CHAPTER II

LITERATURE REVIEWS

Dengue viruses belong to the genus *Flavivirus* in the family *Flaviviridae*. Dengue viruses can be subdivided into 4 serotypes, DENV-1-4 (Lindenbach et al., 2001). Dengue viruses cause mild fever, dengue fever (DF), dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Annually, 100 millions people have been estimated to develop dengue fever. Half a million cases of dengue fever could develop to more severe dengue hemorrhagic fever (Malavige et al., 2004). The dengue hemorrhagic fever is one of major public health problems in South-East Asia (Clyde et al., 2006).

1. Properties of dengue virus

1.1 Structure and components of a dengue virus particle

Dengue virus particle has a spherical shape formed from two structural components: an inner core and an envelope (Zhang et al., 2003). The inner core is composed of nucleocapsid with an icosahedral symmetry (Zhang et al., 2003) about 30 nm in diameter. The nucleocapsid contains a molecule of positive sense, single-stranded RNA and several copies of capsid (C) protein. The nucleocapsid is surrounded by the envelope membrane, which consists of a lipid bilayer embedded with two viral glycoproteins: envelope (E) and pre-membrane/membrane (prM/M) (Lindenbach and Rice, 2001; Zhang et al., 2003; Burke and Monath, 2001). The diameter of the viral particle is approximately 50 nm (Zhang et al., 2003; Burke and Monath, 2001). The buoyant density of the particle is about 1.19 to 1.23 g/ml (Smith et al., 1970).

The RNA genome of dengue virus, about 11 kb long, possesses one large open reading frame. This single reading frame encodes three structural protein and

seven non-structural (NS) proteins. The order of these proteins in the genome is C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Gruenberg et al., 1992; Yaegashi et al., 1986; Zhao et al., 1986). In addition to the coding region, there are 2 untranslated regions (UTR). One, about 100 nucleotides in length, is located at the 5′ end and the other, about 400 nucleotides, is at the 3′ end of the genome (Figure 1) (Smith et al., 1985; Lee et al., 1992). The 5′ end is tagged with the type I cap structure, which is m^7GpppAmp (Vaughan et al., 2002) while the 3′ end lacks a polyadenylate tail.

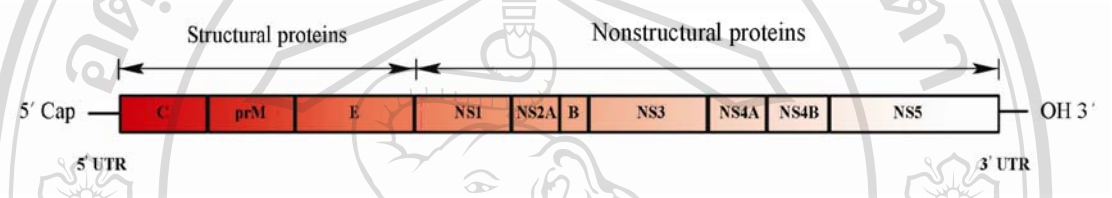

Figure 1. Dengue virus genome and gene organization.

1.2 Viral proteins

Viral proteins are synthesized as a stretch of polyprotein. The polyprotein is cleaved by host-encoded and virus-encoded proteases, resulting in 3 structural and 7 non-structural proteins (Figure. 2). Properties and functional roles of these proteins are described, as follows.

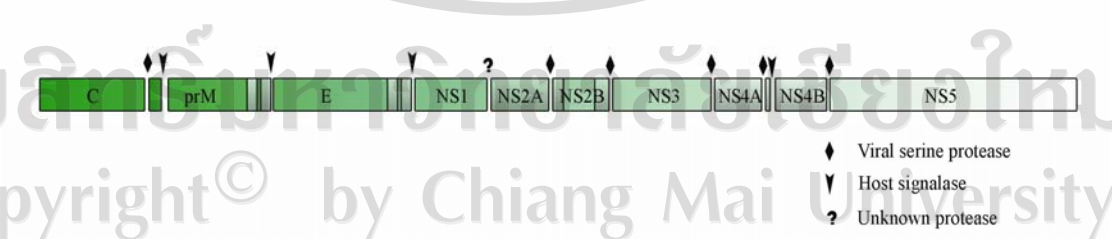

Figure 2. Dengue virus polyprotein and cleavage sites

Structural proteins

Structural proteins including C, prM/M and E proteins, are essential components of dengue virus particle.

