CHAPTER II
LITERATURE REVIEWS

1. Nature of virus

The *Flaviviridae* family consisting of three genera, *Flavivirus*, *Pestivirus* and *Hepacivirus* includes important human pathogens such as hepatitis C virus (HCV), west nile virus (WNV), and yellow fever virus (YFV), dengue virus (DV) [7, 10]. Genus *Flavivirus* comprises over 70 viruses, many of which are arthropod borne viruses [6]. More than 50% of all known *flaviviruses* are associated with human diseases, including some of the most important human pathogens, such as DV, YFV, WNV, Japanese encephalitis virus (JEV) and tick-borne encephalitis viruses (TBEV). DV is classified into four serotypes: DV1, DV2, DV3, and DV4 (Figure 1) [11]. DV1–4 are transmitted from one human host to another by the major vector is the mosquitoes of the genus *Aedes* principally, with *Aedes aegypti* and *Aedes albopictus* (Figure 2) [11-14]. *A. aegypti* was firstly reported in Thailand in 1907 by Theobold [15].

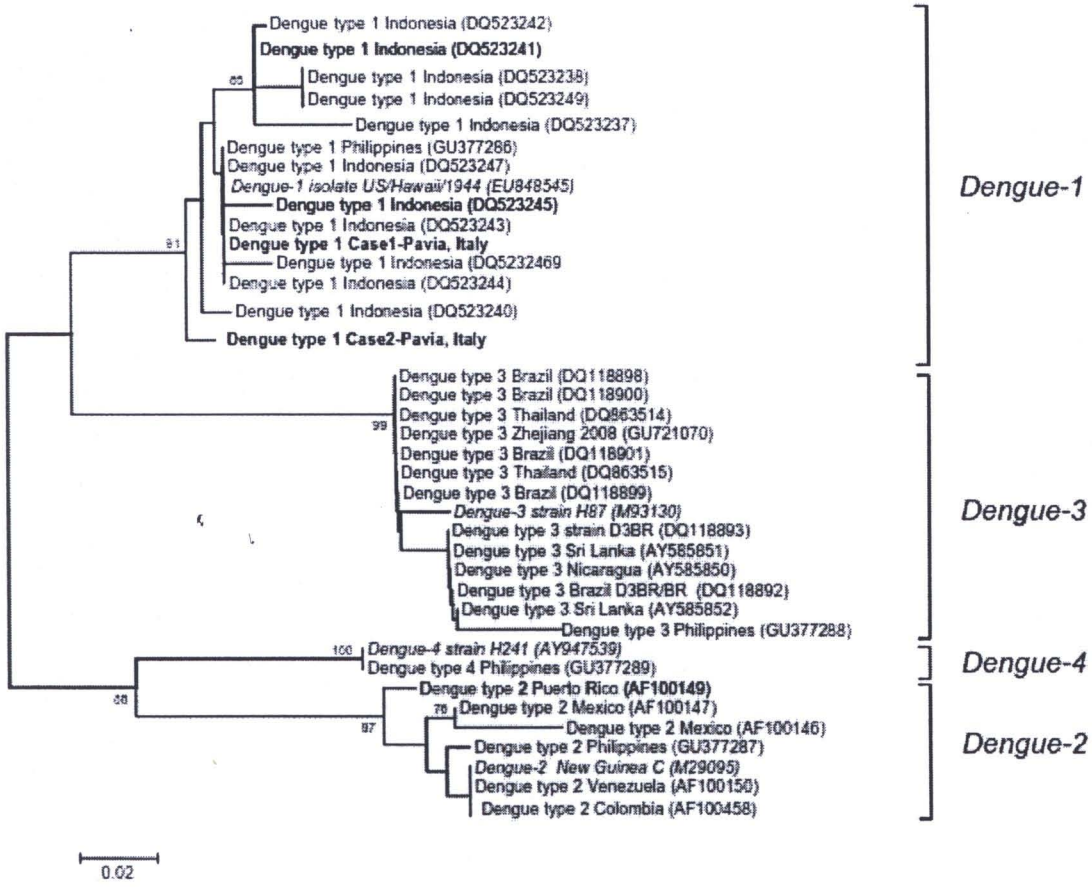

Figure 1 A neighbour-joining phylogenetic tree analysis of a fragment of 3'UTR region (nt 121) of DV. The cases identified in Pavia are reported in bold and the sequences of four prototype strains (DV1, US/Hawaii/1944; DV2, strain New Guinea C; DV3 strain H87; DV4 strain H241) are reported in italics [11].

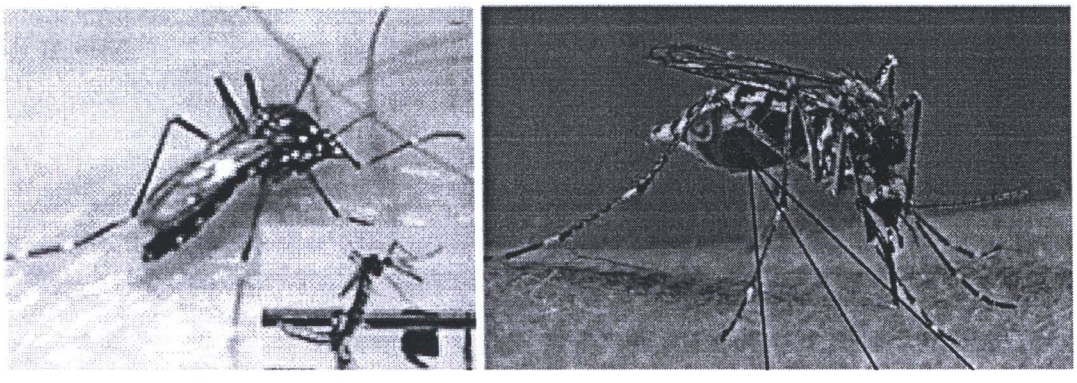

Figure 2 *Aedes aegypti* adult [15].

Structure and genome

Electron micrographs show that dengue virions are characterized by a relatively smooth surface and small enveloped virus, with a spherical shape about 40 -50 nm in diameter, and an electron-dense core surrounded by a lipid bilayer (Figure 3). There are three structural proteins that occur in stoichiometric amounts in the particle: core, membrane (M), and the envelope (E), present in mature virions [16]. The crystal structure of the E protein showed that it has three domains, the N-terminal structurally central domain I, the dimerization domain II, and the C-terminal, Ig-like domain III (Figure 5)[17].

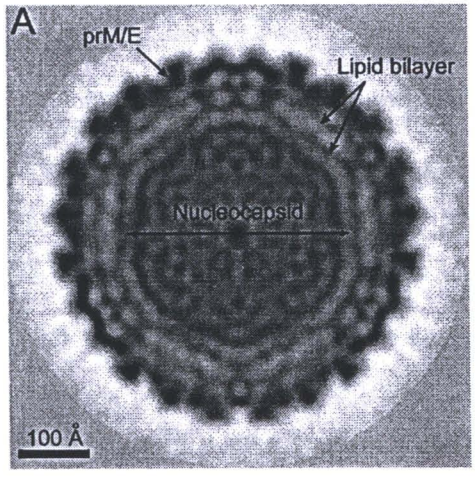

Figure 3 DV structure.

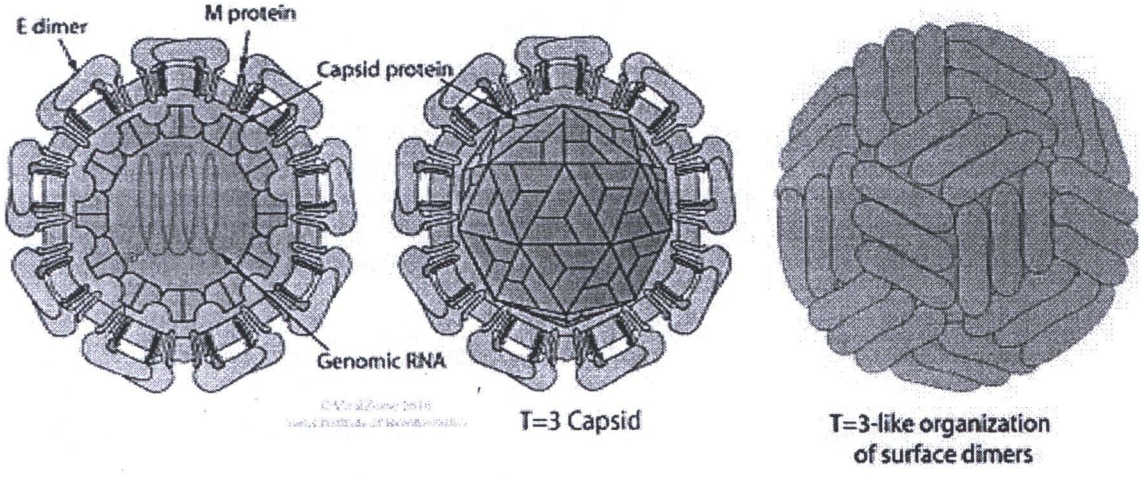

Figure 4 Drawing DV structure.

