (Age ≥ 15 years old) coming to the hospital with other febrile illness were enrolled as a negative control group. Exclusion criteria were accepted for immunodeficiency or immunocompromise patients, patients with hematologic related diseases, patients experiencing with immunosuppressive drugs and lost-to-follow-up patients as well as patients having only one time point of specimen collection. Inform consent was firstly signed by each patient in both groups before participating in this project.

Clinical diagnosis and confirmed DENV infection were investigated by using WHO criteria for dengue diagnosis (2009) and standard ELISA, which referred to results of our previous project described by Laosakul *el al.* [30, 97]. A value (unit) ≥ 40 is positive for IgM and ≥ 100 positive for IgG. The ratio of IgM: IgG ≥ 1.8 is considered as a primary infection whereas < 1.8 is considered as a secondary infection [52].

EDTA blood, saliva and urine were collected from patients in both groups during febrile period, early convalescent (1st day of recovery to day 25 of illness) and late convalescent (day 26 to day 90 of illness) periods. At least two time points (duration ≥ 7 days) of specimen collections were accepted for studying in this project.

Blood was drained from each patient in 3 ml K_3EDTA tube (2 tubes). Saliva was collected in a sterile covered container in volume of 5 ml. Midstream urine was

collected in a sterile covered container in volume of 20 ml. All specimens were sent to laboratory for processing immediately. If specimens could not be brought to laboratory, they were kept at 4 °C and sent to laboratory in a next day.

Sample size calculation

Due to the lacking of previous studies about the monitoring of DENV infection, we used the sample size calculation as a pilot study accounting for 30 patients [130].

Lab equipments and instruments

1. PCR tube size 0.2 µl (Axygen, USA)
2. Micro tube size 1.5 ml (Axygen, USA)
3. 15 ml and 50 ml sterile tube (Corning, USA)
4. Plastic petri dish (sterile) 90 mm (Sterilin, UK)
5. Spreader (Corning, USA)
6. Forceps (Hilbro, Pakistan)
7. Filter tips size 10 µl, 20 µl, 100 µl, 200 µl and 1000 µl (Axygen, USA)
8. Blue pad (SOFT 'n SECURE. Thailand)
9. Alcohol flame lamp (Suksapan, Thailand
10. Sterile tips size 10 µl, 20 µl, 100 µl, 200 µl and 1000 µl (Axygen, USA)
11. Tissue paper (RiverPro, Thailand)
12. Plastic bags (Tesco, Thailand)
13. PCR racks and 1.5 ml racks (Axygen, USA)
14. Bottle top filter size 500 ml (Corning, USA)
15. Auto pipettes size 0.1-2.5 µl, 0.5-10 µl, 2-20 µl, 10-100 µl, 20-200 µl and 10-1000 µl (Eppendorf, Germany; Biohit, Finland)
16. Pipette Aid (Biohit, Finland)
17. Toothpicks (BigC, Thailand)
18. Water bath (WiseBath, Korea)
19. Heat box (Biosan, Latvia)
20. Incubator (Memmert, Germany)

21. Incubator shaker (Biotek, France)
22. Micro centrifuge (Labtech, Korea)
23. Mini centrifuge (WiseSpin, Korea)
24. Refrigerated centrifuge (Eppendorf, Germany)
25. Vortex mixer (WiseMix, Korea)
26. Gel chamber (SciePlas, UK)
27. Power supply (Biorad, USA)
28. Microwave (Sharp, Thailand)
29. Balance 2 and 4 points (Ohaaus, Switzerland)
30. Autoclave (Hirayama, Japan)
31. Hot oven incubator (Memmert, Germany)
32. Plastic boxes (Axygen, USA)
33. Duran bottles size 50 ml, 200 ml, 250 ml, 500 ml and 1000 ml (Schott, Germany)
34. Flask size 250 ml and 500 ml (Pyrex, USA)
35. PCR thermal cycle (Eppendorf, Germany)
36. Rotor Gene 6000 Real time PCR machine (Corbett Life Science, QIAGEN, Germany)
37. Beaker size 250 ml (Pyrex, Germany)
38. Biohazard Safety Cabinet class II (Esco, Singapore)
39. Safety Cabinet for PCR preparation (Augustin, Thailand)
40. Stop watch (Canon, Thailand)
41. Hot plate and Magnetic stirrer (Labtech, Korea)
42. Autoclave tape (3M, Thailand)
43. Para film paper (Parafilm M, USA)
44. Thermometer (Precision, Germany)
45. Sterile plastic serological pipettes size 5 ml and 10 ml (Costar, USA)
46. Sterile pasture pipette (Suksapan, Thailand)
47. Gel Doc (Quantum ST4 100, France)
48. Refrigerator 4°C (Sanyo, Japan)
49. Freezer -30 °C (Sanyo, Japan)
50. Deep freezer -80 °C (Sanyo, Japan)

51. pH meter (Schott, Germany)
52. Nano drop spectrophotometer (Thermo, USA)

