CHAPTER V
DISCUSSION

There are many studies mentioned about persistent infection and genetic variation in different specimens and time points of flaviviruses such as WNV, HCV and SLEV [5, 8-11]. Due to DENV, there are few studies to determine the persistent infection, the genetic characterization and the viral load in different specimens and time points. To elucidate whether DENV persisted for a long period, how the genetic characterization was and how the viral load in various compartments and time points in DENV-infected patients was, 23 dengue and 5 non-dengue-infected adult patients were enrolled in this study. Both groups of patients were selected from our previous project "Survival of dengue virus in blood, urine, saliva and buccal mucosa in complete- recovery dengue patients" [30, 97]. This study mainly focuses on adult patients because we have proper specimens in our laboratory. Importantly, the prevalence of DENV in adults becomes increasing in Thailand and the pathogenesis of DENV in adults is still unclear due to the lacking of enough dengue studies in adult patients. In this study, all DENV-infected patients were diagnosed as secondary infection. However, one patient a foreigner from India was diagnosed as primary infection. These findings suggest that most symptomatic adult patients are secondary infection consistence with previous studies mentioning about secondary dengue infection in symptomatic patients [145, 146]. Moreover, prolonged IgG antibody was found in our secondary dengue-infected patients until day 90 of illness (See Appendix C). Our finding is similar to the study of Imrie *et al.* who studied the presence of IgG antibody in patients experiencing with DENV infection during World War II and found that dengue specific IgG and neutralizing antibody to DENV1 were prolonged up to 60 years[147]. In addition, Okuno *el al.* and Halstead *el al.* also showed the persistent of neutralizing antibodies to DENV until day 48 of illness in DENV-infected patients [148, 149]. The persistence of antibodies after viral infection may be from the effective function of memory B cell secreting antibodies to neutralize viral

particles after infection. Moreover, persistent viral particles or antigens may elicit prolonged antibody production.

Plasma, PBMCs, saliva and urine collected during febrile (fever period), early convalescent (first day of fever recovery until day 25 of illness) and late convalescent (day 26 to day 90 of illness) periods from both dengue and non-dengue infected patients were taken to monitor the persistent DENV in each specimen and time point (using nested RT-PCR and qRT-PCR), to explore the live DENV by using negative strand detection (tagged qRT-PCR) and to study the genetic characterizations (serotypes, genotypes, strains and genetic variations) in different specimens and time points. The duration of specimen collections was defined basing on our arbitrary criteria, clinical symptoms of infected patients and the suitable time for specimen collections in our previous project. Due to the lacking of scientific model about persistent DENV infection, the duration time of 90 days was specified as the pilot time to monitor DENV infection in this study. There were 20 patients infected with single dengue serotype in each specimen and time point. More patients were infected with DENV2 than with DENV1 and DENV3. There was no patient infected with single serotype of DENV4. From this finding, the high prevalence of DENV2 was found in our patients. However, we cannot conclude that the epidemiology of DENV2 infection is higher than other serotypes in that year due to the small sample size in this study and the incidence of DENV infection is not consistent in different areas. Generally, multi or mixed serotype infections are found in a hyperendemic area of DENV and there are many studies purposing about this evidence in both patients and mosquitoes in many countries such as in Sri Lanka, Brazil, Indonesia and Thailand [45, 46, 89, 135, 150-157]. Our results showed that multi serotype infections were found in 3 patients (DENV2+DENV4, DENV1+DENV3 and DENV1+DENV2), limited in only one among all specimens in each patient. These findings relate to previous studies showing mixed DENV infections in plasma, PBMCs, platelets, saliva, buccal mucosa and urine of infected patients [46, 89]. Multi serotype infections in each patient may be from previous DENV infection, coinfection or superinfection of different dengue serotypes. The presence of different numbers of clones representing each serotype in mixed-DENV-infected specimens can indicate which major or minor serotype is and suggests that the competition among dengue

serotypes may occur. This finding is supported by the study of Pepin, Lambeth and Hanley [158]. They studied the competitive suppression between DENV2 and DENV4 in C6/36 model and concluded that the suppression of viral replication was found in DENV2 than in DENV4 when the condition was set up as either coinfection or superinfection. In addition, the dominant serotype in each specimen is confirmed by direct sequencing and blasting with Genbank. Moreover, in mixed-serotype-infected specimens, there was no evidence of genetic recombination between 2 serotypes along the length of PCR products in this study after analyzing by SimPlot program (version 3.5.1) (data not shown) [159]. However, the genetic recombination of 2 serotypes may occur in other regions of DENV genome, which is not explored in this study.