C protein, a protein component of nucleocapsid, is a small protein containing 113 amino acid residues (Kuhn et al., 2002). This protein is first synthesized as an anchor C protein with a C-terminal hydrophobic stretch, which serves as a signal sequence for prM. This C-terminal signal sequence is later removed by the viral NS2B-NS3 protease to generate a mature form of C protein (Markoff et al., 1997). The mature form is a highly basic protein since it contains large numbers of Lys and Arg residues (Mandl et al., 1988, Yamshchikov and Compans 1995, Amberg et al., 1994). These positively charged residues, mainly located at the N- and C-termini, contribute to the ability of C protein to bind with the negatively charged groups on the viral genomic RNA (Ma et al., 2004; Zhu et al., 2007). Another significant feature of C protein is that it contains conserved hydrophobic regions which are believed to play a role in interaction with envelope membrane. These two features might contribute to the function of C protein as a carrier molecule for encapsidating the viral genome into viral particle (Ma et al., 2004).

Two other structural glycoproteins, prM/M and E, are components of the viral envelope. The prM protein contains 166 amino acid residues with the MW of 18-26 kDa. This glycoprotein contains three potential N-linked glycosylation sites and six conserved cysteine residues (Rice et al., 2007). All conserved cysteine residues form intramolecular disulfide bonds. In the C-terminus, double transmembrane stretches are found. The prM protein initially functions as a chaperone protein for E protein folding (Lorenz et al., 2002). Later, the prM protein protects E protein from premature acid-induced conformational changes that may occur during the transport of virus particles through the secretory pathway. Before the virus particle is released; prM protein is cleaved by host furin/furin-like serine protease at the pr-M junction (Chang et al., 1997). After the cleavage, a pr portion is released from the virus particle while an M portion (about 7-9 kDa) is retained with the particle which is now called a mature form of virus.

The E glycoprotein contains 495 amino acids and is about 53 kDa in molecular weight (Kuhn et al., 2002). It possesses the cell receptor binding moiety and the membrane fusion ability. There are 12 conserved cysteine residues that form intramolecular disulfide bonds (Lindenbach et al., 2007) and two conserved N-linked glycosylation sites (Mondotte et al., 2007). With two membrane-spanning regions at

the C-terminus, E is characterized as a type I membrane protein (Zhang et al., 2003). E protein is composed of three domains. The domain I is formed as a central structure which is flanked with domains II and III. The domain II carries a fusion peptide which mediates membrane fusion (Modis et al., 2004). The domain III bears an immunoglobulin-like domain and acts as a cell receptor-binding domain (Zhang et al., 2003).

Non-structural proteins

These proteins are important for RNA synthesis, translation of the polyprotein and polyprotein processing.

The NS1 glycoprotein is about 46 kDa. NS1 is directed into the ER compartment by the second transmembrane region of E protein, which serves as a signal peptide. Following its synthesis, NS1 is released from E protein by a host signal peptidase which recognizes and cleaves at the E-NS1 junction. However, which of the ER resident proteases is responsible for the cleavage at the NS1/NS2A junction is unclear. NS1 contains three potential N-linked glycosylation sites and 12 conserved cysteine residues, which form intramolecular disulfide bonds (Lindenbach et al., 2001).

NS1 molecules are found in various sites, including the ER compartment, the surface of infected cells, and the culture supernatant, but its function is still unclear. A significant role of NS1 on viral genomic RNA replication has been shown (Lindenbach et al., 2003). Mutations of the N-linked glycosylation sites and temperature-sensitive mutation in NS1 were found to reduce the amount of viral genomic RNA as well as viral particle production (Lindenbach et al., 2003). It is strongly believed that the NS1 protein plays an important part in the early event of viral RNA replication.

The NS2 protein is cleaved to the NS2A and NS2B proteins. The NS2A protein is about 22 kDa, and the NS2B protein is about 14 kDa. The NS2B protein is a membrane associated protein, and it is cofactor of NS3 protease activity. The function of the NS2A protein is still unknown (Chambers et al, 1989).