Chemicals and reagents

1. Tris (hydroxymethyl) aminomethane (Research organic, USA)
2. Ethylenediaminetetraacetic acid (EDTA) (Bio Basic, Canada)
3. Boric acid (Research organic, USA)
4. Sodium hydroxide (NaOH) (Merck, Germany)
5. Hydrochloric acid (HCl) (Merck, Germany)
6. Isopropyl alcohol (Sigma, Germany)
7. Sodium chloride (NaCl) (Merck, Germany)
8. Potassium chloride (KCl) (Merck, germany)
9. Potassium phosphate monobasic (KH_2PO_4) (Merck, Germany)
10. Sodium Phosphate, dibasic, anhydrous (Na2HPO4) (Merck, Germany)
11. Sodium bicarbonate (Na_2CO_3) (Merck, Germany)
12. Sodium hydrogen carbonate ($NaHCO_3$) (Merck, Germany)
13. Ethidium bromide (Bio Basic, Canada)
14. Absolute ethanol (Merck, Germany)
15. UltraPure DNase/RNase-Free Distilled Water (Gibco, Invitrogen, USA)
16. Tryptone powder (Bio Basic, Canada)
17. Yeast Extract powder (Bio Basic, Canada)
18. Sodium chloride (NaCl) (Merck, Germany)
19. Spectinomycin (Bio Basic, Canada)
20. Glucose ($C_6H_{12}O_6$) (Bio Basic, Canada)
21. Magnesium chloride ($MgCl_2$)(Merck, Germany)
22. Magnesium sulfate($MgSO_4$)(Merck, Germany)
23. Agarose (Bio Basic, Canada)
24. DNA marker 50 bp, 100 bp and 1 kbp (Fermentas, EU)
25. 6X loading buffer (Fermentas, EU)
26. ExoSAP-IT® for PCR product clean up (USB, Affymetrix, USA)
27. QIAquick Gel Extraction Kit (Qiagen, Germany)

28. Distilled water (DW) and reverse osmosis (RO)water (ELGA, UK)
29. Ficoll-Paque [tm] Plus (GE Healthcare, Sweden)
30. Sequencing service (1st BASE, Malaysia)
31. QIAamp Viral RNA Mini Kit (Qiagen, Germany)
32. Oligonucleotide primers (DEUL, DEUR, DENUL, DENUR, 5'UTR-F, 5'UTR-R, Tag 5'UTR-F, Tag, D1, D2, TS1, TS2, TS3, TS4, D1L, D1R, D2L, D2R, D3L, D3R, D4L and D4R) (1st BASE, Malaysia)
33. QIAGEN OneStep RT-PCR Kit (QIAGEN, Germany)
34. HotStarTaq DNA polymerase (QIAGEN, Germany)
35. SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, US)
36. StrataClone ™ Solopack® Competent Cells (*E. coli*) (Agilent Technologies, US)
37. pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen, USA)
38. NucleoSpin Plasmid QuickPure (Macherey-Nagel, Germany)
39. Invisorb® Fragment Clean up (Stratec, Germany)
40. EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Invitrogen, USA)
41. SuperScript® III First-Strand Synthesis System (Invitrogen, USA)
42. BioScript™ One-Step RT-PCR Kit (Bioline, UK)
43. dNTP mix (10 mM each) (Fermentas, EU)
44. *Taq* DNA polymerase (recombinant, 500u) (Fermentas, EU)
45. RNase Inhibitor (TOYOBO, Japan)

Stock DENV for positive control and standard curve construction

All 4 DENV stocks were kindly provided from TROP MED Dengue Diagnostic Center (TDC), Faculty of Tropical Medicine, Mahidol University (Thailand).

1. Dengue virus serotype 1 (DENV1) strain Hawaii (5×10^4 PFU/ml)
2. Dengue virus serotype 2 (DENV2) strain 16681 (4.75×10^6 PFU/ml)
3. Dengue virus serotype 3 (DENV3) strain H87 (2.75×10^5 PFU/ml)

4. Dengue virus serotype 4 (DENV4) strain 814609 (2.5 x 10^5 PFU/ml)

Specimen processing

EDTA blood was centrifuged at 3000 rpm for 10 minutes at 4 °C to separate plasma and cell compartments. Cell pellet was diluted 1:2 with phosphate buffer saline (PBS), pH 7.4. The cell mixture was overlaid on Ficoll-Paque ^{tm} Plus in 15 ml centrifuge tube. PBMCs fraction was isolated using refrigerated centrifuge at 2,200 rpm for 20 minutes at 4°C. After centrifugation, the white ring (PBMCs) on the top of solution was washed with PBS, pH 7.4 by centrifuging at 1,500 rpm for 10 minutes. The supernatant was discharged and PBMCs pellet was suspended in 600 µl of PBS, pH 7.4. Plasma and PBMCs were divided in small volume (140 µl/tube) and kept at deep-freezer (-80 °C) until doing an experiment.

Saliva was diluted 1:2 with oral buffer, pH 7.0. The mixture was centrifuged at 3,000 rpm for 20 minutes at 4 °C. The supernatant was discharged and the pellet was dissolved in 600 µl of oral buffer and divided in small volume (140 µl/tube), kept at deep-freezer (-80°C) until doing an experiment.

Urine was centrifuged at 1,500 rpm for 10 minutes at 4 °C. The supernatant was discharged and the cell pellet was dissolved in 500 µl of PBS, pH 7.4. Cell suspension was also divided in small volume (140 µl/tube), kept at deep-freezer (-80°C) until doing an experiment.

Viral RNA extraction

Viral RNA was extracted from plasma, PBMCs, saliva and urine pellet of each patient in different time points using the QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturing handbook. Briefly, the 560 µl of buffer AVL-carrier RNA was added into 140 µl of each specimen. Solution was mixed vigorously using vortex mixer for 15 seconds and incubated at 25 °C for 10 minutes. Then, 560 µl of absolute ethanol was added into solution and mixed gently before it was applied to a spin column. The spin column was centrifuged at 8,000 rpm for 1 minute. Solution in each collection tube was discharged. Then, 500 µl of buffer AW1

was added into the column and each column was centrifuged at 8,000 rpm for 1 minute. After that, solution in the collection tube was discharged and 500 µl of buffer AW2 was added into each column and it was centrifuged at 14,000 rpm for 3 minutes. Each column was dry by centrifuging at 14,000 rpm for 1 minute. After that, each column had been replaced with a sterile 1.5 ml tube before 40 µl of elution buffer (AVE) was added to elude viral RNA. Before centrifuging at 8,000 rpm for 1 minute, each column was incubated at 25°C for 1 minute. Eluted RNA was collected in small volume and kept at -80°C until use. Concentration and purity of extracted viral RNA were determined by using NanoDrop spectrophotometer. The good purity of RNA extraction should be 2.0 (A260/A280).

