CHAPTER II

REVIEW OF RELATED LITERATURE

The virology of dengue virus (DENV)

DENV is one of the members in flaviviruses, family *Flaviviradae*, genus *Flavivirus*. There are many viruses in genus *Flavivirus* closely related to DENV and causing human diseases such as JEV, TBE, and WNV. According to the serological study and the molecular phylogenetic, flaviviruses are classified into serocomplex groups, clades and clusters (Figure 1) [27].

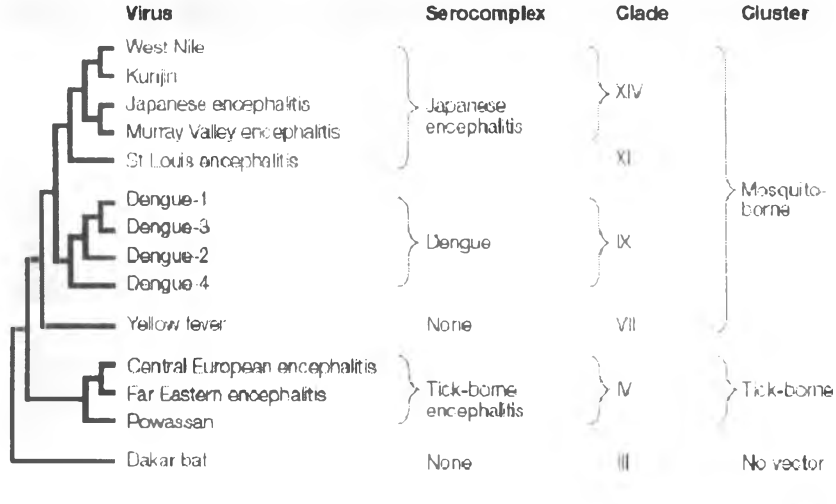

Figure 1: The diagram of flavivirus classification based on the different criteria to classify the specific group of viral members [27].

DENV is an arthropod-borne virus transmitted by *Aedes aegypti* and *Aedes albopictus*. It is divided into four serotypes (DENV1-DENV4). In the same serotype, it is classified into genotypes and strains, which relates to geographic distribution and genetic variation. Each serotype contains genotypes and each genotype consists of various strains. The serotype classification is based on the difference of antigenic sites or the 25-40% difference of amino acid sequences. Genotypes or subtypes are classified when the difference of nucleotide and amino acid sequences is

approximately 6-8% and 3%, respectively [31, 32]. The genotype classification using complete E gene sequence presents that each serotype (DENV1-DENV4) contains 5, 6, 4 and 4 genotypes, respectively (Table 1) [33, 34]. There is no criterion for strain classification but the nomenclatures of DENV are usually depended upon geographic distribution and the characterization of an isolated virus from clinical specimen. Moreover, the strain classification may be based on the different nucleotide sequences such as the difference of nucleotide sequences of E gene between 2 strains is approximately 2-3%. The genome organization of DENV is approximately 11 kilobases (kb) in length with one open reading frame (ORF) and composes of 3 structural and 7 non-structural genes (Figure 2). Structural genes are capsid (C), premembrane (prM) and envelope (E) genes, which is translated to polyproteins for constructing viral particles. Non-structural genes are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, which are translated to multi enzymes for viral assembly and mature virion construction [35].

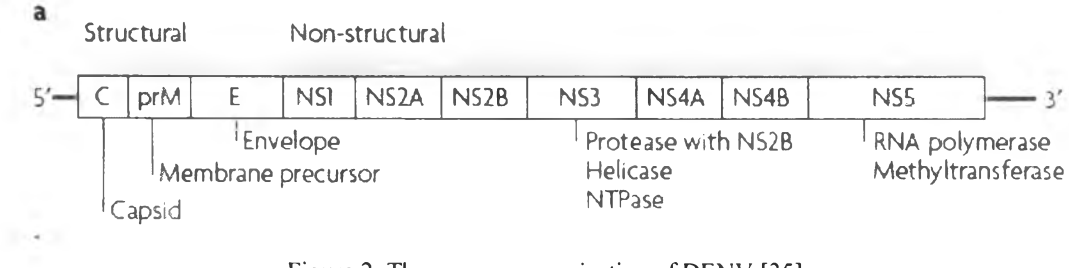

Figure 2: The genome organization of DENV [35].

Table 1: The summary of genotype classification of DENV based on complete E gene sequences (Adapted from Weaver *et al.* and Chen *et al.* [33, 34])

Serotype	Genotype	Geographic distribution
DENV1	I	Southeast Asia, China and East Africa
	II	Thailand collected in the 1950s and 1960s
	III	Sylvatic strains collected in Malaysia
	IV	West Pacific islands and Australia
	V	America, West Africa, Asia
DENV2	Asian I	Thailand, Malaysia, Cambodia, Myanmar, Vietnam and Australia

Serotype	Genotype	Geographic distribution
	Asian II	China, Indonesia, Philippines, Taiwan, Sri Lanka, India, Honduras and Mexico
DENV2	Southeast(SE) Asian/American	Southeast Asia, Central and South America, the Caribbean
	Cosmopolitan	East and West Africa, the Middle East, the Indian subcontinent, Indian and Pacific Ocean Islands, Australia and Mexico
	American	Central and South America, the Caribbean and older strains from Indian subcontinent and Pacific Islands
	Sylvatic	humans, canopy-dwelling arboreal mosquitoes and non-human primates
DENV3	I	Indonesia, Malaysia, Philippines and South Pacific Islands
	II	Thailand, Vietnam and Bangladesh
	III	Sri Lanka, India, Africa, and Samoa
	IV	Puerto Rico, Latin and central America
DENV4	I	Thailand, Philippines, Sri Lanka and Japan
	II	Indonesia, Malaysia, Tahiti, the Caribbean and America
	III	Thailand
	IV	Sylvatic strains from Malaysia

DENV transmission, replication and pathogenesis

The transmission of DENV changes from forest cycle or enzootic stage to rural cycle and urban cycle via the increasing of *Aedes spp.* and the different susceptible mosquito species in each cycle (Figure 3) [2]. The primitive host of DENV is primates in a forest. Nowadays, the transmission cycle of DENV is generally found in both rural and urban areas.

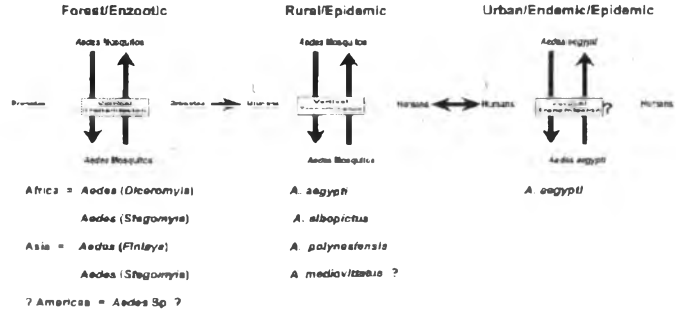

Figure 3: The diagram of DENV transmission [2].