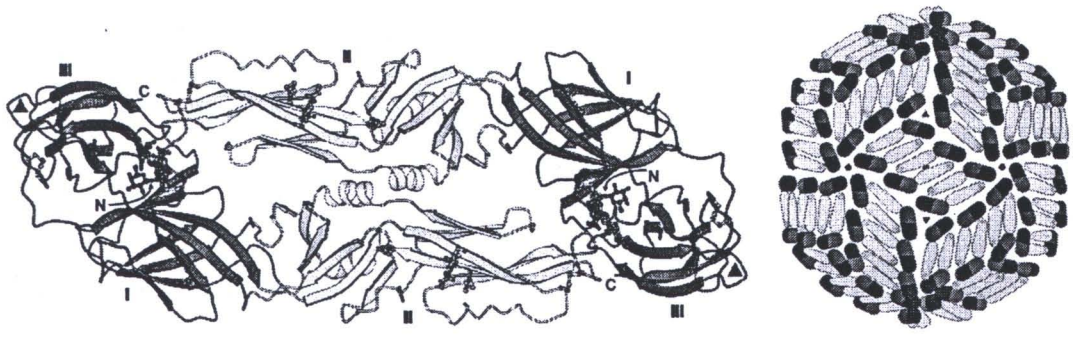

Figure 5 Structure of Dengue E protein. This is the conformation of E in the mature virus particle. Domain definition of dengue E. Domain I is red, domain II is yellow, and domain III is blue [17, 18].

The DV genome is a single stranded RNA molecule of positive polarity or messenger sense about 11,000 nucleotides in length that encode a single open reading frame encoding a polyprotein which is flanked by 5'and 3'untranslated regions (UTRs) that is translated in a cap-dependent manner at the rough endoplasmic reticulum (rER) [19]. The genomic RNA includes 5' (100 nt) and 3' (400–800 nt) UTRs (Figure 6). The length of the UTRs varies among different dengue serotypes (89–101 nt and 432–466 nt for 5'and 3'UTR, respectively) [1, 20]. The exact function of the UTRs has not been completely elucidated; however several studies have suggested that both the 5'and 3'UTRs of flaviviruses are important for virus replication, translation and virulence. The 3'UTR of DV consists of a variable region of approximately 100 nt proximal to the stop codon of the NS5 gene and a distal region of approximately 300 nt. The secondary structure of the dis-tal part of the 3'UTR of DV and other flaviviruses has been studied extensively and is shown to contain conserved structural elements among all flaviviruses, although some differences have been observed between mosquito and tick-borne flaviviruses. Those structures are believed to play an important role in the synthesis of minus strand RNA and consequently in virus replication. The 3'distal part of the UTR forms a long stable hairpin, which stabilizes viral genome and enhances initiation of translation. The proximal part of the 3'UTR shows a high degree of sequence variation, however, the structural elements of this region have not been extensively described [21]. The 3'-UTR includes several conserved sequences and structures that play important roles in

viral replication and translation: (i) a long (90–120 nt) nucleotide sequence that forms the stem-loop (3'-SL), which is the most common shared structural similarity shared among all flaviviruses [22, 23]. Although, the precise function of the 3'-SL is not known, it has been suggested that it may be involved in viral replication [24]. The topology of bulges in the long stem of flavivirus 3'-SL is a major determinant of RNA replication competence [25], regulation of translation and RNA synthesis [26], as well as in interactions with viral and cellular proteins of functional importance [27-29]; and (ii) the well-conserved motifs CS1, CS2, RCS2 (conserved sequence 1, 2 and repeated conserved sequence, respectively) and a few tandem sequence repeats (R) that are found among mosquito-borne flaviviruses [23, 30]. In tick-borne flaviviruses the PR, R3 and interestingly a polyadenylate sequence that is found in some tick-borne viruses, constitute the common conserved motifs of the 3 0-NCR [31]. Translation of the single ORF results in a single polyprotein that is cleaved by host and virus derived proteases to produce the three structural proteins (the capsid protein C (C, 100 amino acids), a small nonglycosylated M protein (M, 75 amino acids) and the E glycoprotein (E, 495 amino acids)) and seven non-structural (NS1-NS2ANS2B-NS3-NS4A-NS4B-NS5) proteins [32, 33]. Of these, only NS3 and NS5 have known enzymatic activities. The polyprotein precursor is processed in the rER where the action of the cellular signal peptidase results in three structural proteins, the components of the virion, the C protein, precursor membrane protein (prM), and the E protein. The remainder of the polyprotein codes for at least seven nonstructural proteins that are generated by the combination of the cellular signal peptidase and the virally encoded, two component serine protease, NS2B/NS3 [34, 35]. The 5' and 3'-SL, 3'termini of the DV genomes include untranslated sequences important for viral RNA replication and translation, and probably interact with cellular factors involved in these functions. The C protein, an 11 kDa homodimer protein with an unusually high net charge [36], is essential for RNA genome encapsidation [37]. The prM/M protein, a 27–31 kDa N-glycosylated protein cleaved in the trans golgi network by a cell-encoded furin-like protease during the late stages of virus assembly, to release mature virions [1, 38, 39].The E protein is a 53 kDa class II N-glycosylated dimeric membrane fusion protein that mediates virus binding and fusion to host cell membrane [18, 40, 41], as well as confers protective immune responses by eliciting

neutralizing, antifusion, and replicationenhancing antibodies [42, 43]. As mentioned above, each of the monomer subunits of the E protein is composed of three distinct domains: domain I, ab-barrel structure oriented parallel to the viral M; domain II, a finger-like structure composed of a pair of discontinuous loops one of which is highly conserved among all flaviviruses functioning as an internal fusion peptide and is stabilized by three disulfide bridges; and the C-terminal domain III, located in the outer lateral surface of the dimer. The flavivirus domain III is believed to interact with cellular receptors for entry [44-47] and has been suggested to contain residues that are responsible for the determination of host range, tropism and virulence among flaviviruses [41]. It also includes most important epitopes that bind to neutralizing antibodies. Mutations within domain III, such as N390D which is located within the putative glycosaminoglycan binding motif (386L411M) responsible for the binding of DV onto the host cell membrane via a non-Fc receptor [42], have been implicated as a potential virulence determinant in American.

The N-terminal domain of NS3, together with NS2B, contains a serine protease activity; the C-terminal domain functions as an RNA helicase, an RNA triphosphatase, and an NTPase [48-50]. The N-terminal domain of NS5 contains a methyltransferase activity; the C-terminal domain serves as an RNA-dependent RNA polymerase (RdRp) [51-54]. Other nonstructural proteins are required for RNA replication, among which NS2A, NS2B, NS4A, and NS4B are transmembrane proteins that form the scaffold for the viral replication complex [55-57]

DV2 genotype [58], and has been shown to alter virulence in mice [59]. Among the non-structural proteins, NS1 is a 46 kDa dimeric Nglycosylated glycosylphospha-tidylinositol anchored protein that exists in both intra- and extracellular forms [60, 61]. NS2A, is a 22 kDa protein involved in the coordination of change between RNA packaging and replication [62] and possibly antagonism of interferon (IFN) [63, 64]. Several recent analyses have indicated that the gene encoding for NS2A are under weak selection pressures, a process that influences virus evolution during natural transmission [65-67]. NS2B is a membrane-associated 14 kDaprotein which associates with NS3 to form the viral protease complex and serves as a cofactor in the structural activation of the DV serine protease of NS3 [68, 69]. NS3 is a 70 kDa multifunctional protein with trypsin-like serine protease, helicase, and RNA

triphosphatase enzyme activities [70, 71] and is involved in the processing of the viral polyprotein, as well as RNA replication. NS4A and NS4B are small hydrophobic proteins of 16 and 27 kDa, respectively, with the latter functioning as an IFN-signaling inhibitor [63, 64]. Recent evidence demonstrated a concentration of putative positive selection in NS4B of sylvatic DV [66], which suggests a possible role in distinguishing endemic and sylvatic DV genotypes. Lastly, NS5 is a large multifunctional, well-conserved protein of 103 kDa with RNA capprocessing (Encoded by amino acid residues 1–269), RNA dependent RNA polymerase (RdRp) (encoded by amino acid residues 270–900) [51, 52], interleukin-8 induction (IL-8) [72] and nuclear localization (encoded by amino acid residues 387–404) [73, 74] activities [7].

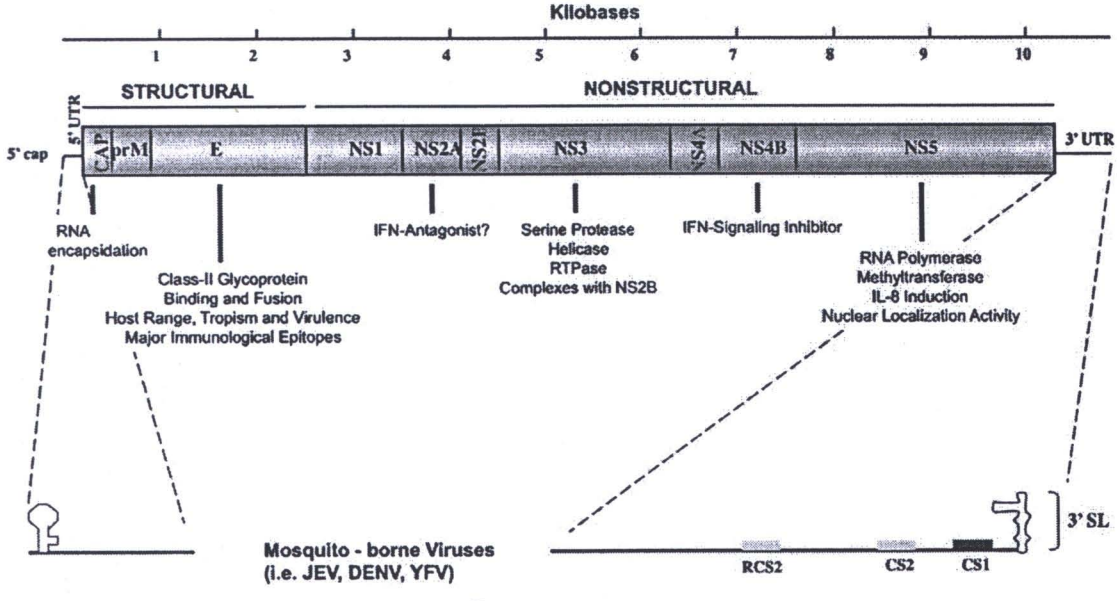

Figure 6 The DV genome and the major functions of the gene products. CS, conserved sequence; RCS, repeated conserved sequence; 3'-SL, 3'-SL; R, repeat [7].