To ensure that mixed-serotype infections are not from cross contaminations in our laboratory during an each RT-PCR step, all RT-PCR processes were done in the separated area and suspected results were repeated by using new RNA extraction. Moreover, PCR products were sent for sequencing and aligned with stock DENV controls in our laboratory. Even though some sequences were similar to DENV stocks, they were not from the contamination because of the high genetic variation between DENV control and specimen sequences after doing pairwise alignments.

Genotype and strain analysis of DENV in infected patients are based on the nucleotide alignment of E gene with DengueDB and GenBank databases. There are many studies indicating that E gene is widely used and more suitable for genotype and strain classifications [34, 143, 160-165]. Other regions such as C/PrM and E/NS1 junction can be used as well [141, 166, 167]. The DENV genotype results indicate that detected DENV in our patients circulates in Asian countries including Thailand because the blast results were clustered in genotype I (DENV1), genotype Asian I (DENV2), genotype Cosmopolitan (DENV2), genotype II (DENV3) and genotype I (DENV4) [34]. Interestingly, co-circulation of 2 dengue genotypes in our DENV2 infected patients implies that epidemic of mixed genotype infections was found in that year. Similar to dengue genotype results, strains of DENV circulating in our infected patients are found in Asian countries. There were 6 strains of DENV1 and DENV2, 3 strains of DENV3 and 1 strand of DENV4. All strains in each serotype are previously reported in Thailand, Myanmar, Vietnam, Singapore, Cambodia and Taiwan in the

last decades [160, 161, 164, 165]. This finding indicates that all strains of DENV having circulated in Thailand compose of multi strains originating from Thailand and other countries. The introducing of DENV strains from other countries to Thailand may be from the migration of people, the increasing of mosquito vectors and the climate changes.

Serotypes, genotypes and strains of DENV in different specimens and time points were analyzed in 13 patients positive for nested RT-PCR (E gene) at least 2 time points. The serotypes, genotypes and strains in different specimens and time points in 8 of 10 single-serotype-infected patients were the same. This finding suggests that there is no evidence of mixed genotype and strain infections in those patients. Interestingly, two patients with DENV2 infection illustrated the different strains in various time points. The similar strains were found in all febrile specimens but were different from convalescent PBMCs of both patients. All different DENV2 strains of both patients were clustered in the same genotype. Our findings are related to previous studies describing mixed genotypes and strains of DENV1 and DENV2 in patients [32, 143]. To our knowledge, this is the first study to describe mixed strains of DENV in different time points. The occurrence of mixed strain infections may be from either coinfection or superinfection of multiple DENV strains [32]. The persistence of previous strains and following by a new strain of DENV can possibly occur. The mixed strains found in convalescent PBMCs imply that PBMCs may be the suitable cell type for DENV infection, replication and persistence. The competition among DENV strains may be influenced on the appearance of each strain in different specimens and time points. However, there is not enough data to substantiate this point. In mixed or multi serotype infected patients, the clear results of different serotypes, genotypes and strains were shown. Moreover, the presence of mixed strains of DENV in multi-serotype-infected specimens of 2 patients suggesting that the occurrence of multi serotypes and strains of DENV may be possible in the endemic area of DENV infection.