Nested RT-PCR for detecting DENV using envelope (E) gene primers

To monitor the longest time of DENV detection and to study serotype, genotype, strain and genetic variation of DENV in each specimen and time point, extract viral RNA was subjected to do nested RT-PCR using specific primers for partial E gene (434 bp). These primers were taken from the previous study by Yenchitsomanus *et al.* [56]. Primer summary is listed in Table 2.

Table 2: Primers for nested RT-PCR (E gene)

DEUL	5' TGG CTG GTG CAC AGA CAA TGG TT 3'
DEUR	5' GCT GTG TCA CCC AGA ATG GCC AT 3'
DENUL	5' GAT CTC AAG AAG GAG CCA TGC A 3'
DENUR	5' ATG GAA CTT CCC TTC TTG AAC CA 3'

The volume of 5 µl of extracted viral RNA was done first round of nested RT-PCR using the QIAGEN OneStep RT-PCR Kit (QIAGEN, Germany). The protocol is performed according to Table 3.

Table 3: The reaction setup of first round nested RT-PCR (Yenchitsomanus protocol, E gene)

Reagents	1X (µl)
5X QIAGEN OneStep RT-PCR Buffer	4.0
dNTP Mix (containing 10 mM of each dNTP)	0.8
10 µM DEUL	1.0
10 µM DEUR	1.0
40 U/µl RNase inhibitor (TOYOBO, Japan)	0.2
DNase/RNase-Free Distilled Water	7.2
QIAGEN OneStep RT-PCR Enzyme Mix	0.8

RNA template	5.0
Total	**25 µl**

The PCR thermal cycle (Eppendorf, Germany) was set below.

Reverse transcription:	50	°C	40 minutes	
Initial activation step:	95	°C	15 minutes	
Denaturation step:	94	°C	1 minute	40 cycles
Annealing step:	42	°C	1 minute	
Extension step:	72	°C	1 minute	
Final extension step:	72	°C	10 minutes	

After that, 1 µl of first round PCR was subjected to do nested PCR using HotStarTaq DNA polymerase (QIAGEN, Germany) with DENUL and DENUR primers. PCR reaction is done using the protocol in Table 4.

Table 4: The reaction setup of nested RT-PCR for E gene

Reagents	**1X(µl)**
10X PCR Buffer	2.0
25mM $MgCl_2$	1.6
dNTP Mix (10 mM of each dNTP)	0.4
10 µM DENUL	0.75
10 µMDENUR	0.75
DNase/RNase-Free Distilled Water	13.4
5U/µl HotStarTaq DNA Polymerase	0.1
First PCR product	1.0
Total	**20**

The PCR thermal cycle (Eppendorf, Germany) was set as follows.

Initial activation step:	95	°C	15	minutes	
Denaturation step:	94	°C	1	minute	35 cycles
Annealing step:	58	°C	1	minute	
Extension step:	72	°C	1	minute	
Final extension step:	72	°C	10	minutes	

The PCR product was analyzed by 1.5% agarose gel electrophoresis in 1X TBE and stained with 0.5 µg/ml ethidium bromide. Expected band was 434 bps for all 4 serotypes. The sensitivity of this assay was 0.1-1.0 PFU/ml [56]. Clearly single

band of PCR product was purified using ExoSAP-IT® for PCR product clean up (USB, Affymetrix, USA). If each PCR product showed multiple bands, expected band was cut and purified by using the QIAquick Gel Extraction Kit (Qiagen, Germany). Concentration and purity of purified PCR product were investigated using NanoDrop spectrophotometer before sequencing using DENUL and DENUR primers (1st BASE, Malaysia) or ligating into the pCR®8/GW/TOPO® vector (Invitrogen, USA).

Semi-nested RT-PCR for detecting DENV serotypes (Lanciotti protocol)

This protocol was used to classify serotypes of DENV in each patient. Primers were taken from the previous study of Lanciotti *el al.* [58]. These primers spanned on C/prM of all 4 serotypes given the different bands in each length (482 bp for DENV1, 119 bp for DENV2, 290 bp for DENV3 and 392 bp for DENV4). The summary of used primers is listed in Table 5.

Table 5: Primers for semi-nested RT-PCR (DENV serotype classification)

D1	5' TCA ATA TGC TGA AAC GCG CGA GAA ACC G 3'
D2	5' TTG CAC CAA CAG TCA ATG TCT TCA GGT TC 3'
TS1	5' CGT CTC AGT GAT CCG GGG G 3'
TS2	5' CGC CAC AAG GGC CAT GAA CAG 3'
TS3	5' TAA CAT CAT CAT GAG ACA GAG C 3'
TS4	5' CTC TGT TGT CTT AAA CAA GAG A 3'

The volume of 5 µl of extracted RNA was used to generate cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, USA). Each reaction was done as described in Table 6.

Table 6: The reaction setup of cDNA synthesis for semi-nested RT-PCR serotype classification

Reagents	**1X (µl)**
RNA template	5.0
10 µM D2	1.0
10 mM dNTP Mix	1.0
DNase/RNase-Free Distilled Water	1.0

Each reaction was incubated at 65 °C for 5 minutes. After that, the reaction mixture was placed on ice for 2 minutes. Mastermix solution for cDNA synthesis was prepared by following the reaction setup in Table 7.