Proteins and Properties

DV has three structural proteins as the C protein, prM protein, and the E proteins. Following the E protein is NS1 (NS for nonstructural). NS1 is a glycoprotein and has multiple functions that are only poorly understood. It is found as dimer and higher

multimer in three locations in mammalian cell: intracellular; anchored in the plasma membrane by GPI (glycosal-phosphotidylinositol) anchor; and as a soluble protein secreted from infected cell. It is required for RNA replication, presumably a function of the intracellular form of the protein. For this function, it interacts with NS4A. The cell surfaces-anchored form is capable of antibody-induced signal transduction that may play a role in cell activation. The function of the secreted form is unknown but it has been speculated that it has a role in counteracting immune responses to the virus.

Next in the polyprotein precursor are two hydrophobic polypeptides called NS2A and NS2B. These proteins are cleared by the viral NS2A-NS3 protease. They are associated with the membranes and may serve to anchor parts of the replication machinery to internal membranes in the cell. NS2A has multiple functions. It inhibits the production of INF-α /β by infected cell, the IFN are potent inhibitors of virus replication and most, perhaps all viruses encode products to block interferon action. NS2A also has a role in the production of infectious particle from the infected cell, since certain maturation in this protein block virus assembly but do not affect other aspects of the virus life cycle. These mutants can be suppressed by changes in the NS3 helicase domain, suggesting an interaction between NS2A and NS3. NS2B also interacts with NS3, but with the protease domain. It is a cofactor required for the NS3 protease activity and the central domain of NS2B forms a complex with NS3, which follow NS2B in poly protein precursor. The NS2B-NS3 serine protease cleaves many bond n the polyprotein. NS3 also has at least two other activities-the middle domain of NS3 is a helicase, required for RNA replication, and the C-termina domain has RNA triphoaphatase activity, an activity required for the capping of viral genome.

NS4A and NS4B are hydrophobic polypeptides that associate with membrane. They may function in assembly of the viral replicase on intercellular membrane. Both the viral NS2B-NS3 protease and cellular signalase are required to produce the final cleaved products.

NS5 is the viral RNA polymerase. It appears to be a soluble cytoplasmic protein that associates with the membrane through association with other viral peptides. It also has methyltranferase activity and thus is the capping enzyme that caps the viral genome. Thus, capping requires two flavivirus proteins, NS3 (RNA triphosphatase) and NS5 (capping enzyme). Note the similarities to alphaviruses where the RNA

triphosphatase activity is also on the helicase-protease protein (nsP2, the analogous of the flaviviral NS3), and the methyltransferase or capping activity is different protein (nsP1). However, in alphaviruses the capping enzyme and RNA polymerase (nsP4) are distinct proteins, whereas in flaviviruses they are present in the same polypeptide.

Table1 Properties of DV proteins [75]

Protein	Amino acid	Molecular weight (kDa)		Function(s)
		From calculate	From experiment	
Anchored capsid (C)	113	13.0	-	Precursor transmembrane of protein virion C
Virion capsid (C)	100	11.5	13.5	Constituent of nucleocapsid, to wrap genome of virus, some part is sent to nucleuse but don't know function
Premembrane (prM)	166	18.9	19 – 23	Glycoprotein on shell of particle in host cell (immature virion), protect E protein change in acid condition while transport particle from host cell
Membrane (M)	75	8.4	7 – 9	Cleavage product from cleavage prM protein, found on shell of particle virion out of host cell (mature virion)
Envelope (E)	495	54.4	55- 60	Glycoprotein on shell of virus, bind with receptor molecule on host cell; can change in acid condition that occur shell of virus fuse with membrane of cell, target of Ab that can decrease viral infection & protect infection in animal model

Table1 Properties of DV proteins (Cont.)

Protein	Amino acid	Molecular weight (kDa)		Function(s)
		From calculate	From experiment	
Nonstructural protein 1 (NS1)	352	39.9 86 (two molecules)	46	Glycoprotein that found in cell, membrane & out of cell, anticipate have role in increase genome especially in initiate of infection but unknown mechanism, stimulate immune system to protect infection of virus in animal model
Nonstructural protein 2A (NS2A)	218	23.7	22	Integral membrane protein found in same area with replication complex but unknown role, inhibit IFN-signaling
Nonstructural protein 2B (NS2B)	130	14.0	14	Membrane protein, it is cofactor with NS3 that function is protease enzyme
Nonstructural protein 3 (NS3)	618	69.4	69	Many function: serine protease, RNA helicase, RNA-stimulated nucleoside 5'-triphosphatase & RNA 5'-triphosphatase
Nonstructural protein 4A (NS4A)	150	16.4	16	Integral membrane protein found in same area with replication complex but unknown role, inhibit IFN-signaling
Nonstructural protein 4B (NS4B)	248	26.8	27	Integral membrane protein that have important role to inhibit expression protein stimulate through IFN-signaling, found spread in cytoplasm& nucleus
Nonstructural protein 5 (NS5)	900	103.2	104	Many function: 2'-O-methyltranferase & RNA-dependent RNA polymerase; found in cytoplasm; some molecule is hyperphosphorylated form that will transport into nucleus but unknown function

2. Target cell

DV is transmitted between humans via mosquito vectors. Human cells are the natural targets in which DV reside and produce virus particles, in addition to monocytes and macrophages, B and T lymphocytes, Langerhans cell, $CD14^{+}$dendritic cell precursor, dendritic cells (DCs), resident in skin are the initial target cells of DNV infection [76], hepatocytes, endothelial cells, epithelial cells and fibroblasts have also been reported as potential hosts for DV [16].

DV can infect in cell line in laboratory including neuroblastoma cell line, diversified mononuclear cell line and hepatoma cell line from human [77-79] and from animal such as an *Aedes albopictus* C6/36 cell (mosquito cell) and vero cell which was isolated from African green monkey kidney epithelial cells, D.Mel-2 cells, which are derived from S2 cells, and used for propagation of DV in laboratory [9].

DV enters these cells via receptor-mediated endocytosis. However, the primary receptors of DV are still unknown. In the body of human expect principal target of DV infection has been presumed to be blood monocytes and tissue macrophages. However, myeloid DCs residing in the epidermis (Langerhans cells) and dermis are the predominant cells of the innate immune system that DV encounters following the bite of an infected mosquito. The studies show that DV can infect and replicate in immature DCs better than differentiated CDs, monocytes and macrophages [76, 80-82].

From patients with DHF/DSS, viral antigens were detected in many tissues, including liver, spleen, lymphnode, thymus, kidney, lung, and skin, but mainly in mononuclear phagocytic cells. The more presence of viral antigens within cells, especially phagocytic cells, does not necessarily mean that the cells in question support viral replication, since antigens may represent phagocytized, killed virus or sequestered immune complexes in the process of being degraded. Evidence from in vitro studies suggested that other cells (e.g., hepatocytes, B and T lymphocytes, endothelial cells, and fibroblasts) could be potential targets for virus infection and replication, but relatively little is known about the involvement of these cells in in vivo infections. Anticipate endothelial cell, hepatocyte and megakaryocyte may have relevant with pathogenesis of DF and replication of viral but unknown mechanism [83].

3. Replication of DV

The DV consists of an outer glycoprotein shell and an internal host derived lipid bilayer that surrounds the capsid and viral RNA. DV particles bind to cells via interactions between the surface viral glycoprotein and one or several poorly defined cellular receptors (Figure 7A). A specific receptor for internalization of these viruses into host cells has not yet been identified. Several cellular molecules capable of mediating virus attachment are known, but none has been conclusively shown to function as virus receptors. In addition, viral particles may enter cells via Fc-receptor upon opsonization. During virus entry, E proteins forming the glycoprotein shell bind to cell surface receptors that assist in internalizing the virus through clathrin-mediated endocytosis. Virions are internalized by receptor-mediated endocytosis, the low pH of the endosome triggers structural rearrangements in the viral glycoproteins that drive fusion of the viral and endocytic membranes resulting in release the viral RNA into the cytoplasm (Figure 7B). Like other positive-strand RNA viruses, DV translate and replicate in the cytoplasm of susceptible cells. After infection, viral proteins induce re-arrangements of intracellular membranes forming distinct structures that have been designated vesicle packets and convoluted membranes. It appears that vesicle packets are sites of RNA replication that is probably catalyzed by a multi-protein complex composed of viral proteins, cellular membranes and presumably also cellular proteins. This RNA (~11 kb) is directly translated as a single polyprotein that is processed by viral and cellular proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A/B, NS3, NS4A/B and NS5). The non-structural proteins actively replicate the viral RNA in replication complexes associated with cellular membrane (Figure 7C). DV RNA replication occurs in close association with cellular membranes that may serve as a scaffold for the viral replication complexes (RC) (Figure 7D). Viral protein and RNA synthesis occur predominantly in the cytoplasm of host cells. Replication is slow and begins within 15 hrs after infection. DV RNA is replicated via a negative strand intermediate that serves as a template for the production of excess amounts of positive strand progeny. Newly synthesized RNA and C protein are enveloped by glycoproteins prM and E to assemble immature virus particles that bud into the ER (Figure 7E). These immature particles are transported through the secretory pathway to the Golgi apparatus. In the low pH environment of

the Trans-Golgi, furin-mediated cleavage of prM to M drives maturation of the virus (Figure 7F). Maturation is also accompanied by significant structural rearrangements of the glycoprotein shell and then mature virus is released into the cytoplasm (Figure 7G). Following maturation, virus particles migrate along the surface of naive cells until they encounter clathrin-coated pits that assist in virus entry. Because of differences in the cellular environments and the non-lytic nature of the infections, the entry, replication and assembly of these viruses may differ in mosquito cells versus vertebrate cells [84]. Low amounts of DV are released into the supernatant fluid [8].