The presence of DENV in plasma, PBMCs, saliva and urine in the same and different periods of infection suggests that both blood and non-blood compartments are the target sites for DENV infection, replication and persistence. Our findings are similar to previous studies in other flaviviruses such as in WNV and SLEV as well as

in other hemorrhagic fever viruses such as CCHFV and Ebola virus [7, 8, 94, 108, 118]. Moreover, these findings are consistent with previous studies in our laboratory demonstrating DENV detection in plasma/serum, PBMCs, saliva, buccal mucosa and urine during febrile and post febrile periods, and the study of Hirayama *et al.* presented the prolong DENV detection in urine up to day 16 of illness [12, 19, 28, 70, 71]. Although, our recent work had mentioned the persistent DENV in PBMCs and urine, the use of 2 alternative protocols in this study was done to confirm the feasibility of DENV detection in various specimens and time points [30, 97]. In this study, qRT-PCR with SYBR Green I detection is the main protocol for monitoring the persistent infection due to the higher sensitivity than nested RT-PCR and other real time RT-PCR techniques with different detection systems [168, 169]. However, the latest day of DENV detection in PBMCs was found when using nested RT-PCR. To ensure that positive RT-PCR results did not from contaminations in pre, during and post PCR assays, all experiments were set in the separate area and following the guidelines "How to reducing false positive results" by Kwok and Higuchi [170]. The latest times of DENV detection varying each specimen and time point (day 8 of illness in saliva, day 22 of illness in plasma, day 27 of illness in PBMCs and day 46 of illness in urine) indicates that viral load, viral dynamics and efficiency of viral clearance depend on each cell types. Moreover, these findings also imply that kidneys may be the main target site than PBMCs and salivary gland for DENV persistence. However, we cannot rule out that salivary gland is not the target site for DENV persistence. The short duration time of DENV detection in saliva may be from the low viral load resulting from the less blood containing viral particles circulates via salivary gland. Generally, salivary gland is an exocrine gland which does not secrete an amount of body fluid via large blood stream like other endocrine glands. Therefore, viral particles in salivary gland may be from blood circulation via capillaries and a number of viral particles are depended on the high dengue viral load in plasma enough to penetrate from capillaries into salivary gland. The virus in saliva may be from the blood contamination in saliva as well. In addition, the incomplete DENV genome due to the nature of specimen and viral RNA extraction may affect the efficiency of DENV detection. The presence of DENV in saliva indicates that salivary gland may be the target sites for DENV as same as in kidneys. However, the

pathogenesis in saliva is still inconclusive. Secreted viruses in urine may be from the blood circulation or the reactivation of virus in kidneys. Luby *et al.* supposed that SLEV in urine was from the viral movement across capillaries at glomerular or renal tubular during a viremic phase of infection. The presence of SLEV antigen in uroepithelial cells suggested the viral multiplication in a urinary tract of each patient [189]. Moreover, DENV was carried into glomeruli by monocyte-like cells in the form of immune complex [171]. In addition, Chuansumrit *et al.* presented that the presence of NS1 protein in urine might be from plasma leakage or higher production of viral antigen in kidney cells infected with DENV [190]. The presence of DENV in salivary gland and kidneys may also be from the escape population from host immune response. To date, the mechanism for describing persistent DENV in both blood and non-blood compartments is not well defined. Nevertheless, there are a few studies describe that the persistent DENV may be the slow rate clearance of viral load resulting from the virus-immune complex formation and the mediating of suppressive cytokine (interleukin 10, IL-10) during each infection [95, 96, 171].

The comparison of viral loads in different body compartments and time points are reported in many viruses such as in HCV, CCHFV, RSV, HSV and influenza virus [66, 77-79, 131, 173]. In DENV, there are a few studies focusing on the different viral loads in the same and different specimens during febrile period [13, 19, 174]. To our knowledge, this is the first study to describe dengue viral load in different specimens and time points of each patient. The spectra of dengue viral load of our DENV-infected patients depend upon each specimen and time point. The viral load in mixed-serotype-infected specimens was reported as a total viral load because universal primers were used in this project. The unit "PFU/ml" was used to express the viral load in all specimens in both blood and non-blood compartments. The use of PFU/ml to describe viral load in plasma and other tissues was reported in the study of Goncalves and his research team [185]. Up to date, there is no standard unit for determining viral load in clinical specimens, especially in cell, cell-free compartments or tissues in each patient. Our results revealed that the viral load in plasma or PBMCs was higher than in urine and/or saliva if specimens had been collected during febrile period. In contrast, the viral load in urine was higher than in other specimens when specimens were collected during early convalescent period. These findings suggest