After humans are bit with DENV-infected mosquitoes, DENV enters susceptible cells such as dendritic or Langerhans cells by using receptor-mediated endocytosis (RME) mechanism. There are many types of cell receptors involving in DENV entry such as heparan sulfate, heat shock protein 70 (Hsp70), Hsp90 and DC-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN). After that, the endosome formation of DENV particle continuously occurs. The acidic endosome affects a viral particle by changing the conformation of envelope (E) protein and nucleocapsid is uncoated to release the viral genome into cytoplasm of infected cells. DENV RNA acts as mRNA translating into polyproteins containing 3 structural and 7 nonstructural proteins, and then the viral replication occurs by synthesizing the negative strand for generating the new positive strand of DENV. The replicative form of DENV can be detected at least 3 hours post-infection [36]. Viral assembly using a new positive strand and translated proteins occurs in endoplasmic reticulum (ER) and mature or infectious virions are modified in Golgi complex before released from infected cells into blood stream using exocytosis mechanism. The summary of DENV life cycle is shown in Figure 4 [37].

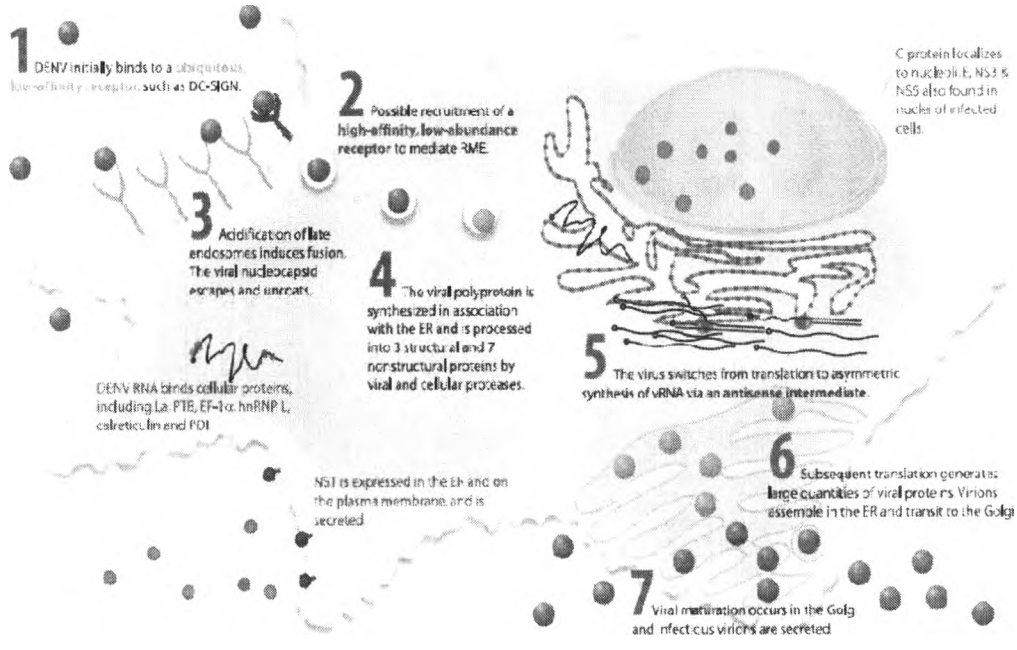

Figure 4: DENV life cycle [37].

During viremic stage, DENV spreads via blood circulation into many secondary target organs and tissues for a new replication and persistence. There are many susceptible organs and tissues for DENV such as macrophage cells (Kupffer cells), hepatocytes and sinusoidal endothelial cells in liver; macrophages, multinucleated cells, and reactive lymphoid cells in spleen; macrophages and vascular endothelium in lung; kidney cells and bone marrow [14, 15]. Infection with one serotype provides lifelong immunity to first serotype but not against other serotypes. However, the first immune response to previous serotype is cross-protective to other serotypes only first several months after infection [37]. In secondary infection with a heterologous serotype, patient is usually suffered from non-neutralizing antibodies to a latter serotype and may get more severity of disease. The pathogenesis of DENV infection belongs to the complexity of interaction between viral and host factors generating various clinical symptoms from asymptomatic to DSS. Viral factors are the difference of serotypes, genotypes and strains, the modification of viral proteins such as glycosylation in E and NS1 proteins. The host factors belong to the different genetic backgrounds and host immune response to DENV infection. Moreover, other factors such as age of patient and nutritional status are reported to influence on DENV

pathogenesis [2, 37-39]. There are 2 theories explaining pathogenesis of DENV infection, especially the development of DHF and DSS. The first theory is "the secondary infection or immune enhancement hypothesis". In brief, patients suffered from heterologous serotype infection during secondary infection have a significantly high risk for developing DHF and DSS via antibody-dependent ADE mechanism occurring when mononuclear phagocytes are infected with the immune complex of DENV and non-neutralizing antibodies (antibodies to previous infection) via Fc receptor or the maternal transmission of DENV antibodies to an infant [3]. The other factor depends on the genetic variation of DENV resulting from host selection pressure to generate some variants having a potential to disseminate, enhance the efficiency of replication and increase in viral virulence [2].

Most DENV infected patients are asymptomatic but some patients may be suffered from symptomatic infection. A latter group results from the infection with heterologous serotype. Based on WHO criteria (2009) referring to the criteria in 1997, clinical symptoms are grouped into 3 categories. There are undifferentiated fever, DF and DHF. Moreover, DHF is classified into four severity grades, (Grade I-IV, grades III and IV being noted as DSS). Although the criteria have been adopt by grading the severity of DHF, the conventional classification of symptomatic infection as DF, DHF and DSS without grading severity remains used [40, 41].

Epidemiology of DENV infection

The first report of DENV infection (dengue liked illness) was described in Batavia (Jakarta) and Cairo in 1779, then the outbreaks had been reported in many areas during that period (1780-1901) such as in Philadelphia, Zanzibar, the West Indies and Hong Kong [36]. At present, many countries in the world, especially in Asia, Africa and South America are endemic areas of DENV transmission and infection (Figure 5).