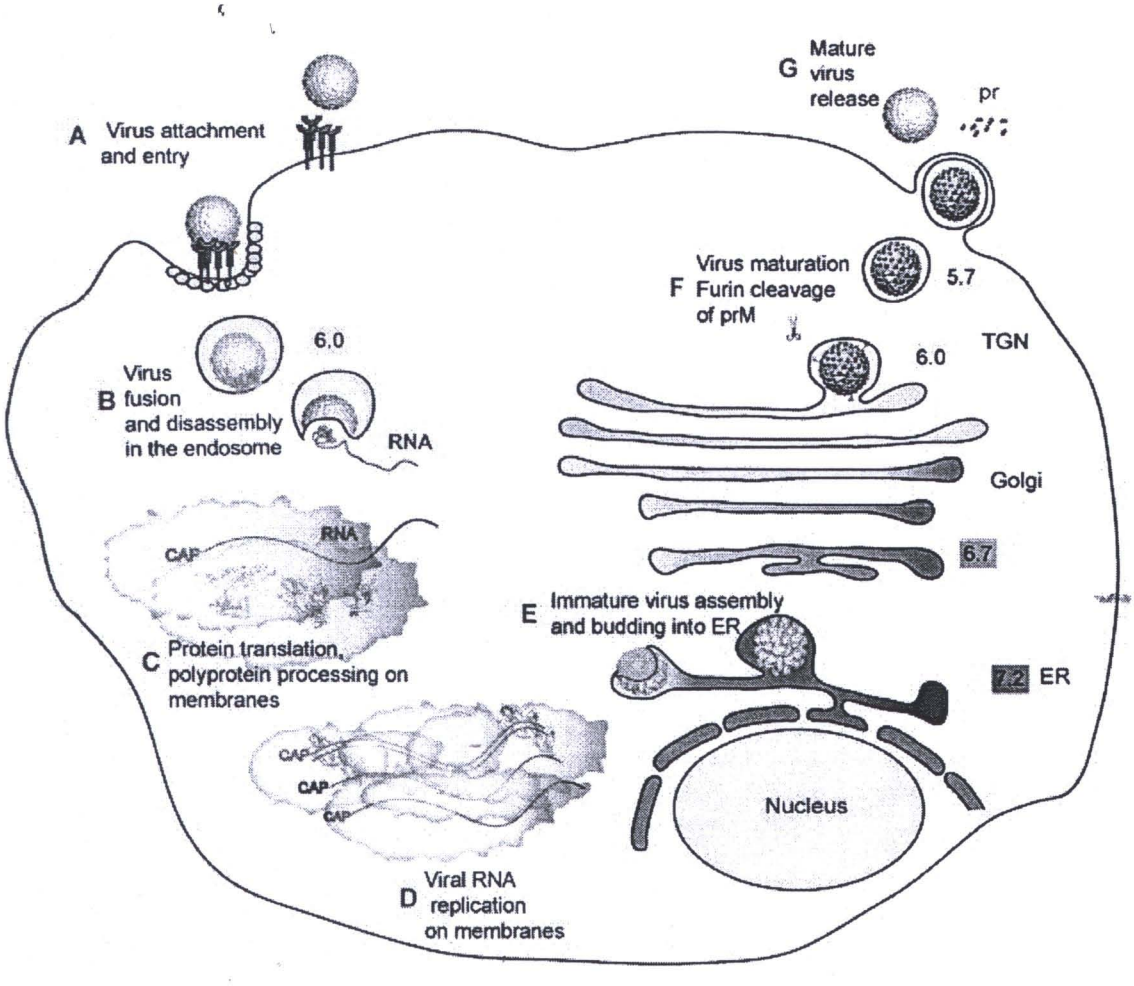

Figure 7 The flavivirus life cycle. (A) Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis. (B) In the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of RNA into the cytoplasm. (C) Viral RNA is translated into a polyprotein that is

processed by viral and cellular proteases. (D) Viral non-structural proteins replicate the genome RNA. (E) Virus assembly occurs at the ER membrane, where C protein and viral RNA are enveloped by the ER membrane and glycoproteins to form immature virus particles. (F) Immature virus particles are transported through the secretory pathway. In the low pH of the Trans-Golgi network (TGN) furin-mediated cleavage of prM drives maturation of the virus. (G) Mature virus is released into the cytoplasm. Numbers shown in colored boxes refer to the pH of the respective compartments [84].

Attachment molecules

In the initial event of flavivirus infection has not been described in detail and little is known. A multitude of cellular surface components that act as receptors for DV have been identified. Virus receptors are defined as cellular (surface) proteins which, in addition to their normal physiological role, specifically bind viruses resulting in attachment of virus particles to the cell. Thus, they mediate a physical interaction between extracellular virions and target cell [84]. Receptor of DV on cell surface can classifies in to 4 types.

Heparan sulfate is the most ubiquitous member of molecules of the glycosaminoglycan family and used by more than 17 of viruses to bind to target cells and lipopolysaccharide binding CD14-associated molecules have been implicated in the DV-attachment process to various non-human and human cell types.[85, 86]. It could act directly as a receptor or help to concentrate these viruses on the cell surface to facilitate the interaction with specific high-affinity receptors. In summary, for infection in cell culture, viruses make initial contact with their target by interaction of virion E glycoprotein with cell surface heparan sulfate proteoglycans and then interaction with other receptors ensues which is essential for entry [87].

Heat shock proteins 90 and Heat shock proteins 70 have an important role as receptor of DV on monocytes and machophages [88]. These proteins are derived from tumors or viral infected cells can stimulate antigen specific $CD8^{+}$T cell responses in vitro and in vivo [89]. DV bind with this receptor may interrupt antigen presentation with helpful DV in monocytes.

The C-type lectin, dendritic cell-specific dendritic cell–specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) ICAM3 grabbing nonintegrin or DC-SIGN has been shown to be essential for DV infections through its interaction with carbohydrate moieties on the E protein. Depending on the virus from which it is derived, one or two N-linked glycosylation sites are found on the E protein. Asn153 is conserved among all flaviviruses while Asn67 is unique to DV. N-linked glycosylation of Asn67 is required for DV growth in mammalian cells. Another C-type lectin receptor, the mannose receptor (MR), has recently been shown to bind DV, JEV and TBEV through a mechanism similar to that of DC-SIGN. However, the ligand specificity of MR (terminal mannose, fucose and *N*-acetyl glucosamine) differs from that of DC-SIGN (high-mannose oligosaccharides and fucosylated glycans). The authors propose that MR has the potential for being a DV receptor (rather than just an attachment molecule), because it is constitutively internalized and found mainly in the endocytic pathway, in contrast to DC-SIGN, which is mainly localized in the plasma membrane [90]. High-Affinity Laminin Receptor as a DV1 receptor that is a specific entry of DV into liver cells [91].

Entry

The initial steps of DV entry has been divided into attachment/adsorption and penetration step. The attachment is a temperature-independent process that occurs at both 4°C and 37°C, whereas viral penetration proceeds only at 37°C indicated that in step of penetration require energy metabolism from cell [92]. DV can penetrate by membrane fusion directly in mosquito C6/36 cells [93] or by receptor-mediated endocytosis pathway in monocytes and Kuffer cell then fusion membrane with membrane of endosome will occurs [92, 94].

The fusion between envelopes of DV with membrane of endosome via low-pH in endosome stimulates E protein conformation change. Exposure to the acidic pH of the endosomes triggers conversion E homodimers a major conformational change of the virion's surface, involving dissociation of the native protein complexes (which destroys the surface icosahedral lattice), and the formation of homotrimers of the fusion proteins . The energy released during the transition from the metastable, which is the ability of a non-equilibrium state to persist for some period of time. Dimers at

the viral surface to the very stable target-membrane-inserted homotrimers are used to drive the merging of the viral and cellular membranes [18, 95].

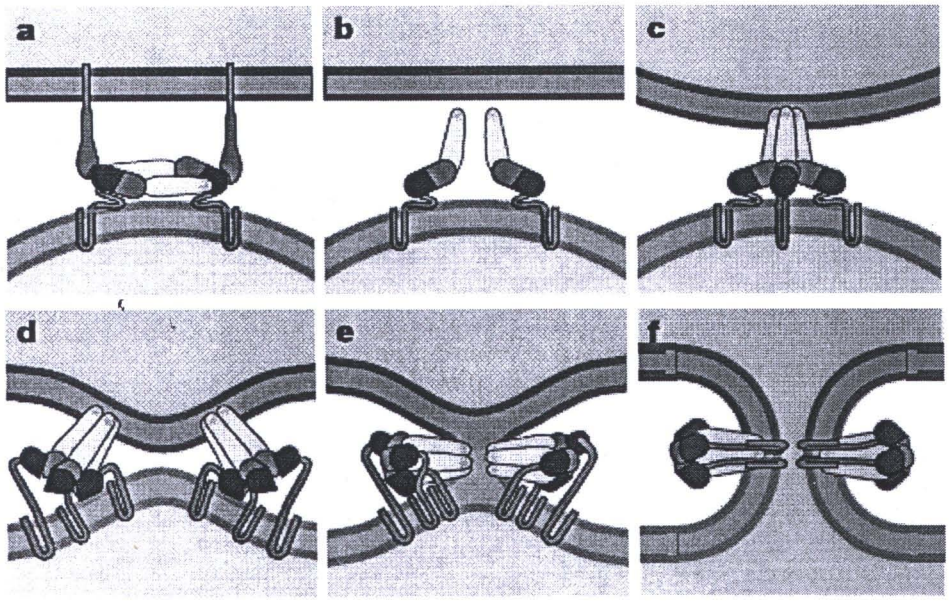

Figure 8 Proposed mechanism for fusion mediated. Full length E is represented as in this figure, with the stem and viral transmembrane anchor in green part (host membrane). **a.** E binds to a receptor on the cell surface and the virion is internalized to an endosome. **b.** Reduced pH in the endosome causes domain II to hinge outward from the virion surface, exposing the fusion loop, and allowing E monomers to rearrange laterally in the plane of the viral M. **c.** The fusion loop inserts into the hydrocarbon layer of the host-cell membrane, promoting trimer formation. **d.** Formation of trimer contacts spreads from the fusion loop at the tip of the trimer, to the base of the trimer. Domain III shifts and rotates to create trimer contacts, causing the C-terminal portion of E to fold back towards the fusion loop. Energy release by this refolding bends the apposed membranes. **e.** Creation of additional trimer contacts between the stem-anchor and domain II leads first to hemifusion and then **f.** to formation of a lipidic fusion pore [18].