that the day of specimen collections and the different body compartments may involve in the different levels of viral load. The high viral load in plasma or PBMCs during febrile period is consistent with the previous study mentioning that the viral load of DENV in blood specimen was higher than in non-blood specimens (saliva and urine) at day 2 after onset of illness and undetectable in all specimens at next 7 days [19]. Other study also supports our finding about the viral load in different time points. Wang *el al.* presented that the viral load in plasma was higher at the early day of illness and it decreased at the later time of specimen collections. The presence of high viral load during early convalescent period may indicate the active viral replication, the escape viral population from host immune response or the slow rate of viral clearance. Moreover, the presence of high viral load in urine during convalescent period strongly implies that kidneys may be the non-hematopoietic site for DENV replication and persistence. The viral load in different time points cannot be studied in one patient (N9) because the cycle threshold (Ct) of all specimens was out of the range of standard curve. In specimens presenting low viral loads, the results were confirmed by melting curve analysis with positive control and gel electrophoresis. Moreover, all RT-PCR processes were under the guidelines "How to reducing false positive results" by Kwok and Higuchi to ensure our results did not from contamination [170]. Due to the difference of DF, DHF and DSS patients in this study, we cannot compare the association between dengue viral load and different clinical appearances in patients. However, the study of Wang and his colleague pointed out that the effectiveness of viral clearance was found in DF patients than in DHF [13]. Moreover, there are many reports demonstrating the correlation between dengue viral load and clinical symptoms in patients. They supposed that the different viral loads did not associate with disease severity and either primary of secondary infection while other study argued that the viral load in plasma and PBMCs was related to disease severity [172, 175]. However, additional studies need to be performed to clarify these controversial points.

The presence of replicative, intermediated form (RF or RI) or negative strand of positive single strand RNA viruses suggests the evidence of active viral replication. In our previous work, the active viral replication in all positive specimens was explored by using the mosquito inoculation technique. The results were mostly

negative although some specimens were mostly collected during febrile period (data not shown). This may be from the presence of high neutralizing antibodies during febrile period because most patients were diagnosed as the secondary dengue infection. Therefore, viral particles were rapidly neutralized and decreased until undetectable by using this technique. Moreover, the use of long-term storage specimens may also affect the efficiency of viral isolation. To solve this problem, the evidence of live DENV was determined by negative strand detection using tagged qRT-PCR. This technique is widely used to explore the evidence of HCV replication in both hepatic and non hepatic sites [83, 86-88, 176, 177]. More researchers claim that this method increases sensitivity although the low level or the absence of viral replication is found. Moreover, this technique is suitable for finding out the evidence of viral replication in viral infected tissue [82, 83, 177]. In this study, we used tagged qRT-PCR with the same primers done in qRT-PCR for detecting the negative strand of DENV to avoid the bias of specificity and sensitivity due to the different target regions of primers. The presence of negative strand varied in different specimens and time points. During febrile period, the negative strand was mostly found in plasma and PBMCs than in urine. However, in early and convalescent periods, the negative strand was found in urine but not in plasma, PBMCs and saliva. These findings suggest that DENV can replicate in both hematopoitic and non-hematopoitic sites such as in kidneys and notes that the evidence of DENV during persistent infection is found in kidneys as well. In my view point, this is the first study to describe both the persistence of DENV infection and the evidence of DENV replication in non-hematopoietic tissues. The occurrence of negative strand in blood during febrile period suggests that DENV replication mostly occurs in blood compartments before dengue virions spread into secondary organs or tissues. Our findings are similar to previous studies about the presence of negative strand of DENV1-DENV4 in blood compartments (plasma and platelets) during febrile period but they did not mention in non-hematopoitic compartments such as in liver tissue and kidneys, and in different time points [89]. However, the presence of DENV2 negative strand in liver cells was described in animal model (BALB/c mice) suggesting non-hematopoitic compartments not only kidneys but also liver is an alternative site for DENV2 replication [90]. However, there is no evidence of the negative strand detection in