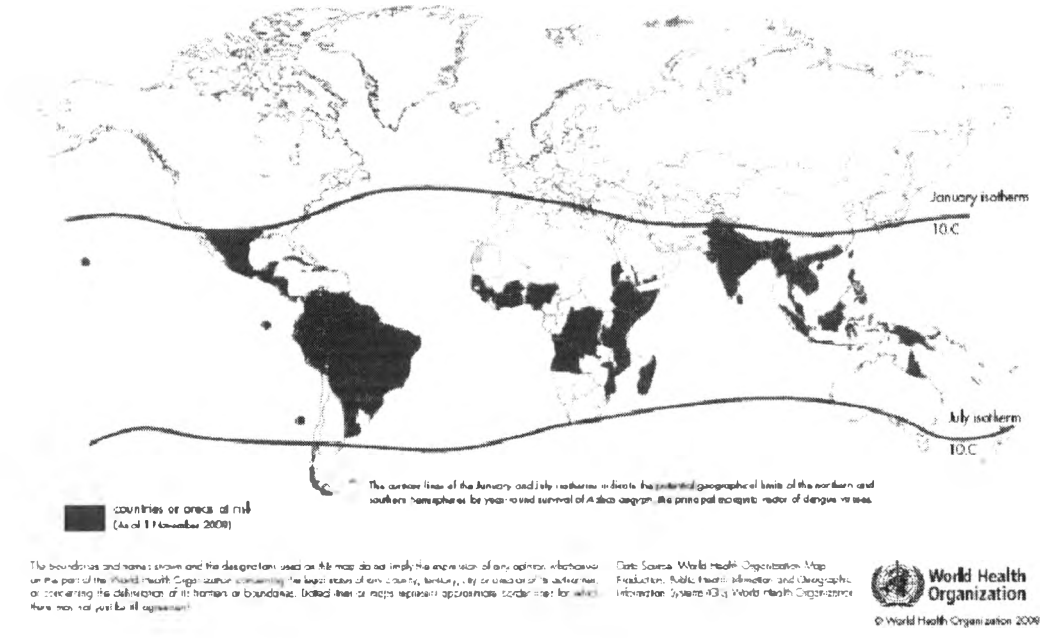

Figure 5: The endemic areas of DENV infection [42].

WHO reports that there are approximately 50 million in the world suffering from DENV infection each year. All four serotypes of DENV have been circulating in endemic areas but the high prevalence of each serotype varies in each year. In the past, the distribution of each DENV serotypes was limited in some areas [3]. For a few decades, the spread of DENV serotype is found many countries of the world (Figure 6). The trend of case reports has been continuously increased every year because of the effect of climate change, the migration of people and the increase of mosquito vector [42]. There are several factors correlating with the spread and transmission of DENV among different regions. First is the uncontrolled population growth. Second is the consequence of convenient transportation. Third is the lack of effective local organization in urban areas such as how to eliminate waste containers for mosquito propagation. Forth is the less effective mosquito control in an endemic area of DENV infection. Fifth is the reemerging of DENV in the same outbreak area that DENV had been reported in the past [2].

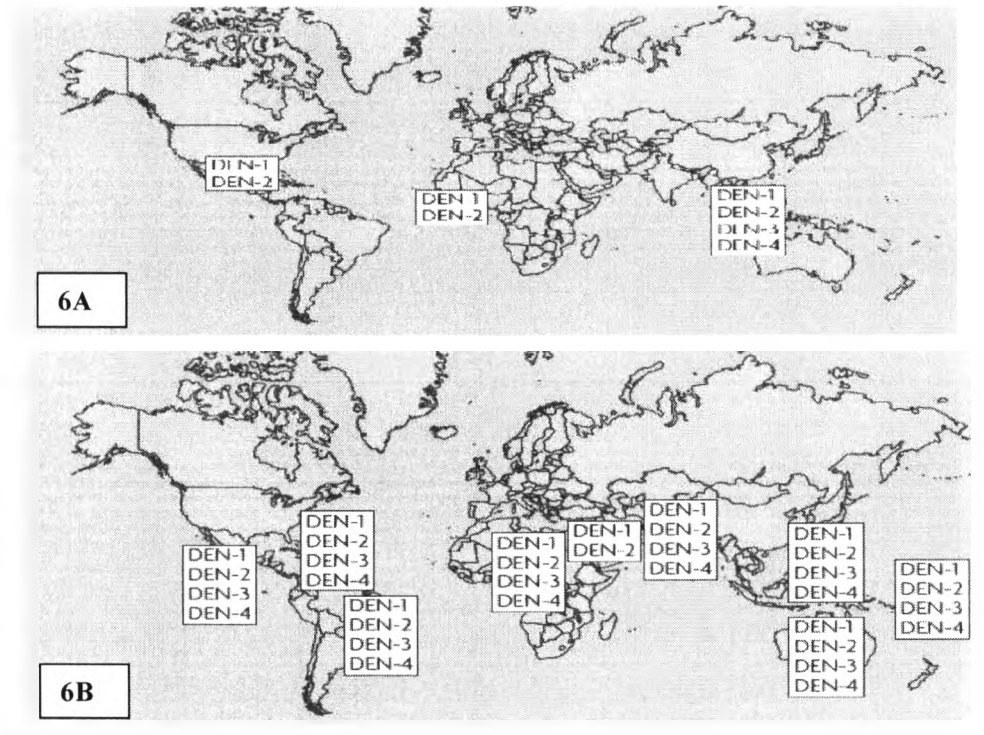

Figure 6: The distribution of DENV serotype in different periods. Figure 6A presents the results in 1970 showing a few countries with DENV serotype infection. Figure 6B presents the DENV serotype distribution in 2004 illustrating the more endemic area of DENV infection. [3].

In Thailand, the first report of DENV infection was described in 1958. The prevalence of infection has been slightly increased every year and is high during rainy season. Infected patients are regularly in the age between 10-14 years old [43]. Nowadays, all ranges of age are the risk of DENV infection. The spread of each serotype is not consistence in each year. All four serotypes can circulate in all areas, but difference in the predominant serotype. The distribution of predominant serotype in each year depends on the climate change, global warming and the density of mosquitoes.

The annual reports from Center of Vector Borne and Infectious Diseases about the prevalence of DENV serotypes during 2008-2011 present that the predominant serotype occurs every two years. Most patients were infected with DENV1, DENV2 and DENV3 than with DENV4 (Figure 7) [44].

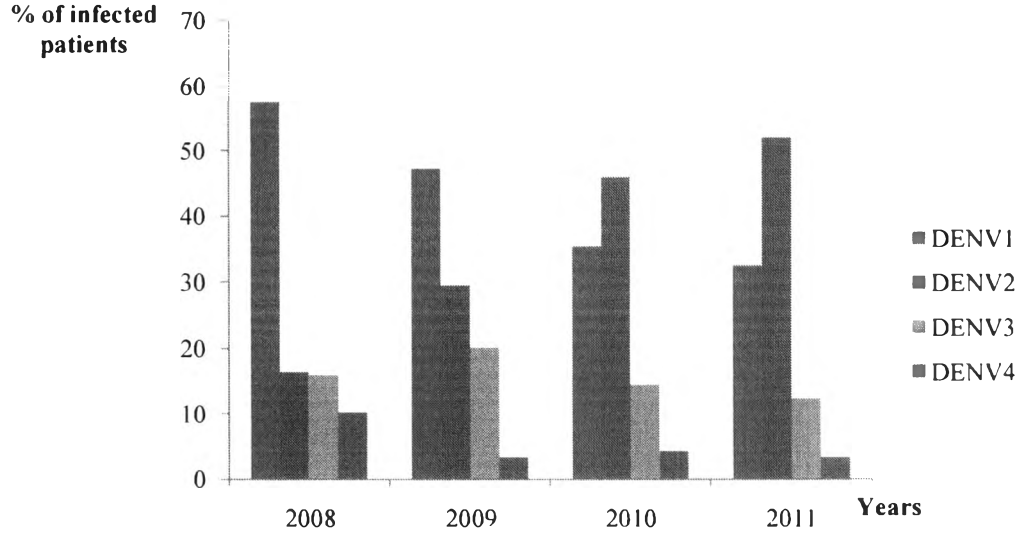

Figure 7: Distribution of DENV serotype in Thailand during 2008-2011
(Adapted from the annual report of DENV serotypes during 2008-2011 from Center of Vector Borne and Infectious Diseases [44]).