Fusion proteins that role in fusion E protein of virus with membrane of endosome in DV and Alphavirus are in group of class II fusion proteins. These 'class II'

membrane fusion proteins II are folded mostly as β-sheets, with the internal fusion peptide (referred to here as the 'fusion loop' or 'cd loop') in a loop between two β-strands. During virus entry, the acidic environment of the endosome triggers an irreversible conformational change in E1, from an E1/E2 heterodimer at the viral surface to a target-membrane-inserted E1 homotrimer. This rearrangement of E1 leads to fusion of the viral and endosomal membranes, releasing the virus genomic RNA into the cytoplasm of the cell E of flaviviruses and alphaviruses are similarities [96].

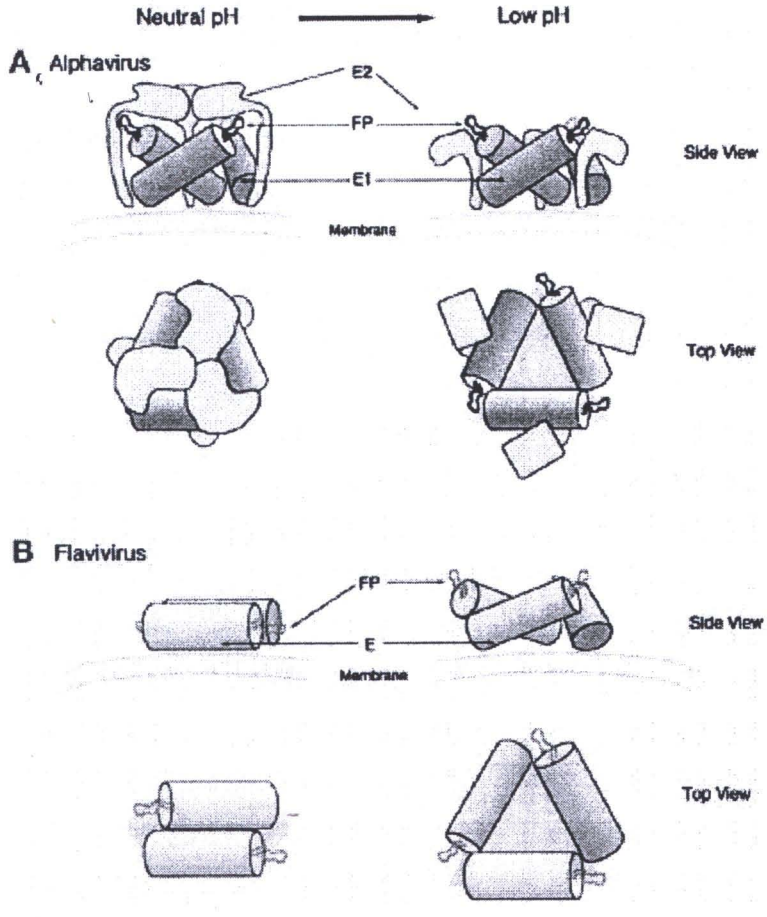

Figure 9 Configuration of glycoproteins of alphaviruses and flaviviruses on the surface of virions at neutral pH and the proposed configuration at acid pH. (A) In the alphaviruses, E1 glycoproteins are shown as green cylinders, E2 glycoproteins as tan shapes, and the fusion peptide (FP) as a black curved line. (B) In flaviviruses, E glycoproteins are shown as yellow cylinders with the fusion peptide as a green curve. In both (A) and (B), the membrane is shown in gray [18].

The discovery mechanism of entry step, in step prefusion structure of dengue E protein has suggested one possible strategy for inhibiting flavivirus entry: by interfering with the fusion transition, inhibited releasing viral RNA to host cell, reduce amount of viral replication [18].

Expression of the viral genome

The genome organization of a typical flavivirus is illustrated in the Figure 10. As for all plus-strand RNA viruses, the genomic RNA is a messenger and in the case of flavivirus serves as the messenger for all of the virus encoded protein. The RNA is capped but lack 3' poly (A). There is a stem loop structure at the 3' end which serves the same function as poly (A) in the other messengers. This structure increases the efficiency of translation of the RNA by about 10-fold. Viral proteins are not required for this effect and therefore cellular protein must interact with the structure in order to increases efficiency of translation. It is known that during translation of mRNAs that are capped and polyadenylated, there is an initiation complex formed that contains both cap-binding protein and poly (A)-binding protein. Thus the complex interacts with both ends of the mRNA to initiate translation. It is assumed that a cellular protein binds the 3'-SL of flavivirus and interacs with the initiation complex so as to perform the same function as the poly A-binding protein. Formation of this complex in the case of flavivirus RNAs could be enhanced by cyclization of the viral RNA described later, although the primary function of cyclization appears to be in replication of the viral RNA.

Viral protein processing

The processing of the long polyprotein produce from the genome is complicate as an example of complex processing events that can occur in viral polyproteins associated with lipid bilayers. The nucleocapsid protein is 5' terminal in the genome and is removed from the precursor polyprotein by the viral NS2B-NS3 protease. Two envelope proteins, prM and E protein are anchored in the rER by C-terminal membrane-spanning domains and glycoprotein. A series of internal signal sequences is responsible for the multiple insertion events required to inserted prM, E and NS1,

into the rER. After separation of these three proteins by signalase, prM and E from a heterodimer. prM is cleaved to M by furin during transport of the heterodimer or during virus assembly.

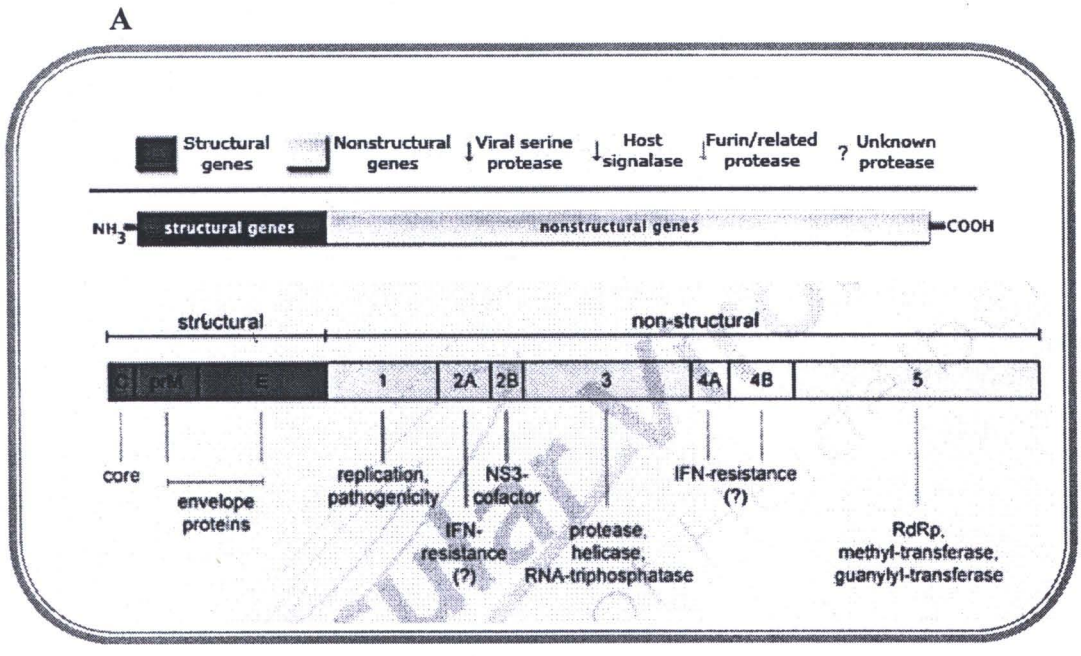

Figure 10 A Structure of DV genome: DV genome structure, polyprotein processing, and cleavage products. The top row is a representation of the initial polyprotein translation product. The middle and bottom rows show the peptide precursors and mature protein products generated by the proteolytic processing cascade. Structural proteins are dark red and nonstructural proteins are white. The arrows indicate protease cleavage sites and are colored according to the particular protease responsible for cleavage (as indicated in the legend).