Table 7: The reaction setup of cDNA synthesis for semi-nested RT-PCR (mastermix preparation)

Reagents	1X (µl)
10X RT buffer	2.0
25 mM $MgCl_2$	4.0
0.1M DTT	2.0
RNaseOUT™ (40U/µl)	1.0
SuperScript™III RT (200U/µl)	1.0

The volume of 10 µl of cDNA synthesis mastermix was applied to RNA/primer mixture. The solution was mixed gently and incubated in The PCR thermal cycle (Eppendorf, Germany) by setting the condition as follows:

50 °C 50 minutes

85 °C 5 minutes

After finishing an incubation step, 1 µl of RNase H was added into each reaction tube. Then, each reaction was incubated at 37 °C for 20 minutes.

First round of PCR was done by using 2.5 µl of cDNA with D1 and D2 primers. The reaction was prepared according to the protocol in Table 8.

Table 8: The reaction setup of first round semi-nested RT-PCR for dengue serotype classification

Reagents	1X (µl)
10X PCR Buffer	2.0
25 mM $MgCl_2$	0.8
dNTP Mix (10 mM of each)	0.4
10 µM D1	0.5
10 µM D2	0.5
DNase/RNase-Free Distilled Water	13.2
5U/µl HotStarTaq DNA Polymerase	0.1
cDNA product	2.5
Total	**20**

The PCR thermal cycle (Eppendorf, Germany) was set as follows:

Initial activation step: 95 °C 15 minutes

Denaturation step: 94 °C 1 minute

Annealing step: 55 °C 1 minute } 35 cycles

Extension step: 72 °C 2 minutes

Final extension step: 72 °C 10 minutes

Semi-nested PCR was done by using 1 µl of first round PCR product with D1 primer combined with TS1-TS4 primers (multiplex PCR reaction) by following the reaction setup in Table 9.

Table 9: The reaction setup of semi-nested RT-PCR for dengue serotype classification

Reagents	1X (µl)
10X PCR Buffer	2.0
25 mM $MgCl_2$	0.8
dNTP Mix (10 mM of each)	0.4
10 µM D1	0.5
10 µM TS1	0.5
10 µM TS2	0.5
10 µM TS3	0.5
10 µM TS4	0.5
DNase/RNase-Free Distilled Water	12.2
5U/µl HotStarTaq DNA Polymerase	0.1
First round of PCR product	1.0
Total	**20**

The PCR thermal cycle (Eppendorf, Germany) was set as follows:

Initial activation step:	95	°C	15	minutes
Denaturation step:	94	°C	1	minute
Annealing step:	55	°C	1	minute } 30 cycles
Extension step:	72	°C	2	minutes
Final extension step:	72	°C	10	minutes

The PCR product was analyzed by 1.5% agarose gel electrophoresis in 1X TBE and stained with 0.5 µg/ml ethidium bromide. Expected bands were 482 bps for DENV1, 119 bps for DENV2, 290 bps for DENV3 and 392 bps for DENV4, respectively. The sensitivity of this assay was 10-100 PFU/ml [58].

Nested RT-PCR for detecting DENV serotypes (Yenchitsomanus protocol)

Serotype specific nested RT-PCR was used as an alternative protocol to classify the serotype of DENV in each patient. Primers and protocol were adapted

from previous study by Yenchitsomanus *et al.* [56]. These primers were designed to cover partial E gene sequences. The primers are listed in Table 10.

Table 10: Primers for nested RT-PCR serotype (Yenchitsomanus protocol)

DEUL	5' TGG CTG GTG CAC AGA CAA TGG TT 3'
DEUR	5' GCT GTG TCA CCC AGA ATG GCC AT 3'
D1L	5' GGG GCT TCA ACA TCC CAA GAG 3'
D1R	5' GCT TAG TTT CAA AGC TTT TTC AC 3'
D2L	5' ATC CAG ATG TCA TCA GGA AAC 3'
D2R	5' CCG GCT CTA CTC CTA TGA TG 3'
D3L	5' CAA TGT GCT TGA ATA CCT TTG T 3'
D3R	5' GGA CAG GCT CCT CCT TCT TG 3'
D4L	5' GGA CAA CAG TGG TGA AAG TCA 3'
D4R	5' GGT TAC ACT GTT GGT ATT CTC A 3'

Extracted RNA in the volume of 5 µl was done first round nested RT-PCR using DENUL and DENUR primers with the QIAGEN OneStep RT-PCR Kit (QIAGEN, Germany). The protocol for first RT-PCR product was similar to the condition of nested RT-PCR (Yenchitsomanus protocol) as described previously.

Nested PCR was done by using 1µl of first round PCR with inner specific primers (D1L-D4R). The PCR reaction was set using the protocol in Table 11.

Table 11: The reaction setup of nested serotypic RT-PCR (Yenchitsomanus protocol)

Reagents	**1X (µl)**
10X PCR Buffer	2.0
25mM $MgCl_2$	1.0
dNTP Mix (10 mM of each dNTP)	0.5
10 µM D1L	0.5
10 µM D1R	0.5
10 µM D2L	0.5
10 µM D2R	0.5
10 µM D3L	0.5
10 µM D3R	0.5
10 µM D4L	0.5
10 µM D4R	0.5
DNase/RNase-Free Distilled Water	11.4
5U/µl HotStarTaq DNA Polymerase	0.1
First PCR product	1.0
Total	**20**

The PCR thermal cycle (Eppendorf, Germany) was set as follows:

Initial activation step:	95	°C	15	minutes
Denaturation step:	94	°C	1	minute
Annealing step:	55	°C	1	minute
Extension step:	72	°C	1	minute
Final extension step:	72	°C	10	minutes

30 cycles

The PCR product was analyzed by 1.5% agarose gel electrophoresis in 1X TBE and stained with 0.5 µg/ml ethidium bromide. Expected bands were 504 bps for DENV1, 346 bps for DENV2, 198 bps for DENV3 and 143 bps for DENV4, respectively. The sensitivity of this assay was 0.1-1 PFU/ml [56].