Not only single serotype infection but also multi serotype infections have been reported in Thailand. For example, Khawsak *et al.* monitored DENV in Thailand during 2000-2001 using RT-PCR. They found mixed serotype infections of DENV in all regions including in Central, Northeast, North and South of Thailand presenting of 2 or 3 serotypes mixing in each patient. Mixed serotypes of DENV3 and DENV4 was frequently found during that period and mixed infections with 3 serotypes (DENV2+DENV3+DENV4 and DENV1+DENV2+DENV4) are also found in patients, as well [45]. Kulwichit *el al.* used nested RT-PCR for classifying the serotype of DENV in plasma, PBMCs, saliva and urine. They found multi serotype infections of DENV with 2 or 3 different serotypes in both plasma, PBMCs, saliva and urine such as DENV1+ DENV4, DENV2+DENV4 , DENV1+DENV2+DENV4 and DENV1+DENV3+DENV4, respectively [46].

After focusing on the prevalence of DENV infection based on clinical diagnosis (DF, DHF and DSS or DHF grade IV) in Thailand, data collected during January 2012 to early September 2012 shows that there were 41,254 patients suffering from DENV infection and 44 patients were die. Most patients were 15-24 years old. DF was frequently found in patients according to 50.78% whereas DHF and DSS

were also found in proportions of 47.24% and 1.98% respectively. The predominance of DF, DHF was found in the South region in age of 10-14 and 15-24 years old, respectively while DSS predominated in the Northeast region in age of 10-14 years old [47].

Laboratory diagnosis of DENV infection

In some cases, clinical diagnosis by physician is not enough to confirm DENV infection. Laboratory diagnosis is an essential tool for confirming DENV infection. There are 3 main assays for verifying DENV infection, viral isolation, viral nucleic acid or antigen identification and serological assay. The use of each assay is based on the day of specimen collection and each specimen type. The suitable time for DENV detection is during the onset of fever to 10 days post-infection [48]. Various specimens such blood, urine, saliva and tissues are able to use for DENV detection. The diagram of appropriate protocols for DENV detection is shown in Figure 8.

The gold standard of DENV detection is viral isolation but the limitations of this technique were time consuming and requiring the facilities for culture system [49]. Viral genome and antigen detections demonstrate the evidence of infection in infected cell or tissues. Specific antibody assays, especially the seroconversion of IgM and IgG antibodies are widely used for confirming DENV infection [3]. The details of 3 principles are briefly discussed below.

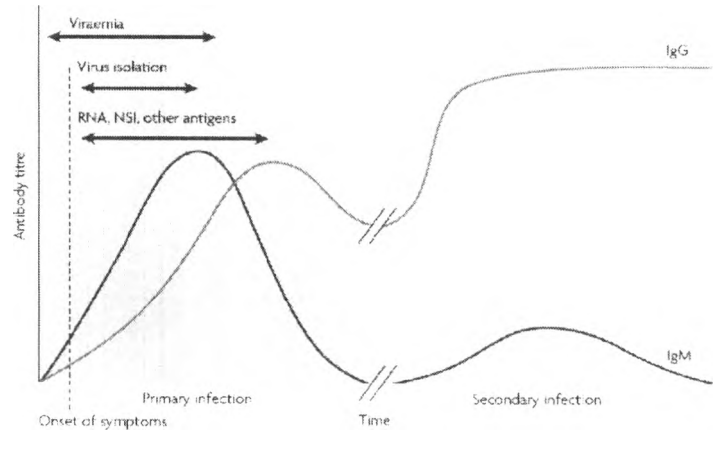

Figure 8: Diagnostic protocols for DENV detection in different times of specimen collections [48].

Both animal models and cell culture systems are used for DENV isolation. For animal model, plasma or serum of infected patient is intracerebral inoculation into suckling mice. However, this assay is not been widely used because of the ethical problem and the facility limitation. Mosquito inoculation technique is one of choices for viral isolation and is done by intrathoracic inoculation of plasma or serum into *Aedes* or *Toxorhynchites* mosquitoes [50, 51]. The limitations of mosquito inoculation technique are labor and requiring isolation areas for mosquito feeding and inoculation. Cell culture system becomes an appropriate and a well-known technique for DENV isolation. There are many cell types used for DENV isolation including vertebrate and invertebrate cell lines such as Vero cells, LLC-MK_2 cells, BHK21 cells, AP61 (*Aedes pseudoscutellaris*) and C6/36 (*Aedes albopictus*). Confirmed viral isolation in cell culture system is done by using specific dengue monoclonal antibody detections by immunostaining assay, ELISA test in cell culture supernatant or using PCR assay [3]. The suitable time of specimen collection for viral isolation is the first 3-5 days of fever or using acute phase specimen. Moreover, fatal tissues can be used as specimens for viral isolation as well.

Molecular diagnosis of DENV infection has been developed to detect DENV genome in clinical specimens and tissues. Conventional RT-PCR (single tube RT-PCR, nested RT-PCR, multiplex RT-PCR and NASBA) and real time RT-PCR (qRT-PCR) are widely used to detect DENV relying on specific primers with the target gene resulting in the increasing specificity and sensitivity of detection [3, 48]. Advantages of this assay are high sensitivity and specificity. It can be used although there is a small amount of specimen. The disadvantage of this assay is a contamination of RT-PCR product generating a false positive result. The molecular assay is suitable for DENV detection in specimens collected during acute illness or viremic phase until the earlier day of convalescent period. Dengue antigen detection is used to detect DENV protein in infected cells or tissues such as lymphocytes, spleen and lymph nodes. It can be used to detect DENV antigen in blood stream such as NS1 protein. Enzymatic and colorimetric substrate assay after using the specific monoclonal antibodies to DENV antigen such as immunohistochemistry staining, immunofluorescence assay or immunochromatographic technique is the main principle of antigen detection. The

suitable time of specimen collection for antigen detection is during acute illness or using fatal tissues [3].