The NS3 protein is a large multifunctional protein, which it is about 70 kDa. At the N-terminal region, it contains a serine protease activity. This activity is

important in the processing of polyprotein. In the C-terminal region, it contains NTP-dependent helicase activity, which is involved with the viral RNA synthesis and the unwinding of RNA (Bazan et al., 1989; Brinton, 1986).

The NS4A protein and the NS4B protein are about 16 and 27 kDa, respectively. They contain hydrophobic domains which allow them to associate with to the membrane. These proteins are believed to interact with the other nonstructural proteins at the viral replication site. The enzymatic activity of these proteins is unclear yet (Chambers et al, 1989).

The NS5 protein is a large protein of about 103 kDa. This protein has an RNA-dependent RNA polymerase activity, which is involved in viral RNA synthesis. It also exhibits the methyltransferase activity required for the generation of 5'cap RNA structure (Lindenbach and Rice, 2001; 2003).

2. Replication of dengue viruses

2.1. Host range and cell lines

Natural hosts of dengue virus are mosquitoes, especially *Aedes aegypti* and *Aedes albopictus,* and primates including humans. An infection of dengue virus in lower primates, such as chimpanzees, rhesus, gibbons and macaques, leads to viremia but it does not cause any clinical signs. Therefore, these primates are helpful in studying immunological responses to dengue virus infection (Henchal and Putnak, 1990).

Different laboratory host cells have been developed for the propagation and experimental studies of dengue viruses. Host cells from mammalian and mosquito origins are more popular than others. Useful mammalian cell lines include LLC-MK_2 cells (monkey kidney cell line), PS (porcine stable kidney cell line), FRhL (fetal rhesus monkey lung cells), and PDK (primary dog kidney) cells. LLC-MK_2 and PS cells are usually used for the infectivity titration assay and the plaque reduction neutralization assay whereas FRhL and PDK cells are suitable for propagation of the viruses, especially the candidate attenuated vaccine strains (Henchal and Putnak, 1990). Cultured mosquito cells, such as *A. albopictus* (C6/36) and *A.*

pseudoscutellaris (AP-61), are used for viral propagation and virus isolation. Replication of dengue viruses in mosquito cells causes cytopathic effects such as rounding shape of cells and cell fusion. The latter change is less apparent in infected mammalian cells.

2.2 Replication cycle

Dengue viruses are an obligate intracellular parasite, which replicates only within living cells. Dengue virus starts its replication cycle by binding to specific receptors, and then the virus replicates in the following steps: internalizing, uncoating, translation and polyprotein processing, RNA synthesis, particle assembly, budding, transport and release, respectively (Figure 3).