DENV detection and viral load using real time RT-PCR (qRT-PCR)

Viral RNA extracted from plasma, PBMCs, saliva and urine in different periods of infection was taken to do one step qRT-PCR for monitoring DENV infection and quantification of DENV genome. Primers and protocol used in this study were taken from the previous study by dos Santos *et al.* [61]. These primers annealed 5' UTR of all 4 serotypes and are presented in Table 12.

Table 12: Primers of qRT-PCR for DENV detection and quantification

5' UTR-F	5' AGT TGT TAG TCT ACG TGG ACC GA 3'
5' UTR-R	5' CGC GTT TCA GCA TAT TGA AAG 3'

The volume of 4 µl of extracted RNA from various specimens was taken for detecting DENV genome using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, USA). The RT-PCR reaction was run on the Rotor Gene 6000 Real time PCR machine (Corbett Life Science, QIAGEN, Germany). The mastermix preparation was done according to the protocol in Table13.

Table 13: The reaction setup of qRT-PCR for DENV detection

Reagents	**1X (µl)**
SuperScript™III RT/Platinum® *Taq* Mix (includes RNaseOUT™)	0.5 µl
2X SYBR®Green Reaction Mix	12.5 µl
10 µM 5' UTR-F	0.5 µl
10 µM 5' UTR-R	0.5 µl
RNA template	4.0 µl

DNase/RNase-Free Distilled Water	7.0 µl
Total	**25 µl**

The Real time PCR machine was set as follows:

cDNA preparation:	50	°C	20	minutes	
Initial activation step:	95	°C	5	minutes	
Denaturation step:	95	°C	15	seconds	45 cycles
Annealing step:	54	°C	40	seconds	
Extension step:	72	°C	30	seconds	

The melting curve analysis was run using the ramp parameter from 60°C - 90 °C, rising by 0.2 °C for each step. Tm of expected product was 80.57 °C - 81.73 °C for all serotypes whereas primer-dimer was 75.14 °C – 76.31 °C. PCR products were confirmed by 2% agarose gel electrophoresis in 1X TBE and stained with 0.5 µg/ml ethidium bromide. The expected band was approximately 149 bps. The sensitivity of this assay was 2.55 PFU/ml [61].

Standard curve construction for determining DENV viral load

To determine the viral load or viral concentration, the results of each specimen were calculated with standard curve generated by using 10-fold dilutions of each extracted RNA from known viral concentration of DENV1-DENV4 (PFU/ml) [61, 62]. DENV1 was diluted from 5 x 10^3 PFU/ml to 5 x 10^{-2} PFU/ml. DENV2 was diluted from 4.75 x 10^5 PFU/ml to 4.75 x 10^{-2} PFU/ml. DENV3 was diluted from 2.75 x 10^3 PFU/ml to 2.75 x 10^{-2} PFU/ml and DENV4 was diluted from 2.5 x 10^4 to 2.75 x 10^{-3} PFU/ml. The volume of 4 µl of viral RNA in each dilution was done qRT-PCR using the same protocol as done in clinical specimen. The value of R^2 in each standard curve at least 0.98 was acceptable for DENV quantification. Because of the standard curve was generated from virus stock in a unit of PFU/ml, viral load in all specimens were expressed as PFU/ml [61, 131, 132, 185]. If some results were out of the range of standard curve at the low level, they were reported "less than the last concentration of standard point".

Partial E gene cloning and sequencing

Specimens in the patient positive for RT-PCR of partial E gene (434 bp) at least 2 time points were continuously studied the diversity of DENV population by cloning and sequencing. PCR cloning was done by using the pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen, USA).

The volume of 4 µl of purified PCR product was ligated into the pCR®8/GW/TOPO® vector according to an instruction manual in Table 14.

Table 14: The reaction setup of PCR ligation into pCR®8/GW/TOPO®

Reagents	1X (µl)
Fresh PCR product	4.0
Salt solution	1.0
DNase/RNase-Free Distilled Water	0.0
TOPO® Vector	1.0
Total	**6.0**

Each ligation tube was mixed gently using auto pipette (up and down) and incubated at 25 °C for 5 minutes. After that, the reaction tube was placed on ice. The volume of 2 µl of ligation reaction was transformed into StrataClone™SoloPack® Competent Cells (Agilent Technologies, US) using the chemical transformation protocol (heat shock at 42 °C for 45 seconds) and followed by adding SOC medium before the reaction tube was incubated at 37 °C with shaking 200 rpm for 1 hour. The volume of 100 µl of each transformation reaction was spread on LB agar contained with antibiotic (100 µg/ml spectinomycin) and incubated that plate at 37 °C overnight. Ten to fifteen colonies of each specimen were picked using sterile toothpicks and checked the insertion gene (434 bps) by colony PCR. The PCR reaction was set according to the instruction manual (*Taq* DNA polymerase, Fermentas, EU) as described in Table 15.