Serological assay is based on the detection of specific antibodies to DENV especially the production of IgM, IgA and IgG to viral protein (mainly E protein) [48]. There are 5 common diagnosis tests for DENV infection; hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA) and immunoglobulin G ELISA [2]. Two latter protocols are mostly used for serological assay in patients. Antibody response for DENV infection, especially IgM and IgG is essential for serodiagnosis and classification in either primary or secondary infection. Plasma or serum was collected two times during acute and convalescent periods to monitor the seroconversion of IgM and IgG. IgM is useful for diagnosis and surveillance purposes. It expresses earlier than IgG and can be detected between 3-5 days of illness. IgM in primary infection expresses at the high level in the first 2 weeks after illness and becomes undetectable in next 2-3 months. Moreover, dynamics of IgM is variable in each patient. IgG is used for exploring the previous DENV infections and current infection in suitable pair serum collections. The rising of IgG is found in a latest day of IgM expression or during the decrease of viremia. The low titer of DENV-specific IgG is detected at the end of first week of illness, and then it slightly increases. The high level of IgG is found in febrile period, and then it dramatically increases over the following 2 weeks and can persist for a long period [3]. IgM:IgG ratio is used to distinguish either primary or secondary infection depending on each criteria of classification and protocol for ELISA assay. For example IgM: IgG ratio greater than 1.8 is considered primary infection whereas the value less than 1.8 is considered secondary infection [52].

Neutralization assay known as plaque reduction neutralization technique (PRNT) or micro-neutralization assay is also used for DENV assay. This method can detect the previous DENV serotype infection and define either primary or secondary DENV infection by using the different DENV antibodies to each serotype. Nevertheless, this assay is limited in a specific purpose such as vaccine study [3, 48].

DENV detection in blood specimens

Generally, DENV is commonly detected and isolated from blood specimens such as plasma/serum and PBMCs as well as platelets during viremia or febrile stage [49]. Viable or live DENV particles are usually detected in plasma/serum or PBMCs during febrile period before the rising of neutralizing antibodies [53]. DENV detection in blood specimen can be done by using molecular techniques such as RT-PCR and real time RT-PCR. For instance, Yenchitsomanus *et al.* developed nested RT-PCR and serotype nested RT-PCR for DENV detection in both cell culture supernatant and serum using specific primers to E and NS1 genes, respectively [56]. Lanciotti *et al.* and Harris *et al.* developed serotype-specific RT-PCR for DENV detection in clinical specimens such as plasma/serum and mosquitoes as well as cell culture supernatant [57, 58]. DENV in blood specimen could also be detected by real time RT-PCR based on SYBR Green I detection or TaqMan probes for both pan dengue detection (given the same PCR size) and serotype specific detection during febrile or acute period [59-62]. All assays were mostly developed to detect DENV in plasma/serum or PBMCs during febrile period. Additionally, the comparison of viral isolation and molecular techniques in blood specimen detection was compared. The researcher reported that viral isolation was effective when blood specimen was collected within day 5 of illness. If specimens were collected later than day 5 of illness, RT-PCR became more sensitive than viral isolation [63].

These are example reviews of DENV detection in blood specimen. In 1980, Scott *el al.* demonstrated the isolation of DENV from PBLs in acute period during the high level of DENV-specific antibodies by using LLC-MK2 cells, and detecting DENV in cell supernatant by plaque assay [54]. King *et al.* stated that DENV could be isolated from PBMCs during acute period and they showed the presence of DENV antigen in PBMCs by using immunocytochemistry staining. They also added that the main type of DENV infection in circulating PBMCs was B lymphocytes [55]. The persistence of DENV in PBLs during convalescent period was described by Wang *et al.* [53]. They studied DENV detection in acute plasma/serum and PBLs during acute ($\leq$ day 7) and convalescent ($>$ day 7) periods. They found that the efficiency of DENV detection was found in all specimens during acute period than in convalescent

periods. Interestingly, DENV antigen could be detected in PBLs during convalescent periods suggesting persistent DENV infection.

DENV detection in non-blood specimens

Most virus infections cause systemic infection due to the spread of viral particles into many organs via blood stream. This results in the presence of viral particles or antigens not only in blood specimens but also in other tissues or organs. In other flaviviruses such as WNV, HCV and SLEV, there are many reported suggested the presence of viral particles in non-blood specimens (saliva and urine) in both animal models and patients [8, 10, 64]. For example, the study of Tonry *el al.* presented the prolonged and culturable WNV from urine of infected hamsters up to 2 months after infection [10]. In human, WNV could be detected in urine of encephalitis patients as late as day 8 of illness [65]. In non-flaviviruses, viral detections in non-blood specimens were described as well. For example, the study of Bodur and his colleague presented the detection of Crimean-Congo hemorrhagic fever virus (CCHFV) genome in saliva and urine of infected patients [66]. In DNA viruses, Rossi *el al.* also showed the presence of human polyomavirus JC in urine of healthy patients when they studied the correlation between viral shedding and the difference of cellular urine sediment [67].

Recently, some studies have purposed the detection and isolation of DENV in non-blood specimens and tissues, especially in saliva and urine of DENV infected patients. The objective of previous studies was mostly to evaluate the use of saliva and urine for DENV diagnosis instead of using blood specimen. There are many techniques for DENV detection in saliva and urine such as viral isolation, immunodiagnosis, viral protein detection and molecular technique. In 2007, Chakravarti *et al.* presented dengue-specific IgM and IgG detections in saliva and suggested that saliva might be used as a specimen for dengue diagnosis by ELISA test [68]. Chuansumrit *el al.* showed that NS1 protein antigen could be detected in urine of both DF and DHF patients during febrile period and gave an explanation about the presence of NS1 antigen from plasma leakage or more viral antigen production in infected cells [69]. Torres *el al.* reported the DENV1 detection by RT-PCR and viral isolation (using C6/36 cell line) in plasma and saliva from a patient with DF causing

acute parotitis [70]. Published articles about DENV detection in saliva and urine have been launched continuously [12, 19, 71]. Mizuno *el al.* published a case report about DENV detection in urine as late as day14 of illness and in saliva up to day 7 of illness of DENV1-infected patient. Moreover, DENV1 could not be isolated from both specimens by using C6/36 cell line [71]. Other studies by Poloni *el al.* and Hirayama *et al.* also presented the evidence of DENV detection in saliva and urine by using real time RT-PCR technique [12, 19]. The latter study noted that DENV was detected in urine as late as day 16 of illness. Moreover, many articles suggest that DENV can be detected in various tissues such as liver, spleen, kidney, lung, bone marrow, brain, heart and lymph node using immunohistochemistry staining, *in situ* hybridization, RT-PCR, real time RT-PCR and viral isolation in C6/36 cell line [15, 72-74].