liver tissues of DENV-infected patients. According to tagged qRT-PCR, the non-priming of modified sequence at 5' site of forward primer with DENV genome was confirmed by blast with GenBank and cDNA purification process before doing qRT-PCR. Moreover, the melting curve analysis and gel electrophoresis were done to confirm suspected PCR results. It is importantly to note that the presence of negative strand does not reflect the current viral replication. It reveals the evidence of viral replication at the time point of specimen collection. In addition, the undetectable of negative strand does not mean there is no evidence of viral replication in suspected specimen. It may be from the low level of negative strands in that specimen or tissue. The different proportions between positive and negative strands is approximately 10-1000 times in flaviviruses and the levels of positive strands may influence on the sensitivity of negative strand detection although the high sensitivity technique is used [81, 82, 87, 176]. If a number of positive strands are very low, the negative strand may be undetectable in some cases. Other reason that describes the disappearance of negative strand is the time of specimen collection. The replicative form occurs when viruses amplify themselves or are under the active replication which is different in each virus. In DENV, previous studies described that the earliest time of replicative form detection was 20 minutes post infection in Vero cells [178]. However, we cannot estimate the earliest time of negative strand detection in our patients due to the lacking of data about the first time of DENV injection by mosquito bite. If the time of specimen collections is not consistent with the viral replication stage, a negative strand may not be detected. In addition, the presence of heterogeneous population or "quasispecies" may correlate with the presence of negative strand in different body compartments [86]. The correlation of negative presentation and clinical symptoms in DENV is still controversial because of the lacking of published studies. In HCV, there are some pieces of evidence to state the association between negative strand detection and clinical outcomes [88, 179]. In this study, we cannot determine a number of negative strands in each specimen because our tagged qRT-PCR assay did not show the clear melting curve analysis and there is no evidence to prove whether the correlation between negative strand viral load and disease severity in DENV-infected patients. In addition, the validation of this protocol is necessary because it may become an important tool for detecting the replication of DENV. Moreover, we

cannot confirm that the presence of negative strand in urine suggesting the active viral replication in kidneys. The presence of negative strand in kidneys may be from either the replicative form of DENV population from blood compartments or genuine viral replication in kidneys resulting from the viral selection mechanism. Finally, we still believe that DENV replication can occur in a salivary gland and both positive and negative strands are secreted in saliva according to the evidence of DENV detection in saliva [70].

The genetic variations in different specimens and time points are studies in many viruses such as in HIV, HCV and Ebola virus [103-106, 110, 113, 114, 116-118, 180]. In this study, we focused on 13 DENV-infected patients positive for DENV with E gene primers at least 2 time points. Primers used in this study mostly covered at the domain III of E gene in all 4 serotypes [56, 143]. This gene is chosen to study the genetic variation because it plays a major role in the pathogenesis of DENV infection and is the target for host immune response as well as under the selection of immune pressure [25, 102, 181]. According to the direct sequencing illustrating the major population of DENV, identical nucleotide and amino acid sequences among specimens and time points found in more patients (except mixed-DENV-infected patients) confirm that patients were infected with single serotype, genotype and strain of DENV. However, nucleotide and amino acid variations found in convalescent PBMCs of 2 patients (N2 and N10) and urine of one patient (N20) when comparing with all febrile specimens indicate that 3 patients may be infected with mixed strains of DENV or may be from the variants of single strain or population of DENV in different time points. After referring to blast results in our previous findings, nucleotide variations in different time points of PBMCs (N2 and N10) are most likely from mixed strain infections rather than genetic variation of major dengue strains or population. Nevertheless, the different nucleotide variations in convalescent urine (N20) may be likely from genetic variations of DENV in different time points. Most variations were found at the domain III of E supporting that the high genetic variations commonly occur at this domain and these variations can be widely used for DENV classification [143]. Moreover, these genetic variations may play a role in viral pathogenesis and under selection pressure. The same position of nucleotide variations (position 1113, C→T) found in convalescent PBMCs of 2 patients is not