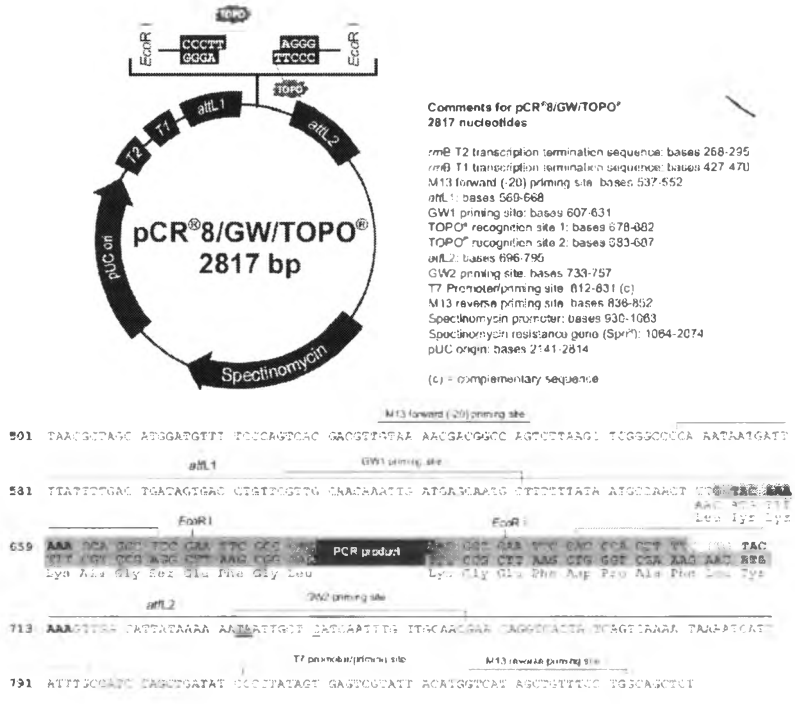

Figure 9: The map and sequence of pCR ®8/GW/TOPO® vector (Invitrogen, USA).

Table 15: The reaction setup of colony PCR screening

Reagents	1X (µl)
10X *Taq* buffer with $(NH_4)_2SO_4$	2.5
25 mM $MgCl_2$	2.0
10 mM dNTP Mix	0.5
10 µM DENUL	1.0
10 µM DENUR	1.0
DNase/Rnase-Free Distilled Water	17.87
5U/µl *Taq* DNA polymerase	0.13
Total	**20.0**

The PCR thermal cycle (Eppendorf, Germany) was set as follows:

Initial activation step:	95	°C	3	minutes
Denaturation step:	95	°C	30	seconds
Annealing step:	58	°C	30	seconds
Extension step:	72	°C	1	minute
Final extension step:	72	°C	5	minutes

30 cycles

Each PCR product was analyzed by 1.5% agarose gel electrophoresis in 1X TBE and stained with 0.5 μg/ml ethidium bromide. Expected bands were 434 bps for all serotypes.

After picking interested colonies into PCR reaction mixture, each toothpick was placed into the 5 ml tube contained LB broth with 100 μg/ml spectinomycin and incubated by shaking at 200 rpm, 37 °C for 16-18 hours. Plasmid DNA extraction was done using the NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel, Germany). Purified DNA plasmid was determined concentration and purity by NanoDrop spectrophotometer and sent for sequencing using universal primers (M13 forward and T7 promoter) (1st BASE, Malaysia).

Strand specific real time RT-PCR (tagged qRT-PCR) for detection the replicative form of DENV

This protocol was used to characterize whether detected DENV in different specimens and time points was alive. Only positive specimen with real time RT-PCR was enrolled in this experiment. Primers in this protocol were taken from studies of dos Santos *et al.* and Peyrefitte *et al.* [61, 82]. The 5' modified forward primer (tag5'UTR-F) was used for cDNA synthesis and, then cDNA was amplified using tag and 5'UTR-R primers (Figure 10).

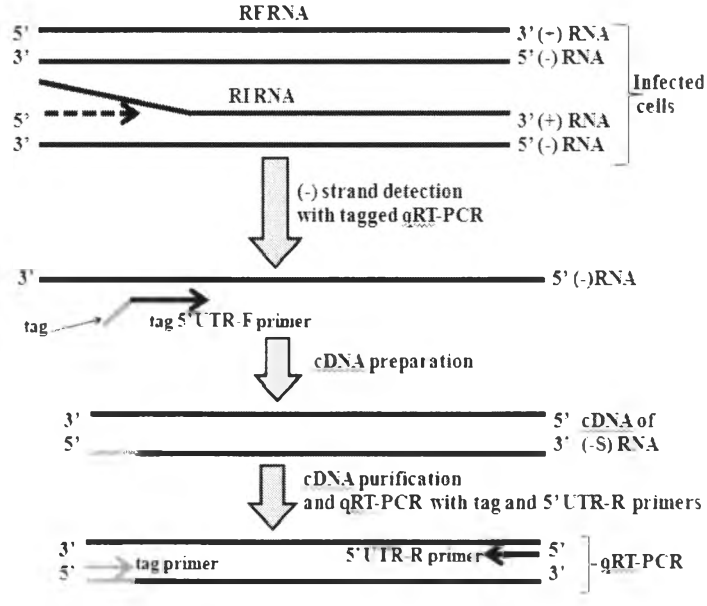

Figure 10: Schematic pattern of tagged real time RT-PCT (Adapted from Peyrefitte *et al.* [82]).

The primers in this study are presented in Table 16:

Table 16: Primers for tagged qRT-PCR

Tag 5'UTR-F	5' CGG TCA TGG TGG CGA ATA AAG TTG TTA GTC TAC GTG GAC CGA 3'
Tag	5' CGG TCA TGG TGG CGA ATA A 3'
5'UTR-R	5' CGC GTT TCA GCA TAT TGA AAG 3'

cDNA of each specimen was generated using tag 5' UTR-F primer with the SuperScript® III First-Strand Synthesis System Kit (Invitrogen, USA). The reaction setup was performed as described in Table 17.

Table 17: The reaction setup of cDNA synthesis for tagged qRT-PCR

Reagents	**1X (µl)**
RNA template	5.0
10 µM Tag F	1.0
10 mM dNTP Mix	1.0
DNase/RNase-Free Distilled Water	1.0

Each reaction tube was incubated at 65 °C for 5 minutes and placed on ice for 2 minutes. After that, cDNA synthesis mastermix was prepared according to the reaction setup in Table18.

Table 18: The reaction setup of cDNA synthesis for tagged qRT-PCR (mastermix preparation)

Reagents	1X (µl)
10X RT buffer	2.0
25 mM $MgCl_2$	4.0
0.1M DTT	2.0
RNaseOUT™ (40U/µl)	1.0
SuperScript™III RT (200U/µl)	1.0

The volume of 10 µl of cDNA synthesis mastermix was added to RNA/primer mixture. Each reaction tube was mixed gently and incubated in the PCR thermal cycle (Eppendorf, Germany) by setting the condition as follows:

50	°C	50	minutes
85	°C	5	minutes

After finishing incubation step, 1 µl of RNase H was added into each reaction tube and incubated at 37 °C for 20 minutes.