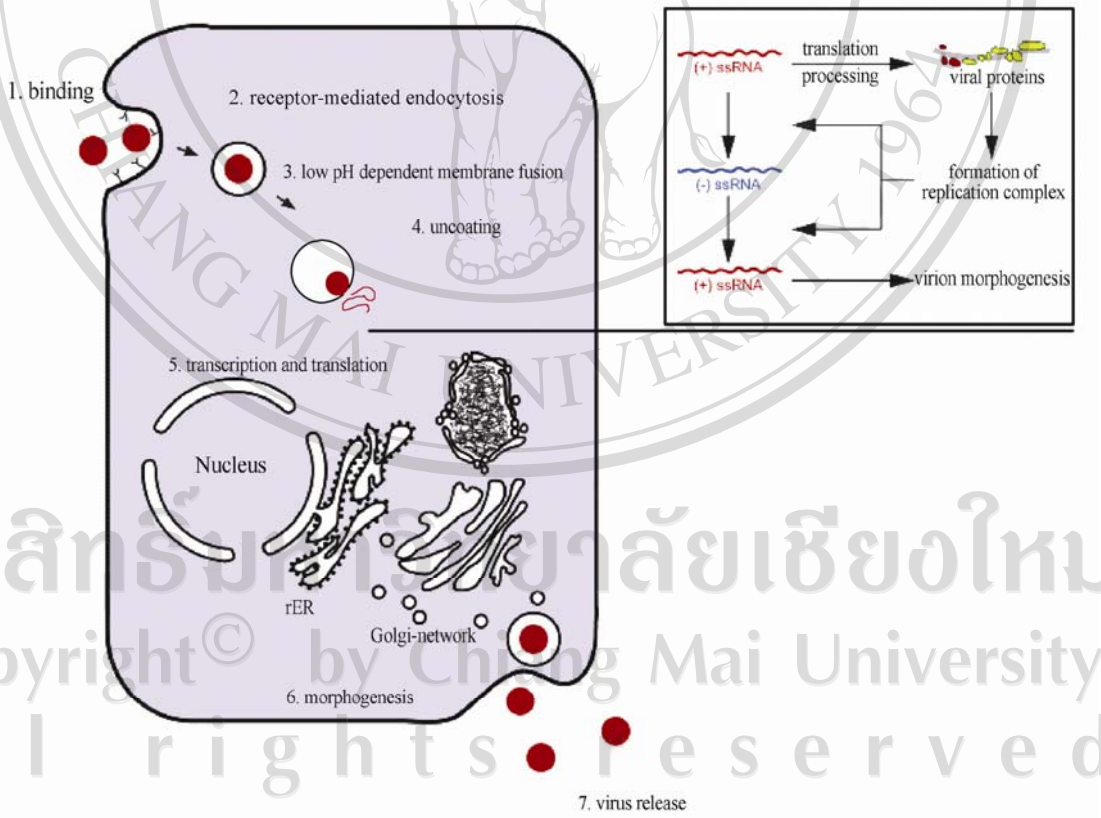

Figure 3. Dengue virus replication cycle (adapted from www.molecular virology. uni-hd.de/rsr/dengue/2_replication_cycle.htm).

Dengue virus attaches to the host cell surface by binding to cell surface receptors. Specific receptors for dengue virus include heparan sulfate, dendritic cell specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), GRP78/BiP and CD14 (Modis et al., 2004; van der Schaar et al., 2007). Binding of E protein to its receptors induces viral internalization by endocytosis (van der Schaar et al., 2007; Gollins and Porterfield, 1984). The Fcγ receptor on the surface of monocytic cells can also mediate the internalization of dengue virus by antibody-dependent enhancement mechanism (Chanas et al., 1982; Gollins et al., 1984; Halstead and O'Rourke, 1977).

After the attachment step, virus particle is internalized by endocytosis. The adherence of particle to its receptors induces plasma membrane thickening by clathrin protein and a clathrin-coated vesicle is formed. This vesicle is formed and a dengue virus particle is wrapped inside (Ishak et al., 1988; Mossquera et al., 2005).

The uncoating step is mediated by a series of changes in E protein induced by changes in the acidity of the endosomal environment. The endosome containing the virus particle is transported to the cytoplasm. During the transport, maturation of the endosome occurs and the pH of the endosomal lumen is reduced (Ishak et al., 1987; van der Schaar et al., 2007). E protein homodimers on the envelope dissociate and rearrange into homotrimeric form, this changes are triggered by low pH in the endosome (Heinz et al., 1994; Kuhn et al., 2002; Zhang et al., 2003). This results in a movement of the fusion loop at the tip of domain II of E protein to the endosomal membrane (van der Schaar et al., 2007). Then, this loop inserts itself into the outer leaflet of the endosomal membrane. The E protein trimer creates a bridge between the viral envelope and the endosomal membrane. The folding of the domain III and rotation of domain II lead to the envelope and the endosomal membrane coming close together. This allows fusion to occur between these two membranes. As a result of the membrane fusion, the nucleocapsid is released into the cytoplasm (Modis et al., 2004).

When the nucleocapsid enters the cytoplasm, the single-stranded RNA genome dissociates from the C proteins. The details of this uncoating process remain

unclear. The free genomic RNA in the cytoplasm, with helps from cellular components, is able to initiate the viral polyprotein synthesis.

Synthesis of dengue virus polyprotein is a cap-dependent process. The initiation complex is composed of three host proteins, including eIF4F protein, eIF4B protein and 43S subunit protein, which bind to the cap structure. This complex can unwind the RNA secondary structure at the 5' UTR and scans for a correct start codon. Then, translation of the polyprotein is initiated (Clyde et al., 2006). During translation process, the polyprotein is cleaved by host-encoded proteases as well as the viral serine protease. Ten different structural and non-structural proteins are generated (Cleaves, 1985).