significantly in DENV adaptation or persistent infection because this variation causes silent mutation (not affecting the phenotypic change of DENV). Amino acid sequence variations at domain III found in 2 patients (S376G in N2 and V358A in N20) state that nucleotide variations in this domain result in a change of antigenic site or a protein structure. Moreover, we can imply that these amino acid variations are under host selection pressure and the evidence of mixed DENV populations is found in both patients. The amino acid variations in both positions have not been previously reported and there is no studies claimed that these positions are the hot spot for DENV pathogenesis such as in the previous report of Sistayanarain *el al.*, and Sanchez and Ruiz purposing that amino acid variations in E protein (A173V and D390H) associated with neurological disorders in patients and mice, respectively [128, 129]. Some of our findings agree with previous studies of Hirayama *et al.* in that nucleotide variations between blood and non-blood specimens during the same and different periods were identical [12]. However, our findings add more interesting points by presenting the nucleotide variations in convalescent PBMCs and urine.

The genetic diversity of viral population in different specimens and time points is widely studies in HCV and HIV [103, 104, 108, 111, 116]. In previous studies about DENV, the genetic diversity of DENV population in clinical specimen is previously investigated but the results limited only in a single specimen and time point [25, 26, 125, 143]. In this study, 10-15 colonies derived from each specimen in all patients were used to investigate the extent of genetic diversity. A number of these clones are enough to study the genetic variation of DENV as described in previous studies [25, 26]. Nucleotide and amino acid alignments presented that each single serotype and mixed serotype infected specimens composed of major and minor populations confirming the presence of DENV quasispecies. The minor population or variant was different from major population by the presence of both nucleotide and amino acid variations, which randomly occurs. Nucleotide variations composed of single nucleotide not only mutation but also deletion (resulting in frame-shift mutation) and in-frame stop codon. The nucleotide deletion and in-frame stop codon indicate the presence of defective viral genome and have been reported to involve in viral replication, clinical appearances and persistent infection [25, 106]. In this study, the defective viral genome was found as totally 9.54% in all sequences derived from

each specimen during febrile and convalescent periods. Our findings are similar to the previous study that presents the defective genome of DENV and HCV in clinical specimens [25, 106]. Interestingly, our results demonstrated the high percentage of specimens containing single nucleotide deletion clones (19 in 49 specimens analyzed) resulting in frame-shift mutation confirmed by the bi-directional sequencing and the clear chromatogram after sequencing. Frame-shift mutations were previously described in DENV, HCV and HIV [25, 186,187]. The high percentage of frame-shift mutations in this study may be from our large and different specimen types than in previous study limiting only a small and single specimen type. Additionally, it may be from the viral adaptation in each body compartment. The roles of frame-shift mutations in DENV are rarely studied. In other viruses such as HBV, HIV and HCV, frame-shift mutations were reported involving in the viral adaptation in cell culture system, in the drug resistant mechanism and in the enhancing of viral replication in infected cells [186-188]. Because of the defective viral genome was found in early and late convalescent period, we can imply that defective viral genome may affect the persistent DENV infection by controlling the rate of viral replication and generating the incomplete or changing the antigenic sites to escape from host immune recognition.

According to the primers used in this study annealing E gene sequence, especially at the domain III which plays a major role in DENV pathogenesis, immune recognition and viral persistence, nucleotide and amino acid variations found in domain III of E gene of each specimen indicate that the presence of DENV quasispecies may be influenced by host immune pressure and may present various escape populations by changing the conformation of E protein [102]. This finding is supported by the previous study monitoring DENV3 quasispecies and suggesting domain III of E gene was under the positive selection [25, 125]. However, domain I and II of E proteins are also reported under the positive selection but the selection pressure is not stronger than in domain III [125].