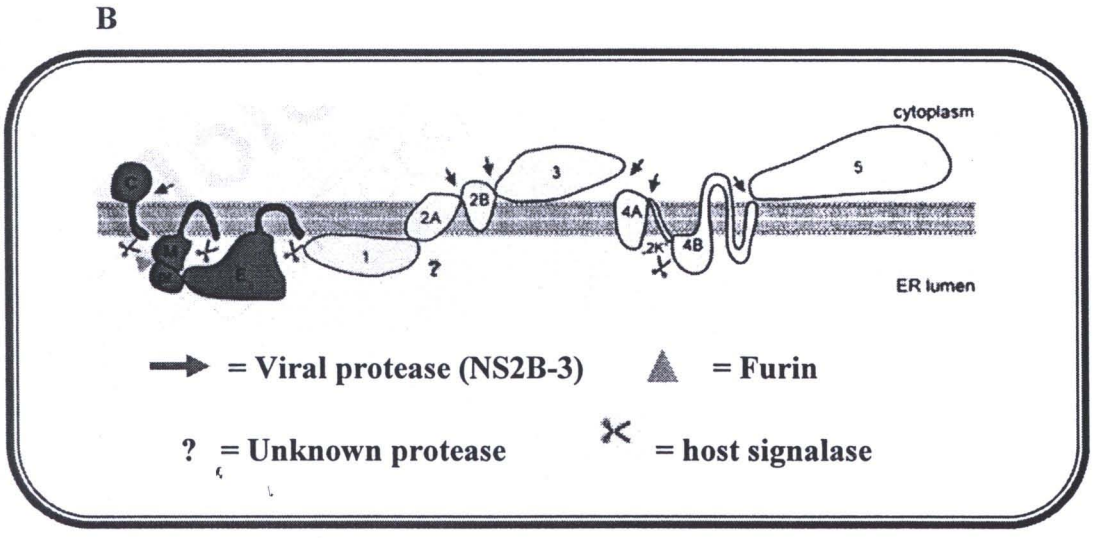

Figure 10 B Putative membrane topology of DV proteins and proteinases involved in polyprotein cleavage.

Replication of the viral RNA

RNA replication is associated with the nuclear membrane. The composition of the replicase complex is not understood but is assumed to consist of many of the viral nonstructural proteins with associated cellular protein. Cyclization of the RNA is required for replication. Sequence for the 3' and 5', regions of DV RNA that form a number of stem-loop structure and that also cyclize the RNA. Studies data have shown that the RNA sequence in the C protein downstream of the AUG start codon is involved in cyclization (region mark CS1). Sequences upstream of the start codon are known to be required for cyclization, and the two structures shown different ways that these might be used for cyclization. This figure also illustrates the long stem-loop structure at the 3' end of the RNA, discussed earlier. A stem-loop structure in the 5'region just upstream of the CS1 region has also been shown to be important in translation of the RNA, in this case for recognition of the AUG start codon, which is found in a poor context for a strat codon.

The sequences surrounding CS1 are illustrated for a number of mosquito-borne flavivirus in figure 11. This eight nucleotide sequence is invariant among the mosquito-borne flaviviruses, and studies have shown that sequence is important for

cyclization and replication of the RNA. The 3' sequences complementary to this region are found in the 3' nontranslated region. Changes in these sequences that eliminate cyclization prevent the RNA from replicating, even in model systems in which translation of the RNA is not required for expression of the replicase. Compensating mutations in the partner sequence that restore cyclization restore the ability of the RNA replicate. Thus, cyclization is required for RNA replication.

The identities of the promoters recognized by the RNA replication machinery are as yet unknown, but the requirement for cyclization suggests that sequences at both ends of the RNA are required. The conservation of the 8-nucleotide core sequence suggests that these sequences might be part of the promoter recognized by RNA replicase.

	5'		CS1		3'
YF	147	CCC UGG GCG	UCA AUA UG	GUA CGA CGAG	173
MVE	126	CCC CGG GUCG	UCA AUA UG	CUAAAA CGCG	153
JE	126	AA CC GGGCUA	UCA AUA UG	CUGAAACGCG	153
WN	127	AA CC GGGC UG	UCA AUA UG	CUAAAACGCG	154
SLE	129	AA CC GGGUUG	UCA AUA UG	CUAAAACGCG	156
DV4	127	GA CC AC CUU	UCA AUA UG	CUG AAA CGCG	153
DV2	125	AC AC GC CUU	UCA AUA UG	CUG AAA CGCG	152

Figure 11 Conserved nucleotide sequence element in 5' region encoding the C protein in six different mosquito-borne flavivirus. The number of the first and last nucleotides shown is given in parentheses. The boxed nucleotides in the red are those postulated to be important for cyclization of RNA.

Formation of the virus

DV matures at intracellular membranes. It grows in the culture cell and binding of preassembled nucleocapsids at the plasma membrane is readily seen.

The processing of the structural protein from the precursor polyprotein was described earlier. prM and E form heterodimer shortly after synthesis. A heterodimer

is the first formed, between prM and E. Immature virus particles can be isolated that have uncleaved prM whose infectivity is very low. DV have a triangulation number of 3 and therefore 60 trimeric spike each consisting of 3 heterodimers of prM and E. After cleavage of prM, the M-E heterodimer dissociates and there is a dramatic rearrangement whereby 90 E-E homodimers are formed and the particle shrinks from 60 nm in diameter to 50 nm. Upon infection of a cell and exposure of the mature DV to acidic pH there is another dramatic rearrangement and 60 E-E-E homotrimers are formed that tilt up so that the fusion peptide at the extremity of domain 2 is inserted into the cellular target membrane and fusion results. The DV nucleocapsid is thought to be icosahedral in symmetry, perhaps having a triangulation number of 3. There appears to be no interaction between the E proteins and the C proteins in the DV.

4. Prevalence of Dengue infection

The pathogenesis of severe dengue results from a complex interaction between the virus, the host, and, at least in part, immune-mediated mechanisms [97]. Dengue is one of the most important reemerging viral diseases, promulgated by increase in human population size and the expansion of global travel networks coupled with the possibility that global climatic changes alter the distribution of the mosquito vector [98]. Although most cases of DV infection are subclinical or cause a febrile illness that is rarely fatal (DF), increasingly large numbers of patients experience the more severe form of the illness, DHF, associated with plasma leakage and hemorrhage. With a tendency to progress into fatal shock (DSS), case fatality rates of DHF/DSS vary from 1 to 5%. Epidemiological features of DV infections (DF and DHF) are a serious cause of morbidity and mortality in most tropical and subtropical areas of the world: mainly Southeast and South Asia, Central and South America, and the Caribbean (Figure 12). The WHO estimates that more than 2.5 billion people are at risk of dengue infection [99]. Nearly 100 countries and areas have a risk for domestic DV infections. Dengue cases are estimated to occur in up to 100 million populations annually. A total of approximately 500,000 cases of DHF occur annually, and the case fatality ratio is 1–5%. Thus, dengue presents one of the most serious human infectious diseases. The areas where DV infection is a serious public health problem have been expanding, and the number of DHF cases has been recently increasing.

The incidence of DF is estimated to have increased 30-fold over the last 40 years and DV now infect an estimated 50-100 million humans each year [20, 67].

The average number of DF/DHF cases reported to WHO per year has risen from 908 between 1950 and 1959 to 514,139 between 1990 and 1999. The real figure is estimated to be closer to 50 million cases a year causing 24,000 deaths. Of an estimated 500,000 cases of DHF/ DSS requiring hospitalization each year, roughly 5% die according to WHO statistics. Regional distribution of dengue and its serotypes are described elsewhere. In summary, DF/DHF/DSS is an immediate problem in South and Southeast Asia and Central and South America. Although DF is present in the African region, there are no cases or outbreaks reported to WHO [100].

Half the world's populations live in countries endemic for dengue, underscoring the urgency to find solutions for dengue control. The consequence of simple DF is loss of workdays for communities dependent on wage labour. The consequence of severe illness is high mortality rates, since tertiary level care required for DHF/DSS management is beyond the reach of most of the persons at risk [100].

DF is typically acknowledged to be a childhood disease and is an important cause of paediatric hospitalization in Southeast Asia. There is, however, evidence of increasing incidence of DHF among older age groups. In the past, DF can cause infectious in 2-10 years age. Several studies in both Latin America and Southeast Asia have reported a higher association of DHF with older ages. Three studies in Asia using surveillance data report increasing age of infected patients. In Singapore, surveillance data showed a shift in peak dengue mortality from paediatric ages (1973–1977) to adults in 1982, since which year more than 50% of the deaths occurred in patients older than 15 years. From 1990–96, the highest age-specific morbidity rates were in the 15 to 34 year age groups [99].

Racial predisposition

Race-related susceptibility to dengue has been observed in a few studies and merits further investigation. In a retrospective sero epidemiologic study, Guzmán reported that blacks and whites were equally infected with DV1 and DV2 viruses during the Cuban epidemics of 1977 and 1981, while severe dengue disease was observed less frequently in dengue-infected black persons than whites. Genetic

polymorphism in cytokine profiles and coagulation proteins has been proposed as a factor protecting persons of African origin [99]. In Asia, two studies report racial differences in disease incidence. A 15-year study of the epidemiology of dengue reports a significantly higher incidence of DHF among Chinese compared to Malaysian males. This finding was supported by a six-year surveillance data study in Singapore, which found the race-specific morbidity rate among the Chinese to be three times that of the Malays and 1.7 times that of Indians [99].

Sex differences

Understanding male-female differences in infection rates and severity of disease is important for public health control programs. The studies from epidemics in India and Singapore found nearly twice the number of male patients compared to females, both report male higher than female ratios. However, deaths in this sample, two were female. The authors suggested that either immune response in females are more competent than in males, resulting in greater production of cytokines, or the capillary bed of females is prone to increased permeability. Kaplan in Mexico suggests that an incidence bias in favour of females is related to the timing of the survey interviews, while Goh puts forward that low incidence among women occurs because they stay at home and are less exposed to infection [99].

Rural spread

Historically, DF/DHF has been reported as occurring predominantly among urban populations where density of dwellings and short flying distance of the vector create the right conditions for transmission. However, the literature shows that dengue transmission and, in some cases, outbreaks occur in rural settings in both Asia and Latin America.