In our laboratory, we are interested in the detection and pathogenesis of DENV in blood, saliva and urine. Our pilot results presented that not only plasma/serum and PBMCs but also saliva and urine as well as buccal mucosa could be used for DENV detection by RT-PCR technique [28, 30, 75, 76]. During febrile period, DENV can be detected in all specimens as described above. However, during post febrile period, urine and PBMCs become the useful specimens for DENV detection. Interestingly, Chaiyo *el al.* demonstrated that DENV in urine could be cultured using a mosquito inoculation technique suggesting the evidence of live viral particles in urine as late as day 28 of illness [29]. Moreover, our pilot study about the monitoring of DENV infection in blood, saliva and urine presented that DENV could be detected for a long period by nested RT-PCR up to day 46 in urine [30]. However, other confirmation tests and more details about this phenomenon have not been performed.

The different viral loads in each specimen and tissue

Even though many viruses are detected in various specimens or body compartments, the viral load or viral titer may not be equal in each specimen type because of the dissimilar role in viral replication, survival and pathogenesis. The high viral load is found in permissive or susceptible cells for viral replication. Real time PCR (qPCR) or real time RT-PCR (qRT-PCR) is a major tool for study the viral load in each specimen. Studies of viral load in different specimens and time points are

beneficial because some data will be used for monitoring drug treatment or describing interesting details about viral pathogenesis. There are some studies presenting viral loads in different specimens and/or time points in both animal models and patients. In chronic viral infection such as the study of Pugnale *et al.*, the viral load of HCV was explored in different time points in PBMCs of chronic HCV-infected patients treated with pegylated interferon-α_{2a}. They found that the HCV viral load decreased when patients were monitored for day 42 of illness [77]. In herpes simplex virus type 1 (HSV-1), Burgos *el al.* studied the difference of viral loads in each tissue (blood, gonads, adrenal gland, spinal cord, brain and trigeminal ganglia) during the same period of HSV-1 infected C57BL/6J male and female mice in both acute and latent infections. They found that the viral load varied in sex, specimen and type of infection. In acute infection, the viral load was higher in adrenal gland than in brain, blood and trigeminal ganglia in male whereas the viral load was higher in brain than in other specimens in female. During latent infection, the viral load was higher in brain than in spinal cord of male whereas the viral load was higher in brain than in spinal cord and trigeminal ganglia in female [78].

In acute viral infection, viral loads in different specimens and/or time points are investigated as well. Ngaosuwankul *el al.* studied viral loads in nasopharyngeal aspirate (NPA), nasal swab (NS) and throat swab (TS) of pandemic H1N1, seasonal H1N1 and H3N2 infected patients [79]. They found that the difference of viral loads was depended on each specimen type instead of the different viral strains. The viral load in NPA was higher than in NS and TS. In Crimean-Congo hemorrhagic fever virus (CCHFV), viral loads were compared among blood, saliva and urine. Scientists represented that viral loads varied in different specimens of each patients during acute period. The viral load in plasma was mostly higher than in saliva but in some patients, the viral load in saliva or urine was instead of in plasma [66].

There are a few studies illustrating the viral load of DENV in different specimens and time points. However, previous studies about dengue viral load mostly focus on a single specimen in different time points. Murgue *el al.* studied durations of viremia and viral load using PCR-ELISA technique [80]. They found that the viral load was highest in plasma during early day of fever, and then the viral load decreased when a specimen was collected at the end of febrile period. Wang *el al.* studied the

DENV viral load in plasma collected in different time points [13]. They noted that the viral load in plasma was higher during an early febrile period. After that, the viral load in that patient was slightly decreased until undetectable in convalescent period (day 11 of illness). There is the interesting study of Poloni *et al.* presenting the different viral load in various specimens in the same period of collections [19]. The viral load in serum was higher than in urine and saliva, respectively. However, there has been no study to investigate the viral load of DENV in both different specimens and time points.

Molecular strategies for detecting the evidence of viral replication

As describing above, viral isolation is an essential tool for determine whether DENV is alive. Viable virus detection can be done using molecular technique known as negative strand detection, which can investigate the evidence of viral replication in tissues, organs or specimens, especially in positive strand RNA viruses. When comparing between two techniques, they can suggest whether live virus or evidence of viral replication in each tissue, organ or specimen is found. Viral isolation is an indirect method whereas negative strand detection is direct method. There are various assays to detect negative strand in positive sense RNA viruses such as LiCl-fractionated RNA gradients, classical RT-PCR, competitive RT-PCR or real time RT-PCR [81, 82]. Three latter protocols are easily to perform by using forward primer to do cDNA synthesis and to continuously perform PCR. The disadvantage of all 3 assays is the non-specific priming results. Therefore, tagged RT-PCR and rTth RT-PCR are developed to solve these problems [83, 84]. Tagged RT-PCR is commonly used to detect negative strand of positive strand RNA virus such as HCV and DENV [82, 89]. The forward primers added with 15-20 oligonucleotides at 5`-end is used for cDNA synthesis. Filled oligonucleotides at that primer are called "tag sequence". PCR is done using tag sequence as a forward primer and gene specific sequence as a reverse primer [83]. The principle of tagged RT-PCR was described in Appendix E. Advantages of negative strand detection instead of using viral isolation are high sensitivity and easy to perform when clinical specimens are not suitable for viral isolation. Moreover, in case of incomplete repletion of viruses resulting from the high

defective viral population or genetic variation, the viral isolation technique may not be successful. The use of negative strand detection can fulfill that weak point. In HCV, negative strand detection was done in various specimens and showed the evidence of viral replication in PBMCs, brain tissue, liver tissue and serum of chronic HCV-infected patients as well as in bone marrow of patients suffering from hematologic disease [85-88]. Negative strand detection of DENV is previously performed in many specimen types and cell culture system such as in DENV-infected cell culture supernatant, in hepatocytes and vascular endothelium cells in mouse liver tissues, in DENV-infected mosquitoes, and in plasma or platelets of DENV-infected patients [82, 89-91]. However, no study has mentioned about the negative strand detection of DENV in non-blood specimens such as saliva, urine and other tissues of DENV-infected patients in different time points of infection.