There are many parameters widely used to illustrate the extent of quasispecies such as mutation frequency or mean diversity, pairwise genetic distances or Shannon entropy. In this study, we used Shannon entropy to present the complexity of quasispecies in each clinical specimens of individual patient because this study

mainly focused on a degree of heterogeneous population rather than the frequency of mutations in each sequence. Moreover, the use of this parameter is enough to imply the presence of viral quasispecies. Normalized Shannon entropy (S_n) is commonly used to determine the complexity of viral population such as in HCV and HIV [108, 109, 116, 182]. The value ranges from 0 (no diversity) to 1 (maximum diversity). The high genetic diversity or complexity (sometimes called heterogeneity) demonstrating by the high value of normalize Shannon entropy (S_n) was mostly found in plasma, PBMCs or urine during febrile period and found in PBMCs and urine during convalescent period. These findings imply that the high genetic diversity may depend on each specimen type or body compartment and time point. In addition, the high complexity of DENV population may correlate with the high viral load and the effectiveness of viral replication in each target site. The good example to support this assumption is in early convalescent urine of N12 presenting the high viral load, the occurrence of negative strand and the high genetic diversity when comparing with other specimens in that patient. The heterogeneity of DENV may be from the error of viral replication due to the lacking of proof-reading activity and from the coinfection or superinfection of multiple serotypes, genotypes and strains of DENV as well. The presence of high heterogeneity in PBMCs is similar to the previous study in HCV [116]. Moreover, the presence of mixed strains in PBMCs of 2 patients (N2 and N10) may strongly support that this evidence affects the complexity of DENV population. The complexity analysis of DENV population is not done in the minor populations of mixed-serotype-infected specimens (2^{nd} convalescent urine of N34 and early convalescent PBMCs of N40) because of the lacking of enough sequences to analyze.

To determine whether host immune selection affected the variations of amino acid sequences at E domain III protein, the dN/dS ratio in each specimen and time point was investigated. Sequences from minor serotype of mixed-infected specimens were not calculated because of the inadequate unique sequences to analyze by SLAC method in Datamonkey website. The results showed that amino acid variations of domain III among specimens in each patient were under either positive or purifying selection as well as neutral selection, which varied in each specimen and time point. Purifying selection mostly found in all specimens indicates that nucleotide variations at this domain result in synonymous than in non-synonymous mutations. The

appearance of purifying than positive selection is commonly found in vector-borne RNA virus than other RNA viruses [183, 184]. From our findings, we can infer about the role of genetic diversity of DENV in that DENV population may conserve the protein structure at domain III in order to survive from host immune response even though nucleotide variations were found. In addition, the presence of one purifying selection site at domain III of DENV population in febrile plasma of N17 implies that the amino acid in this position conserve in all populations in that specimen although the single nucleotide variation occurs and can assume that this codon may play a role in viral adaptation and survival. The positive selection of DENV found in plasma, PBMCs and urine during febrile period indicates that more amino acid variations are influenced by host selection pressure. Additionally, positive selection found in PBMCs and urine during convalescent period suggests that the selection pressure of host immune response may affect the persistent DENV infection. The viral adaptation in different specimens of individual patient depends upon each body compartment and time point. The body compartment directly facing with host immune response is mostly under positive selection than other compartments. For example in N12 patient, the positive selection was found in febrile plasma of N12 while purifying selections were found in febrile PBMCs, early and late convalescent PBMCs. These may suggest that DENV population may strongly face with host immune response in plasma than in other body compartments. As a result, viral particles adapt themselves to survive, and then promote the escape populations to other body compartments or secondary target sites. In secondary organs or target sites, the viral adaptation still occurs in the less effect of host immune response. Therefore, the positive selection seem disappeared but instead of either purifying or neutral selection. However, our results point out that positive selection at domain III does not always found in the directly immune-faced specimen. This may rely on the host immune status and the day of specimen collection in each patient. For instance in N2 patient, nucleotide variations of DENV in febrile plasma and PBMCs seem homogeneous and did not affect the amino acid change resulting in neutral selection whereas the strong positive selection was found in febrile urine. This phenomenon infers that the positive selection in plasma and PBMCs might have previously occurred before these specimens were collected. Moreover, the higher immune status in this patient

(secondary DENV infection) may completely select the survival DENV population and reduce the effectiveness of DENV replication. Therefore, the heterogeneity of DENV becomes stable and does not compose of various populations. The positive selection of DENV in febrile urine at the same time point of specimen collections in plasma and PBMCs may be from the immune pressure of neutralizing antibodies penetrating into kidneys or from the high heterogeneity of DENV population from plasma or PBMCs spreading to survive in kidneys.