In the WHO Western Pacific region, WHO has confirmed that disease spread into rural areas from where it had not been reported previously. Rural epidemics occurred as in Indonesia, and outbreak in Laos began in a remote rural district of Nasaithong. Today, Thailand has an incidence rate that is higher in rural (102.2 per 100,000) than urban areas (95.4 per 100,000). Similarly, in India, entomological investigation showed a widespread distribution of *Aedes aegypti*, both in rural and urban areas

during an outbreak in Gujarat in 1988 and 1989. Increase in DF/DHF among rural populations is also observed in Central and South America and identical rates in both populations are reported. Among jungle dwellers in Peru, antibody prevalence up to 67% compared to 66% among the urban population has been found. Increased transport contact, mobility and spread of periurbanisation have been the most frequently cited reasons for spread of dengue to rural areas. While some rural incidence linked to travel contact with urban areas is conceivable, outbreaks and infection rates equal to those in urban areas warrant further investigation [99].

Seasonality and climate variability

The incidence and, in particular, epidemics of dengue have been commonly associated with the rainy season, and the El Niño phenomenon has been associated in the increases of certain vector-borne diseases, including dengue. Despite the relationship between temperature, rainfall, and other condition suitable for increase in number of mosquito have been affect vector-borne disease are increasingly [99]. The prevalence of dengue has been connected to societal changes such as population growth, urbanization, and international travel as well as environmental changes [101].

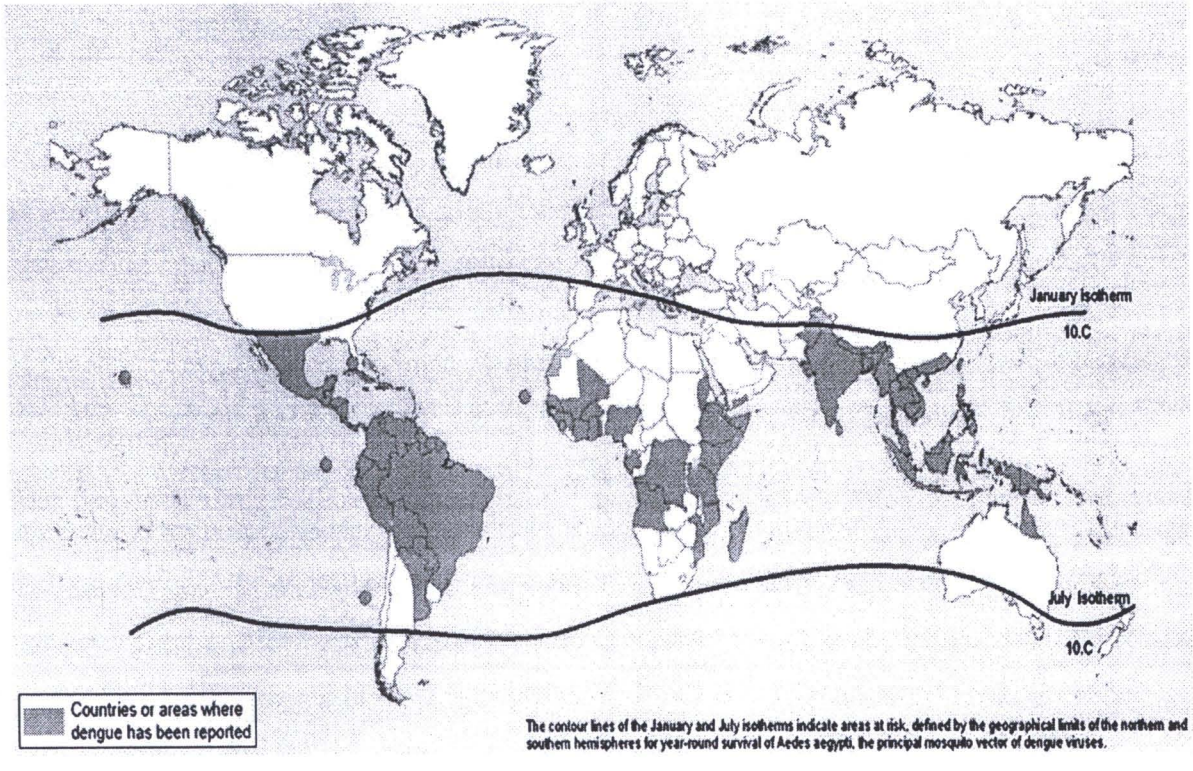

Figure 12 Geographic distribution of Dengue and areas at risk (WHO, 2010) [102].

5. Pathogenesis of DHF

Infection with a DV may be asymptomatic or it can cause a nonspecific viral syndrome, DF or DHF. DF is almost always a self-limited but debilitating illness, generally defined by fever with two or more of headache, retro-orbital pain, myalgia, arthralgia, rash, leucopenia and haemorrhagic manifestations (such as petechiae). DHF is defined by fever, haemorrhagic manifestations, thrombocytopenia and plasma leakage (pleural effusion, ascites, haemoconcentration or hypoproteinaemia) [103].

Major manifestations of DHF include (i) plasma leakage through elevated vascular permeability, (ii) hemorrhage, and (iii) thrombocytopenia. Some of the patients infected with DV develop DHF, while most with symptomatic infections end up as DF. The pathogenesis of DHF has been explained by two theories. One theory is based on the virulence of infecting DV; virulent DV strains cause DHF, while avirulent DV strains cause DF. The other is based on immunopathogenesis. This theory suggests that DHF is mediated by host immune responses including DV-cross-reactive antibodies that augment infections. These two theories have been considered as opposing each other for a long period of time. However, it appears that they represent different aspects of the pathogenesis of DHF [20].

Virus recognition by the innate immune system is a first host defense to prevent viral invasion or replication before more specific protection by the adaptive immune system is generated. Pattern-recognition receptors are the central molecules of innate immune response and recognize molecular patterns specific to microorganisms. Accordingly, tolllike receptors (TLRs) 3, 7, 8 and 9 recognize distinct types of virally derived nucleic acids [104]. Recently, RIG-I and MDA5, members of the RNA helicases family have also been identified as cytosolic receptors for intracellular double-stranded RNA (dsRNA) [105]. This recognition is associated to the induction of inflammatory genes, dendritic cell migration, maturation, and activation of the adaptive immune response, which contribute to the elimination of infection. There are only a few reports demonstrating the involvement of pattern-recognition receptors in DV recognition [106-110]. Even though these studies provided important information to the understanding of the innate immune recognition of DV, further investigation is still required, especially in relation to cells that are not components of the immune system but are actively infected by the virus in vivo, such as hepatocytes.

Naturally acquired immunity

The E glycoprotein plays the most important part in protective immunity to DV, and contains the main epitopes recognised by neutralising antibodies [20]. Robust neutralising antibody responses develop after dengue infection and are believed to provide lifelong protection against reinfection with the same dengue serotype and short-lived protection, of several months, against a heterologous dengue serotype [111, 112]. This naturally acquired immunity provides optimism for the feasibility of a dengue vaccine. The role of dengue-specific cellular immunity in protection against reinfection is less clearly defined. Tcells might limit the expansion of infection by killing infected cells and secreting inflammatory cytokines, and are likely to have a role in viral clearance [113, 114]. The important protective features of the cellular response include a high avidity, homologous response of a T-helper-1-type cell secreting IFN-γ [115, 116]. However, cellular immunity cannot be isolated from the antibody response, and any model of protection against dengue or pathogenesis of severe disease must take into consideration both arms of the immune response [117, 118].

Role of non-neutralizing antibodies in the pathogenesis of DHF

The four DV serotypes are antigenically distinct but cause very similar disease in human. Epidemiologic data have suggested that DHF is mediated by host immune responses. The studies showing an increased risk of severe dengue in people who had been infected with the DV in the past led to the hypothesis of antibody-dependent enhancement (ADE) of infection. The studies done in Thailand demonstrated that up to 99% of DHF cases had heterotypic antibody to the serotype of DV that caused DHF. ADE is believed to happen because pre-existing non-neutralising concentrations of anti bodies and the infecting DV form complexes that bind to Fc receptor-bearing cells, leading to increased virus uptake and replication thereby increasing viraemia and consequent disease severity. These DHF cases were divided into two groups. Nearly 90% of them were children who were older than 1 year and in a secondary infection with a serotype of DV different from that which caused the primary infection. The other 10% were less than 1 year old and undergoing a primary DV infection [111]. They were infants who were born to the mothers with DV

antibodies. ADE is readily shown in vitro using dengue immune sera or monoclonal antibodies and cells of monocytic and B-lymphocytic lineages bearing Fc receptors [119-121]. ADE has also been shown in monkeys infused with human dengue immune serum [122]. Further support for the role of ADE in complex disease is provided by results of infection-enhancement assays in Vietnamese infants [123] implicating maternally derived subneutralising levels of dengue antibody as a crucial risk factor for severe dengue during infancy. In dengue epidemic of DV2 in Cuba in 1981, most DHF cases were those who had acquired dengue antibodies in the previous epidemics by DV1 in 1977 and 1978 [111]. These results suggest that the presence of heterotypic DV antibodies before secondary infections is a risk factor for developing DHF. Cross-reactive antibodies that lack neutralizing activity are induced in the primary infection. In secondary infection, DV and non-neutralizing antibodies form virus–antibody complexes. Virus–antibody complexes bind to Fcγ receptors on target cells and result in enhancement of DV infection. The nonneutralizing, cross-reactive antibodies thus markedly augment DV infection of Fcγ receptor-positive cells [124]. This phenomenon is called antibody-dependent enhancement (ADE). Infants born to the mothers develop DHF in the primary infections. The levels of maternal DV antibodies in the infants needed to decline to the levels that can enhance DV infection and lead to DHF. Antibody-enhanced DV infection has been linked to heightened production of vasoactive cytokines and chemokines that have roles in the vascular dysfunction associated with severe dengue [125-128]. Antibody enhanced infection has also been associated with a caspase-dependent apoptosis of target cells [129, 130]. However, it is rare for a patient with dengue experiencing a secondary infection to progress to more severe forms of the disease, which indicates that other factors must play a part in pathogenesis [131]. In addition, it might be that not all cases of severe disease result from secondary infection. If so, it follows that ADE is neither sufficient nor entirely necessary for severe disease. Indeed, in some studies enhancing antibody activity in pre-illness plasma has been shown not to predict subsequent disease severity or viraemia in secondary infection [132]. These observations are consistent with the idea that enhancing antibodies increase the number of DENV-infected cells and the levels of viremia and lead to DHF [20]. Whatever the role of ADE, it seems that a vaccine inducing a long-lived neutralising

antibody response against all four serotypes simultaneously should not induce any risk in this respect [133-135].