The synthesis of viral genomic RNA occurs after nonstructural proteins are available. The genomic RNA serves as a template for the synthesis of negative strand RNA and then the newly synthesized negative strands serve as a template for the production of viral genomic RNA. During RNA synthesis, three forms of viral RNA can be isolated form virus-infected cells. They are: 1) 20S to 22S RNA "replicative form (RF)", which resists digestion by RNase specific for single-stranded RNA, 2) partially RNase-resistant 20S to 28S RNA "replicative intermediate (RI)", and 3) RNase-sensitive 42S RNA. The replicative form is the full-length double-stranded RNA molecule. This form can be dissociated into the 42S RNA form by heating. The replicative intermediate is generated from the replicative form during the new round of RNA synthesis, with the new strand partially displaces one of the original strands. Heating of the RNA intermediate generates the 42S RNA and smaller RNA molecules (Henchal et al., 1990).

When the viral structural proteins and genomic RNA, which are components of virus particle, are available, particle assembly can occur. The assembly takes place at the ER membrane. The single-stranded, positive-sense RNA associates with several copies of the C protein, leading to the formation of a nucleocapsid (You and Padmanablian, 1999; Barth et al., 1999). At the same time, the E and prM proteins co-associate at the ER membrane, forming lateral lattice structure that may lead to the curving of the ER membrane. The nucleocapsid is enveloped with the ER membrane containing prM and E proteins, leading to the budding of nascent particles into the ER lumen. The mechanism of budding is believed to be similar to that of clathrin-coated

vesicle formation (Lorenz et al., 2003). This assembly first generates the immature particles (Ishak et al., 1988; Op De Beck et al., 2003).

The viral particle in the ER lumen is transported through the secretory pathway for the exit from the infected cells. E and prM proteins are modified by N-glycosylation process during the transport (Johnson et al., 1994). In addition to the glycosylation, prM is also cleaved by furin protease in the trans-Golgi network (Randolph et al., 1990; Stadler et al., 1997). This cleavage is a crucial step in the generation of an infectious viral particle. Finally, this particle is released from the infected cell by exocytosis. For dengue virus, the other type of the particle export has been reported (Hase et al., 1987).

2.3 Structure of immature and mature particle of dengue virus

An immature particle of dengue virus contains three structural proteins; C, E and prM proteins, while the fully mature particle are consisted of C, E and M proteins (Figure.4) (Kuhn et al., 2002). Shortly before particle release, the immature particles are transformed into the mature particles following the cleavage of prM proteins (Figure 3) (Lorenz et al., 2003). When the prM protein is cleaved by furin protease, the pr portion is released from virus particle and secreted out of infected cells (Lorenz et al., 2003).

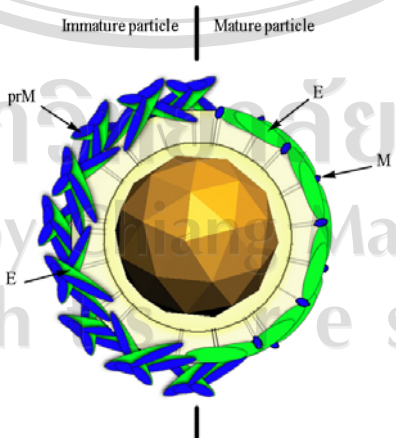

Figure 4. Comparison of immature and mature particle of flavivirus (Adapted form Lindenbach et al., 2007)

3. Dengue virus particle export

The mechanism of the dengue virus particles exporting out of infected cell is unclear. The flavivirus particles transport along the secretory pathway has been well documented; however, the other route of transport has also been documented (Hase et al., 1987; Sriurairatna et al., 1973).

The export of the flavivirus particles, i.e. YFV (Ishak et al. 1988), JEV (Hase at al., 1987) and DENV (Hase et al., 1985; Sriurairatna et al., 1973), had been observed by an electron microscopy. A common mechanism of the particle export was exocytosis through the secretory pathway. The observation of YFV 17D-infected Vero cells under an electron microscope revealed that the nucleocapsids were detected at the ER membrane at 24 hr after infection (Ishak et al. 1988). After that, virus particles were found in the ER lumen and in coated vesicles inside the cytoplasm. These vesicles serve as a cargo for transporting virus particles out of the infected cells by exocytosis (Ishak et al. 1988).