Persistent flavivirus infection

After the rising of neutralizing antibodies, viral particles commonly decrease and are eliminated from each patient. However, some viruses are not completely eliminated and become survival called persistent infection such as HCV and HIV. Persistent infection refers to the presence of both antigen and viral genome in susceptible or non-susceptible cells and organs. Both DNA and RNA viruses can cause persistent infection such as human herpesvirus 6 (HHV-6), varicella zoster virus (VZV), cytomegalovirus (CMV), hepatitis B virus (HBV), HCV, HIV, WNV and SLEV [8, 11, 92]. Virgin *et al.* purposed two mechanisms promoting persistent viral infection [92]. First, persistent viruses must completely evade the host immune response. Second, host immune responses must control the limit of viral replication and inflammatory response to avoid infected cell damage. Moreover, mutations of nucleotide and amino acid sequences, presence of heterogeneous populations or quasispecies, and generation of defective interfering particles (DIPs) are reported to play a role in persistent viral infection [20, 93]. The important factor described here is "quasispecies" promoting viral persistent by generating rapid antigenic variations of escape population, altering in cells or tissues tropism, and influencing on viral virulence, host range and viral gene expression [20]. The duration of viral persistent

varies in each type of viruses. Some viruses persist in a short period (1-2 months) and viral particles are slightly eliminated after the rising of effective immune responses. However, some viruses can persist for a long period and not be eliminated called latent or chronic infection. Moreover, some persistent viruses are most common in healthy patients, which cannot cause disease, even though a human is infected with that virus such as some human polyomaviruses found in urine of healthy subjects collected sequential for 3 months [67].

In flaviviriuses, persistent infection is reported in many viruses in both animal models and infected patients. Well-known studies of flaviviruses are investigated in WNV and SLEV [7-9]. Tesh *el al.* presented the prolonged WNV infection in golden hamsters by detecting secreted and cultureable viruses in urine as late as 8 months after post infection. Moreover, histopathology of kidneys and brain presented viral antigens by immunohistochemistry staining [9]. In WNV-infected patients, this virus could be detected in urine up to 1.6-6.7 years after onset of illness [7]. Persistent viral infection is described in SLEV, as well. Siirin *el al.* reported that SLEV was detected in urine of infected golden hamsters up to 185 days after initial infection [8]. In other virus, Thomas *el al.* reported the persistent CCHFV in patients by detecting the virus in urine and serum at day 36 of illness in the event of high titer of antibodies [94].

In DENV, there are some studies purposing the prolonged infection in both blood and urine specimens. Wang *el al.* illustrated the persistent of DENV antigen by immunocytochemistry staining in PBLs taken from DENV-infected patients [53]. They also implied that the presence of DENV antigen in PBLs suggested the evidence of viral replication in PBLs. In 2003, Wang *el al.* presented the persistent DENV genome in plasma as late as day 8 of illness and suggested the hypothesis describing persistent infection resulting from the formation of immune complex between viral particles and antibodies. This formation interfered with the elimination mechanism called "slow rate clearance of infection", especially in DHF patients [13]. This finding was confirmed by the study of Wang *el al.* and Ubol *el al.* [95, 96]. They showed that patients with DHF had high level of viral load and got a slower rate of viral clearance than patients with DF because of the formation of immune complex of DENV and preexisting enhancing antibodies. The formation of immune complex increased the efficiency of an immune evasion mechanism. Moreover, the immune

evasion was affected by the high level of interleukin 10 (IL-10) during each infection [95]. In non-blood specimens, persistent viral genome is found in kidneys confirmed by the presence of excreting viral particles or antigens in urine. Mizuno *el al.* demonstrated that DENV could be detected in urine of DEN-infected patients by RT-PCR up to day 14 of illness [71]. Interestingly by using real time RT-PCR, DENV became more detectable in urine as late as day 16 of illness [12]. In our laboratory, Laosakul *el al.* presented the prolonged detection of DENV in various specimens by nested RT-PCR [97]. They purposed that DENV could be detected in PBMCs and urine during convalescent period as late as day 21 and day 46 of illness, respectively.

Not only in DENV-infected patients but also in asymptomatic or non-dengue-infected patients illustrate the persistent DENV infection. These projects are conducted in our laboratory. We found that persistent DENV genome was found in bone marrow, lymphoid and kidney tissues of non-dengue-infected patients [98-100].

Genetic variation of flaviviruses

Many RNA viruses commonly present genetic variations because of the lacking proof-reading property during viral replication such as in HIV and HCV [21]. There are 2 processes having an impact on high RNA viral genome variations and replication rates, host's immune responses and the bottleneck at viral transmission [101]. The genetic variation composes of mutation and deletion in nucleotide or amino acid sequences as well as the presence of quasispecies and defective viral genome. Quasispecies is described as genome variations or "closely related not identical genome" in one or a few nucleotides from other sequences during each replication and in each body compartment [20, 21, 102]. There are 2 terms involving in quasispeies, random mutations and selections [102]. Random mutations including the generation of new viral population influenced by each environment are called the selection of viral population (positive, purifying or neutral selection). The existence of heterogeneous population or quasispecies varies in each specimen or body compartment (closely related in an individual site) but segregates or relates to the same single ancestor is called "compartmentalization" [103, 104]. The factors driving compartmentalization have not been clearly described. Philpott *et al.* purposed that

compartmentalization results from the spread of viral population to various tissues or organs during primary infection, then the viral populations in each compartment were under their local evolution and the presence of selective pressure, especially the pressure of host immune response affecting the occurrence of heterogeneous population [103]. Additionally, defective viral genome consisting of either nucleotide deletion or stop codon is one type of genetic variations [25]. The consequential outcomes of this phenomenon involve in pathogenesis and persistent viral infection. There are many methods to study genetic variation such as direct sequencing to demonstrate the different major viral populations. Additionally, cloning and sequencing, single stranded conformation polymorphism (SSCP), heteroduplex gel shift assays and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy are used to study the occurrence of heterogeneous population as well [105]. In this review, nucleotide and amino acid variations as well as the presence of heterogeneous population or quasispecies are mainly discussed.

In Flaviviruses such as WNV, SLEV and HCV, there are many studies describing genetic variations and pathogenesis of infection. Ding *et al.* studied nucleotide and amino acid variations of full length genome after culturing WNV for a long period in hamsters' urine. They found that nucleotide variations in different time points occurred in E, NS1, NS2B, NS5 and 3' UTR genes whereas amino acid variations were found in E, NS1, NS2B and NS5 proteins [6]. Other study in animal model (WNV-infected hamsters) was explored by Wu and his colleague in 2008 [11]. They monitored the nucleotide and amino acid changes in WNV-infected hamsters similar to the study of Ding and his staff. They found that nucleotide variations in urine were mostly found in C, E, NS1, NS2A, NS2B, NS3, NS5 and 3' UTR genes and the amino acid variations presented in C, E, NS1, NS2A, NS2B and NS5 proteins. Variations in each gene depended on a period of urine collection and acted as random mutations. There is the interesting study of Siirin *et al.* in SLEV-infected golden hamsters, which presented genetic variations in different cell types and time points [8]. They presented persistent SLEV infection in brain and kidney tissues as well as the nucleotide and amino acid variations of complete E gene/protein in different time points. They also found that the nucleotide and amino acid sequences between urine and kidney were not change. However, some nucleotide and amino acid variations in

brain tissue were different from urine and kidney. These may suggested the evidence of sequence specificity and viral compartmentalization in each tissue.