To ensure that genetic variations in each specimen and time point are not influenced by RT-PCR and sequencing errors, both forward and reverse primers are used for sequencing and the chromatogram of each sequence was checked and edited by hand before analyzing the data. Moreover, sequences in this study were retrieved directly from direct specimens without *in vitro* amplification by viral isolation technique. The error frequencies of nested RT-PCR with *Taq* polymerase after 60 cycles were between 2.3×10^{-4} and 5.5×10^{-4}/nucleotide/cycle [25]. The % mean diversity among specimens in this study that used as a representative of mutation frequency in all specimens after amplified with nested RT-PCR were between 0.02% - 1.20% (data not shown) mostly larger than the error of nested RT-PCR (data not shown). These findings suggest that the small artifacts from RT and *Taq* polymerase were introduced in this study but they did not affect our findings about genetic variations. The association between the genetic diversity of DENV and disease severity cannot be discussed in this study because of the limitation of sample size. However, there is a previous study demonstrating the correlation between disease severity and genetic diversity of DENV1. They presented that the genetic diversity of DENV1 population was higher in specimens of non-severity-DENV-infected patients [126].

The phylogenetic tree of nucleotide sequences confirmed the presence of genetic diversity or quasispecies and the association of each DENV population in different specimens and time points. The different of DENV population may be from the mixed serotype, genotype and strain infections as well as resulting from variants of major DENV population. Because of each nucleotide sequence represents each DENV population. From this study, there are 3 patterns of DENV population circulating in our patients. First is the similar DENV population in different

specimens and time points. This phenomenon does not present the identical DENV population and may imply the role of viral adaptation to survive than the survival of DENV population for a long period. Second is the mixture of similar and identical DENV populations in the same and different specimens during the same and various time points. In this circumstance, identical sequences in the same specimen refer that each DENV population may be specific in a unique body compartment but not enough to describe the compartmentalization. Identical DENV sequences in different specimens at the same time points may describe the spread of DENV from the first target site to other sites while the identical sequences in different time points indicate the survival of DENV population. However, additional findings to describe this phenomenon are still unclear. Third are the composition of related sequences and the presence of unique DENV population in different specimens and time points. In this study, the presence of unique DENV population in early convalescent PBMCs of N2 may hypothesize the compartmentalization of DENV2 in N2 patient. However, according to the comparison of serotypes, genotypes and strains of DENV in N2 patient (Table 25 in Chapter IV), the presence of unique dengue population in early convalescent PBMCs may be likely from coinfection or superinfection of DENV strains rather than the specific variant populations resulting in compartmentalization of DENV. Our findings are similar to many previous studies in HCV and HIV, which presented the different behavior of each viral population in different body compartment but are different in the occurrence of viral compartmentalization commonly found in chronic viral infection than acute viral infection [103, 104, 110, 111, 114, 116]. Moreover, the presence of unique DENV population in convalescent period may be from the escape viral population from febrile period, and then the survival populations generates their variants during each replication and persist in permissive cells.

Additionally, we continued the further study to identify whether related or identical sequences in each specimen of individual patient were from genetic variations, mixed infections, or composing of both determinants by blasting all nucleotides sequences with GenBank. Our results indicate that the presence of variants in each specimen can be from either mutant spectra from the major DENV population or mixed strain infection as well as come from both causes. The variant

sequences originated from the master sequence in most specimens (strain blast results between direct sequence and variant were identical) indicate single-strain-infected specimens. However, variant sequences of some specimens such as in febrile PBMCs of N21, 29 and 40, early convalescent PBMCs of N2, early convalescent urine of N5, and N29, late convalescent PBMCs of N10, early and late convalescent urines of N12 as well as mixed-serotype-infected specimens (2^{nd} early convalescent urine of N34 and early convalescent PBMCs of N40) composed of sequences with the same and different blast results in each specimen indicating minor populations may be from both variants of major population and mixed-strain infections (data not shown). Moreover, the other pattern of DENV infection in our patients was mixed serotype infections presenting different serotypes in different specimens and time points. The limitation of this study is a small sample size. In a future study, a large sample size and the different age groups of patients as well as the consistent time points of specimen collections should be considered before conducting this project.