The cDNA mixture was purified using the Invisorb® Fragment Clean up Kit (Stratec, Germany) to reduce trace RT reaction by following an instruction handbook. Each cDNA reaction (20 µl) was mixed with the volume of 100 µl of binding buffer. After that, solution mixture was applied to a spin column and centrifuged at 12,000 rpm for 3 minutes at 25°C. After centrifugation, the collection tube was discharged and replaced with a new one. The volume of 20 µl of elution buffer was added to each column and leaved at 25°C for 5 minutes before centrifugation at 10,000 rpm for 1 minute to collect eluted cDNA for doing qPCR. Purified cDNA was taken to do qPCR for detecting the negative strand of DENV using the EXPRESS SYBR® GreenER™ qPCR Supermix Universal Kit (Invitrogen, USA). The PCR reaction was set as described in Table 19.

Table 19: The reaction setup of qRT-PCR for negative strand detection

Reagents	1X (µl)
EXPRESS SYBR® GreenER™ qPCR SuperMix Universal	10.0
10 µM Tag	0.4
10 µM 5'UTR-R	0.4
DNase/RNase-Free Distilled Water	7.2
Purified cDNA	2.0
Total	20

RT-PCR reactions were run on Rotor Gene 6000 Real time PCR machine (Corbett Life Science, QIAGEN, Germany) and set the condition as follows:

UDG incubation:	50	°C	2	minutes
Initial activation step:	95	°C	2	minutes
Denaturation step:	95	°C	15	seconds
Annealing step:	53	°C	40	seconds
Extension step:	72	°C	30	seconds

} 45 cycles

The melting curve analysis was run using the ramp from 60°C - 95 °C, rising by 0.2 °C for each step. PCR products were confirmed by 2% agarose gel electrophoresis in 1X TBE and stained with 0.5 µg/ml ethidium bromide. The expected band was approximately 168 bp.

To validate the protocol before using with clinical specimens, positive control (DENV1-DENV4) viruses were firstly done tagged qRT-PCR using the protocol described above. Each PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany) and ligated into pCR®8/GW/TOPO® vector and transformed the ligation product into StrataClone™SoloPack® Competent Cells (Agilent Technologies, USA) as described in the instruction manual.

Plasmid contained PCR product (pCR®8/GW/TOPO®-3'UTR) was purified using the NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel, Germany) and sent for sequencing (1st BASE, Malaysia) to confirm the insertion product. Plasmid concentration (ng/µl) was determined using NanoDrop spectrophotometer and calculated the copy numbers (copies/µl) by using this formula [133]:

$$\text{Copies}/\mu l = \frac{6.022 \times 10^{23}\left(\frac{\text{molecules}}{\text{mole}}\right) \times \text{DNA concentration } \left(\frac{g}{\mu l}\right)}{\text{Number of bases pairs} \times 660 \text{ Daltons}}$$

6.022×10^{23}: Avogadro's number

DNA concentration: plasmid concentration

Number of bases pairs: the sum of bases pairs (plasmid + insert sequence)

660 Daltons: the average weight of a single base pair

Plasmid of each serotype was done 10-fold dilutions from 10^6 copies/µl to 10^0 copies/µl and did qPCR to check the linearity, the limit of detection and the average of melting temperature for expected PCR products in all 4 serotypes.

Positive qRT-PCR specimens and positive control DENV1-DENV4 were taken to do tagged qRT-PCR using the protocol described above. The results of each specimen was reported as "detected and non-detected" after confirming the expected result with melting curve analysis and 2% gel electrophoresis.

PCR purification using ExoSAP-IT® reagent

The PCR product showing clearly single band on agarose gel electrophoresis was purified before either sent for sequencing or cloning using the ExoSAP-IT® Kit (USB, Affymetrix, USA). Each volume of 10 µl of PCR product was mixed with 4 µl of ExoSAP-IT reagent and incubated the reaction in the PCR thermal cycle (Eppendorf, Germany) by setting the condition as follows:

LID	105 °C
37 °C	15 minutes
80 °C	15 minutes
Hold	20 °C

The purified PCR product was kept at -20 °C until use.

Gel extraction protocol

An expected PCR product was eluted from excised gel by using the QIAquick Gel Extraction Kit (QIAGEN, Germany) by following the commercial instruction. Briefly, the expected band on agarose gel was cut and weighed in a sterile 1.5 ml tube. The 3 volumes of buffer QG were added into 1 volume of gel (1mg of gel = 1 µl of solution). Mixture was incubated at 50°C for 10 minutes or until gel was completely dissolved. The 1 volume of isopropanol was added into gel solution mixture and mixed gently. After that, it was applied into a spin column and centrifuged at 13,000 rpm for 1 minute at 25 °C. Remaining solution in collection tube was discharged. The volume of 500 µl of buffer QG was applied to each column before it was centrifuged at 13,000 rpm for 1 minute. Next, 750 µl of buffer PE was applied and the reaction column was leaved at 25 °C for 2-5 minutes. Then, each column was centrifuged at 13,000 rpm for 1 minute and repeated it one time to eliminate trace reagent in the column. Each column was placed in a new sterile 1.5 ml collection tube and PCR product was eluded from the column by adding 25 µl of elution buffer (EB) following by centrifuging at 13,000 rpm for 1 minute after the column was leaved at 25 °C for 2 minutes. Concentration and purity of purified PCR were determined using NanoDrop spectrophotometer. Eluded PCR product was kept at -20 °C until use.