Similarly, in the infected C6/36 cells and suckling mouse brain, JEV assembled at the ER membrane, and the virus particles were found accumulated in the ER lumen, the intermediate compartment, the Golgi apparatus, and the post-Golgi network (Hase et al., 1987). These evidences reveal that JEV particles are exported out of the infected cells through the secretory pathway (Hase et al., 1987).

In the case of dengue serotype 2 virus, two transport routes have been proposed (Hase et al., 1987; Sriurairatna et al., 1973). These mechanisms include a trans-type export and a cis-type export (Hase et al., 1987). By observing the ultrastructural changes of dengue virus-infected C6/36 cells under an electron microscope, it was founded that the virus particles were transported by the cis-type manner (Hase et al., 1987; Rohman et al., 1998). It was found that viral proteins were translated on the ER membrane, but the viral particles assembled at the membrane of vacuole then the virus particles budded into vacuoles (Hase et al., 1987; Rahman et al., 1998). These vacuoles were present at 24 hours after infection, and their numbers increased with the duration of infection (Hase et al., 1987). The electron micrographs showed the electron dense particles enclosed by a fuzzy envelope in the vacuole (Hase et al., 1987). The structure of the virus-filled vacuole is a large spherical shape

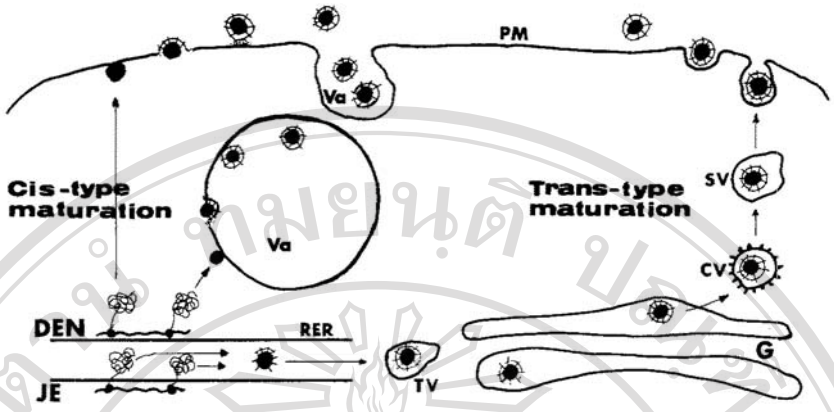

Figure 5. Flaviviruses export by cis-type and trans-type maturations (Hase et al., 1987); Va, vacuole; RER, rough endoplasmic reticulum; TV, transport vesicle; CV, coated-vesicle; SV, smooth vesicle; PM, plasma membrane.

about 166 nm to 566 nm in diameter which was found nearby the plasma membrane. This vacuole was also found to be fused with the plasma membrane to release virus particles to the extracellular compartment (Hase et al., 1987). Moreover, the immunogold electron microscopy revealed that dengue virus particles could assemble at the plasma membrane and then budded out of infected cells (Figure 5) (Hase et al., 1987). The actual transport, either by cis- or trans-type secretions, was found to be dependent on the virus strains and the cell types (Hase et al., 1987; Rahman et al., 1998).

4. Mutations in dengue structural proteins that delay virus particle export

During dengue virus maturation, an internal cleavage of prM by furin is an essential step in the generation of an infectious dengue virion. Various studies have demonstrated that substitutions of conserved Arg or Lys at P1, P2 and P4 are lethal to flaviviruses (Elshuber et al., 2003, 2005; Chaichoun, 2002). The changes of basic amino acids at these positions yield non-infectious particles and trypsin treatment of these immature particles is required to rescue their infectivity (Elshuber et al., 2003). Therefore, the cleavage of flavivirus prM protein is important for virus infectivity.