The genetic variation and quasispecies of isolated RNA viruses from clinical specimens are investigated, especially in HCV. The first report about HCV quasispecies was described by Martell and his coworkers in 1992 [106]. They analyzed nucleotide variations of 5'UTR and NS3/NS4 regions in plasma and concluded that HCV genome was composed of major and minor (variant or mutant) populations by difference of 1 to 4 nucleotides. Mutation types included silent mutation, in-frame stop codons, conservative and nonconservative amino acid substitutions as well as defective genomes. The genetic variation is also investigated in bone marrow of both transplanted patients and donor suffering from HCV infection after monitoring for a long period. The results showed the adaptation of HCV in different hosts and time points by illustrating the amino acid change in partial E2 gene (HVR1 region). Some population survived from donor to recipient up to day 1460 whereas some populations were disappeared [107]. The genetic variations of HCV in different specimens (such as plasma/serum, PBMCs, brain tissue, liver tissue and genital tract) and time points were studied by many scientists [104, 108-117]. They investigated whether genetic variation acted as compartmentalization in different specimens and time points by using phylogenetic analysis. The regions of interest were E2 (IRES and HVR1), E2-NS1 region, E2-NS2 junction, 5'UTR region and E1 region. They found that genetic variations were depended on each tissue and time point. Some HCV population was conserved in a specific tissue or specimen called "compartmentalization" such as the distinguished HCV population between plasma and genital tract, and between plasma and brain tissue, respectively [110, 114]. In longitudinal study of HCV, Cabot and his colleague studied the complexity of viral population in serum and liver tissue in 4 patients in the duration of 2-6 years. They found that the complexity of HCV populations in serum and liver tissue were not stable in the same and different periods. Moreover, they stated that circulating HCV population originated from liver tissue [108].

In other viruses such as Ebola virus and HIV, genetic variations and quasispecies are explored as well. Rodriguez *el al.* stated that the nucleotide and amino acid sequences of nucleocapsid protein (NP) and glycoprotein (GP) of Ebola

virus derived from vaginal swab, rectal swab, conjunctival swab, rectal tissue, tonsil tissue and seminal fluid either in the same or different time points were not change [118]. According to HIV, there are many published articles stating about the genetic variations, especially the presence of quasispecies and compartmentalization in different specimens such as in cerebrospinal fluid (CSF), whole blood, plasma, PBMCs, autopsy, pleural fluid and genital secretion, and in different time points [103, 119-124]. For example, Philpott *el al.* investigated the compartmentalization of HIV in plasma and cervicovaginal lavage (CVL) by analyzing *env* and *gag* genes [103]. Moreover, genetic variations of HIV during acute and chronic HIV infections also investigated. Zhang *el al.* explored the compartmentalization and quasispecies of HIV in PBMCs, tonsil and rectum tissues in patients with acute or chronic infection by analyzing sequences on V1-V2 and V4-V5 domains of envelope glycoprotein [124]. They found that amino acid sequences between PBMCs and tonsil or rectal tissues were identical in patients with acute infection. On the contrary, amino acid sequences between PBMCs and rectum of patients with chronic infection were changed in V3 region. They also purposed the mechanism relating to the occurrence of different HIV population in both acute and chronic stages. During acute infection, homogeneous population was from the completed diversification during late stage of infection in various compartments whereas genetic variations during chronic infection came from the viral adaptation and diversification of dominant population during seroconversion stage.

According to DENV, the genetic variation is investigated in many research groups. Most previous studies mainly focus on a single specimen type during febrile stage. Wang *et al.* studied genetic variations of DENV3 in plasma of DF and DHF patients by analyzing partial E gene (430 bp) locating on domain III. They found that DENV genome in plasma circulated as a heterogeneous population or quasispecies varying in each patient. They also added that defective genome could be found and amino acid variations at domain III of E gene were under positive selection of host immune responses [25]. They also studied the quasispecies structure of DENV in other genes such as C and NS2B to confirm their findings in E gene. The results showed that both 2 genes also presented heterogeneous population or quasispecies and defective genome in plasma of DENV3-infected patients [26]. Chao *el al.* studied

the genetic variation and quasispecies of DENV3 by analyzing a whole genome of the virus. They found that higher sequence heterogeneity was found in C-prM and E than in NS3 and NS5 genes. They confirmed that genetic variations at domain III of E gene was strongly under positive selection as well [125].

The correlation between genetic diversity and clinical outcome is investigated. For example, Descloux and his research team studied intra-host genetic diversity of DENV1 in serum of DF, DHF and DSS patients by investigating the complete E gene sequence (1,485 bp). They found that intra-host genetic diversity was depended on clinical outcome. The genetic diversity was significantly lower in severe cases (DHF and DSS) than in other case (classical DF) [126].

The genetic variation of DENV in different host ranges is also described. Chen *et al.* investigated genetic variations in E/NS1 gene of DENV2 in mammalian cell line (Vero cells) and/or mosquito cell line (C6/36). They found that 2 nucleotides in E gene and 1 nuclotide in NS1 gene changed after the virus had been passaged for 20 times in Vero cells. However, few nucleotide changes in E and NS1 genes were found in C6/36 cells. They concluded that genetic variations were depended on each cell type [127]. Few variations mean the less viral adaptation in a suitable cell or organ for viral survival. Additionally, Lin *et al.* explored the sequence variation at E and C genes of DENV3 in different hosts; human and mosquito by using directly plasma of DENV3- infected patients (human host) and inoculating DENV3 plasma into *Aedes aegypti* mosquitoes. They also collected mosquitoes (naturally infection) in the endemic area of DENV3 infection. They found that the major population of DENV3 in both hosts was the same. The genetic variations and quasispecies of both E and C gene in infected mosquitoes were lower than in plasma of infected patients. In addition, genetic diversity was higher in E gene than in C gene in both patients and mosquitoes [24].

In some studies, genetic variations are correlating with pathogenesis of DENV infection. Sanchez *et al.* demonstrated that the amino acid variation of DENV2 (Maxican strain) on domain III of E gene at position 390 (D390H) resulting from nucleotide variation at position 1168 (G→C) correlated with neurovirulence in mice [128]. Sistayanarain *el al.* showed that the amino acid variation of E gene at position

173 (A173V) locating on domain I could separate enceplalopathy-associted DENV from non-enceplalopathy-associated DENV [129].

As described above about the genetic variations and quasispecies of DENV, previous studies mostly focus on single time point of infection and limited in one specimen type during febrile period. Moreover other dengue serotypes have not been widely investigated. There is one interesting study of Hirayama *el al.* in 2012. They studied the evidence of DENV detection in serum and urine by real time RT-PCR. They also investigated nucleotide variations of partial E gene between serum and urine using direct sequencing. They presented that identical nucleotide sequences were found in serum and urine collected during the same period (febrile period) and different periods (day 2 and day7; day 1 and day 16) of infected patients [12]. However, other genetic characterizations of prolonged DENV infection in various specimens and time points have not been completely investigated.