Plasmid extraction and purification

The NucleoSpin Plasmid QuickPure (Macherey-Nagel, Germany) Kit was used to extract plasmid DNA from optimal growth of *E. coli* competent cells (Solopack)(Agilent Technologies, USA) in 5 ml of LB broth containing 100 µg/ml of spectinomycin for 16-18 hours at 37°C by following the commercial instruction. Briefly, LB media was discharged and cell pellet was collected by centrifuging at 11,000g for 30 seconds. The volume of 250 µl of buffer A1 (containing RNase A) was applied to suspend cell pellet by using vortex mixture until pellet was totally dissolved. After that, the volume of 250 µl of buffer A2 was applied in cell suspension following by mixing it gently (not using vortex mixture). Each reaction was incubated at 25 °C for 5 minutes or until solution seemed clear. Then, the

volume of 300 µl of buffer A3 was continuously added to each solution tube following by mixing it gently (not using vortex mixture) and centrifuged at 11,000g for 5 minutes at 25 °C. Supernatant was applied into Nucleospin plasmid column following by centrifuging at 11,000g for 1 minute. Trace solution in a collection tube was discharged. The volume of 600 µl of buffer A4 (containing ethanol) was applied to each spin column and centrifuged at 11,000g for 1 minute. After centrifugation, each collection tube was discharged and replace with a new one before centrifuging again at 11,000g for 2 minutes to dry a spin column. Plasmid DNA was eluted from each column by adding 30 µl of elution buffer (AE) and centrifuging at 11,000g for 1 minute after leaving the reaction at 25°C for 1 minute. Plasmid DNA concentration and purity were determined using NanoDrop spectrophotometer. The good purity of plasmid was 1.8 (A260/A280).

Sequence analysis and the study of serotypes, genotypes and strains of DENV

Sequencing results of E gene were confirmed read sequences by checking each chromatogram with Chromas Lite (Version 2.01) and editing some errors by hand. The serotype analysis of DENV was depended on results in both RT-PCR and sequencing. Nucleotide sequences of E gene were blasted with GenBank to demonstrate serotypes and strains of DENV in each specimen and time point. If the mixed peaks of sequencing result were found, the PCR cloning and sequencing of E gene sequence were done to select an individual clone at least 3 clones for studying serotype, genotype and strain of DENV [118].

For DENV strain analysis, the blast result showing high similarity at least 98% with sequences in GenBank and the name of similar sequence noted as "strain" of DENV was characterized as similar strain to our input query.

Genotype of DENV was explored by blasting the E gene sequence with Dengue Virus Database (DengueDB) as presented in Figure 11[134, 135].

Figure 11: The webpage of Viral Bioinformatics Research Center (VBRC) for determining the genotype of DENV
(Available from: http://www.denguedb.org/submitGenotypeRequest.aspx?type=Dengue)

The serotypes, genotypes and strains of DENV in different time points were investigated only in the patient positive for nested RT-PCR (E gene) at least 2 time points.

The analysis of genetic variation of DENV in different specimens and time points

This study was done in the patient positive for nested RT-PCR (E gene) at least 2 time points. Multiple nucleotide and deduced amino acid sequence alignments of direct sequencing results (except primers, 388 bp and 129 aa, respectively) and from 10-15 sequences of each specimen using direct sequencing as a reference sequence (to study the genetic diversity in each specimen) were done using ClustalW algorithm in BioEdit (version 7.0) [136]. The position of both nucleotide and amino acid sequences in this study (388 bp and 129 aa, respectively) were based on blasting results with complete E gene sequences in GenBank. The references of nucleotide position for all 4 serotypes were referred to sequences in GenBank with accession no. AY732472.1 (DENV1), DQ181872.1 (DENV2), JF968066.1 (DENV3) and AY786197.1 (DENV4) while the references of amino acid positions were referred to

sequences in GenBank with accession no. ABB70708.1 (DENV1), AFN87732.1 (DENV2), AF171764.1 (DENV3) and AAU89351.1 (DENV4).

The extent of sequence variation or quasispecies was investigated by analyzing a number of identical and non-identical sequences among clones derived from each specimen and time point using the direct sequencing result as a reference sequence and calculating Normalized Shannon entropy parameter (S_n). Moreover, the presence of defective genome in each specimen was also investigated.

Normalized Shannon entropy (S_n) is the marker to measure the size (complexity) of quasispecies at the given time point [105, 108, 111]. The result varies from 0 (no diversity) to 1 (maximum diversity) and is calculated by using the formula described as follows [137].

$$S_n = \frac{S}{\ln N}$$

$$S = -\sum_{i=1}^{n} f_i \, (lnf_i)$$

f_i = the frequency of each sequence in the viral quasispecies (No. of the same sequence type divided by total sequence analyzed)

N = the number of total sequences analyzed

S = Shannon entropy; S_n = Normalized Shannon entropy

To estimate whether amino acid mutations at domain III of E gene sequences of all clones in each specimen and time point were under the selection pressure, the ratio of dN/dS was calculated using SLAC (single likelihood ancestor counting) method in the web-based Datamonkey (www.datamonkey.org) [138, 139]. The ratio of dN/dS more than 1 is considered under positive selection, less than 1 is considered under purifying and equal 1 is considered under neutral selection [139, 140].

Nucleotide sequences derived from all clones in each specimen of individual patient were constructed a phylogenetic tree to demonstrate genetic variations of DENV among specimens and time points. The phylogenetic tree was drawn using MEGA program (version 5.0) based on neighbor-joining (NJ) method with Kimura 2-parameter model (1,000 replicates) [141, 142].

Data analysis

Descriptive study was used to present all results in this study. The percentage of positive results, tables, charts, graphs and figures as well as text describing were mainly used. The genetic variation results were analyzed separately in each serotype and patient. No other statistics was used because of the inadequate sample size.