A stretch of furin consensus sequence (Arg-Xaa-(Arg/Lys)-Arg, Xaa is any amino acid) or the minimal furin motif (Arg-Xaa-Xaa-Arg) is required for furin recognition and cleavage (Nathan et al., 2002). The furin consensus sequences are found at the pr-M junction of flaviviruses. Interestingly, the cleavage of dengue virus pr-M junction has been shown to be less efficient than other flavivirus prM proteins (Keelapang et al., 2004)

A recent study on dengue prM protein has shown that amino acid changes at the pr-M junction could affect dengue virus particle export (Keelapang et al., 2004). When the 13-amino acid sequence just proximal to the pr-M junction of a dengue serotype 2 virus, strain 16681, was replaced with the homologous sequence from three other flaviviruses (Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV)), these chimeric viruses showed differences in the level of prM cleavage, especially in the chimeric virus JEVpr/16681. The level of prM cleavage of the JEVpr/16681 was enhanced to 94% as compared with only about 66-77% as in the parental strain (Keelapang et al., 2004). This analysis reveals that certain amino acids within the 13-amino acid stretch just proximal to the pr-M junction affect the level of prM cleavage of the extracellular dengue virus particles (Sittisombut et al., 2006). Interestingly, an enhanced prM cleavage in JEVpr/16681 is accompanied by the smaller focus size, reduced viral replication *in vitro* as well as a delayed export of infectious virus particles out of virua-infected cells (Keelapang et al., 2004).

A comparison of the 13-amino acid sequence just proximal to the pr-M junction of JEVpr/16681 virus and the parental strain reveals three types of amino acid substitutions: 1) the substitution of uncharged residues at positions P8, P10 and P13 with arginine, 2) the substitution of glutamic acid at positions P3 and P7 with uncharged residues, and 3) the substitution of two uncharged residues at positions P6 and P9 with other uncharged residues. In a previous attempt to determine which of these changes affect the focus size and virus replication, each of the three types of amino acid substitutions was introduced into the full-length cDNA clone of strain 16681 (Sriburi et al., 2001), generating three mutant viruses designated as 16681pr(+7,-2), 16681pr(+4,-0)HS and 16681pr(+7,-0), respectively (Songjaeng, 2004). The determination of focus size revealed that, similar to JEVpr/16681, all three

mutants exhibited smaller focus sizes than that of the parent virus. On the other hand, the multi-step kinetics study revealed that two mutant viruses, 16681pr(+4,-0)HS and 16681pr(+7,-2), replicated to the lower levels than that of the parent strain but higher than JEVpr/16681. The third mutant virus, 16681pr(+7,-0), replicated to the same level as JEVpr/16681 (Songjaeng, 2004). These results lead to a conclusion that the reduction of virus replication detected in JEVpr/16681 represents a combined effect of substitutions at uncharged residues at positions P8, P10 and P13 to arginine and the replacement of glutamic acid residues at positions P3 and P7 with uncharged residues.

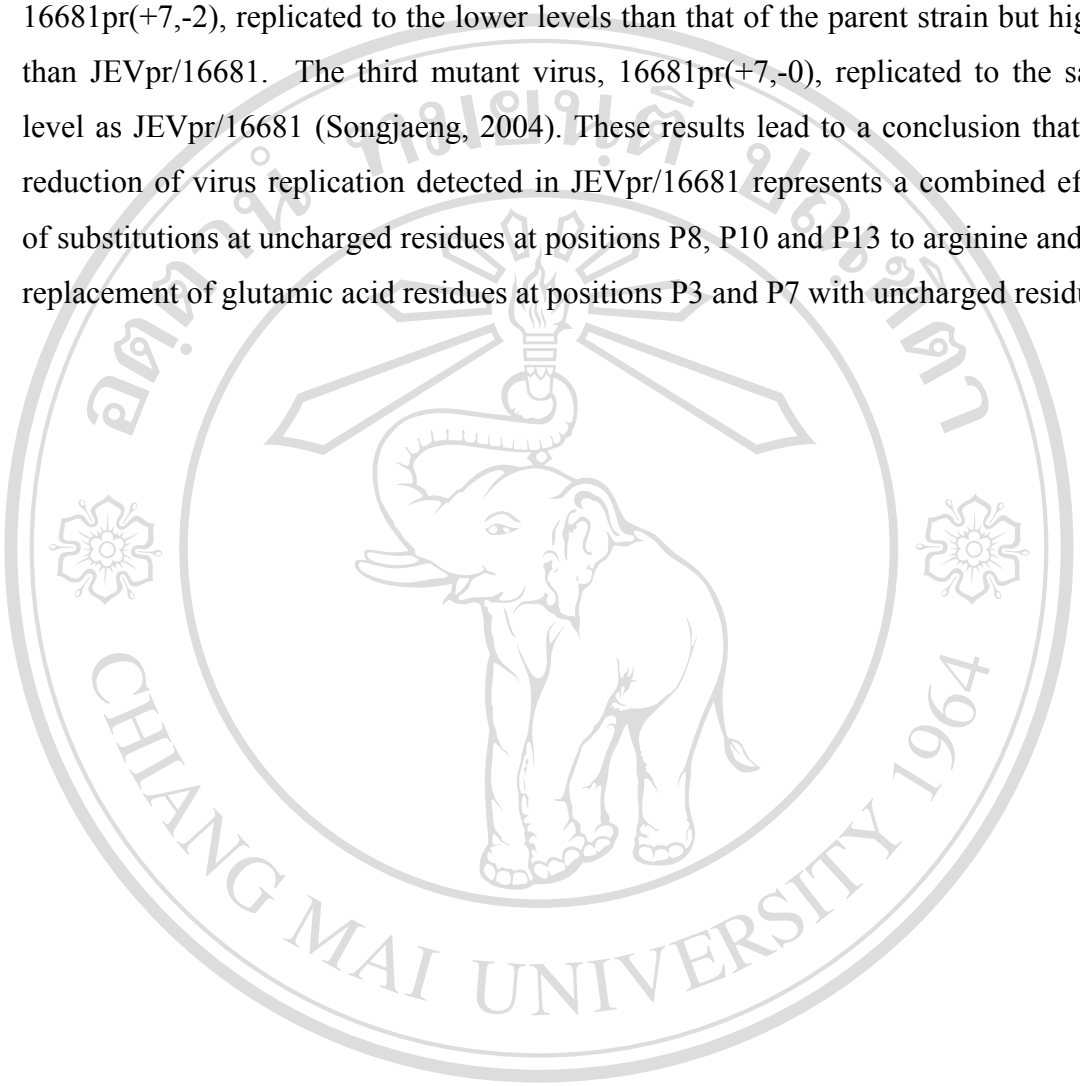

Table. 1 Comparison of 13- amino acid sequence acid sequences adjacent to pr-M cleavage site of flaviviruses (Keelapang et al., Songjeang, 2004).

Virus	Amino acid sequence at the pr-M junction					Accession no.
Dengue virus type 1	YGTCS	QTGEH	RRDKR ↓	SVALA	PHVGL	M87512
Dengue virus type 2	YGTCT	TMGEH	RREKR	SVALV	PHVGM	M20558
Dengue virus type 3	YGTCN	QAGEH	RRDKR	SVALA	PHVGM	M93130
Dengue virus type 4	YGTCT	QSGER	RREKR	SVALT	PHSGM	M1493
TBEV	YGRCG	KQEGS	-RTRR	SVLIP	SHAQG	V27495
Langat	YGRCG	KQEGS	–RTRR	SVLIP	SHAQG	P29837
Sofjin	YGRCG	KQEGS	–RTRR	SVLIP	SHAQG	X03870
KFDV	YGRCG	KPAGG	–RNRR	SVSIP	VHAHS	X74111
Louping ill	YGRCG	KQEGS	–RTRR	SVLIP	THAQG	M59376
Powassan	YGRCG	RQAGS	–RGKR	SVVIP	THAQK	L06436
Yellow fever	YGKCD	SAGRS	RRSRR	AIDLP	THENH	AF094612
JEV	YGRCT	RTRHS	KRSRR	SVSVQ	THGES	M55506
Kunjin	YGRCT	KTRHS	RRSRR	SLTVQ	THGES	D00246
MVEV	YGRCT	RARHS	KRSRR	SITVQ	THGES	AF161266
West Nile	YGRCT	KTRHS	RRSRR	SLTVQ	THGES	NC001563
SLEV	YGRCT	RMGHS	RRSRR	SISVQ	HHGDS	M12294
JEVpr16681	YGRCT	RTRHS	KRSRR	SVALV	PHVGM	
16681pr(+7,-2)	YGRCT	RMREH	RREKR	SVALV	PHVGM	
16681pr(+7,-0)	YGRCT	RMRHH	KRSRR	SVALV	PHVGM	
16681pr(+4,-0)	YGTCT	TMGHH	RRSKR	SVALV	PHVGM	
16681prE203A	YGTCT	TMGEH	RRAKR	SVALV	PHVGM	