Role of cytokines in the pathogenesis of DHF

A series of studies have suggested that plasma leakage, which differentiates DHF from DF, is caused by malfunction of vascular endothelial cells induced by cytokines or chemical mediators rather than by destruction of the small vessels [116]. Plasma levels of various cytokines are significantly higher in DHF than in DF. The cytokines elevated in patients with DHF include TNF-α, IL-2, IL-6, IL-8, IL-10, IL-12, and IFN-γ. The levels of IL-8 and MCP-1 are also elevated in the pleural effusion from DHF patients. However, it is not clearly understood how these cytokines are induced and how these cytokines cause malfunction of vascular endothelial cells and lead to plasma leakage. Of these cytokines, TNF-α has attracted attention because of its well-known activity in inducing plasma leakage. It was reported that DV-infected monocytes and endothelial cells produce some of multiple cytokines including TNF-α [116]. It was also reported that monocytes produce TNF-α when they were infected with DV in the presence of enhancing antibody and that the produced TNF-α induces plasma leakage in an in vitro experimental system using endothelial cells. It has also been reported that mast cell, basophile line infected with DV can produce IL-1 and IL-6. Some of the cytokines, IFN-γ, IL-2, and TNF-α, were also produced by virus-specific T lymphocytes upon activation. It is likely that both DV-infected monocytes and activated specific T lymphocytes are responsible for increased levels of cytokines in DHF [20].

Cell-mediated immunity

The possible involvement of T cells in the pathogenesis of dengue was suggested more than two decades ago and developments in T-cell immunology have refi ned our understanding of the key events and mechanisms involved [136-138]. In this model, following the antibodydependent enhancement of viral replication in monocytes and macrophages, viral antigens are presented, in conjunction with human lymphocyte antigen molecules, to CD4 and CD8 memory T cells, sensitised during a previous infection. This leads to cellular proliferation and release of proinfl ammatory

cytokines such as tumour necrosis factor α (TNFα) that act directly upon vascular endothelial cells and result in plasma leakage (Figure 13) [139] . The T cells generated during a primary dengue infection respond to variant peptides during the secondary infection. In a detailed study in Thai children [140], these T-cell responses showed a lower affi nity for the infecting virus and a higher affi nity for another virus serotype, thus mimicking so-called original antigenic sin. This leads to a selective expansion of lower avidity crossreactive memory T cells that might out-compete the naïve T cells with higher avidity for the infecting serotype [140, 141]. This response would be less effi cient in eliminating newly encountered DV serotypes and could lead to increased virus replication and more severe disease. The extent of T-cell activation is related to disease severity [123, 129] and cross-reactive T cells have been shown to produce much higher levels of both type 1 and certain type 2 cytokines, many previously implicated in dengue pathogenesis [142-145]. There is also evidence that previous infection with other flaviviruses that commonly circulate with dengue might affect the clinical course of subsequent infections with other members of the family. CD4 T-cell clones from patients with dengue infection have been shown to have substantial epitope cross-reactivity among flaviviruses [146]. Regulatory T cells have been studied in acute infection because T-cell immunopathology is thought to play a major part in the pathogenesis of severe disease. Although regulatory T cells are fully functional, able to suppress the production of vasoactive cytokines, and expand in acute dengue infection, this expansion is likely to be insuffi cient or happen too slowly to control the immune response in patients with severe disease [97, 147].

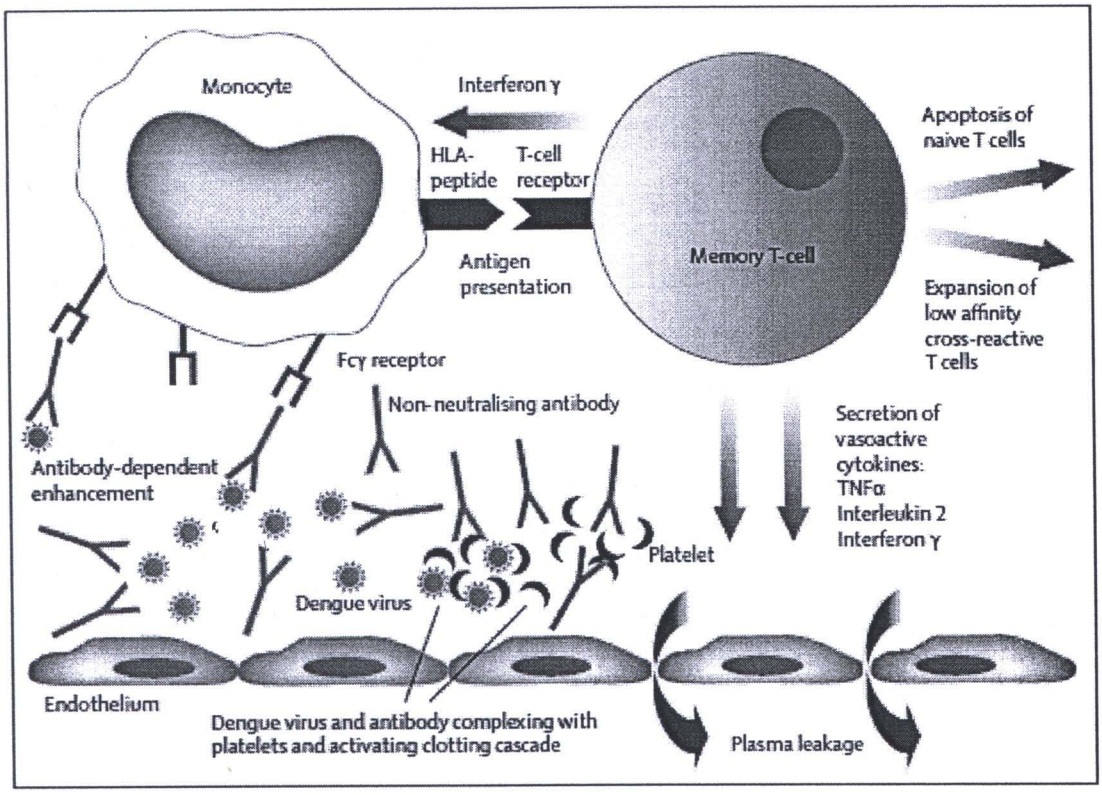

Figure 13 Schematic representation of the immunopathogenesis of severe dengue disease [97].

Cytokine

Studies have suggested that the capillary permeability resulting from dengue infection is caused by the malfunction of vascular endothelial cells induced by cytokines or chemical mediators rather than by destruction of the small vessels [20, 116, 117]. Following massive activation of memory T cells, a cytokine storm targeting vascular endothelial cells might lead to the crucial pathological event of capillary permeability and hence plasma fluid and protein leakage. Patients with severe disease have higher concentrations of cytokines including IFN-γ, TNFα, and interleukins 2, 6, 1, 8, and 10. These findings have been supported by recent studies of sera from severe dengue patients in Vietnam [148], India[149] and Cuba [97, 150].

Upon viral infection of a cell, multiple signaling pathways including those of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and IFNs are activated, and participate in the regulation of gene expression related to inflammation. COX-2 is the rate-limiting enzyme for catalysis of arachidonic acid to prostaglandin

G2, which by peroxidase is further reduced to PGH_2, the precursor of various prostanoids, namely prostaglandins (including PGE_2), prostacyclins and thromboxanes expression of COX-2 leads to increased levels of PGE_2, a central mediator of inflammation [9].

PGE_2 is one of the most important pro-inflammatory cytokines, associated with induction of inflammation, leukocyte chemoattraction, and pain perception. PGE_2 modulates immune function in inflammatory reactions, regulates viral replication and virulence, and participates in a wide range of normal physiological processes.

Expression of COX-2 is regulated by numerous transcription factors, including NF-κB, NFAT/AP-1, cyclic-AMP response element (CRE), PU.1, activating enhancer-binding protein-2 (AP-2), specificity protein-1 (SP-1), GATA box and CCAAT enhancer binding protein (C/EBP), as well as the canonical TATA box at the promoter region. In response to stimulation, activation of transcription factors NF-κB, AP-1 and/or other various transcription factors collaborate to induce transcription of numerous downstream genes as well as the COX-2 gene, which subsequently manipulates immunity.

The cumulative data suggest that, in response to virus infection in most types of cells, COX-2 mRNA accumulation, protein expression, and PGE_2 production are stimulated, however the opposite response has also been shown; in peripheral blood mononuclear cells infected with Epstein–Barr virus, the reactions seem to be virus- and cell type dependent. Activated PGE_2 may protect the cell from invasion but also be used by some viruses to favor viral production. Induced COX-2 expression and resultant prostaglandins may also play an anti-inflammatory role. Although the COX-2 and PGE_2 response to different viral infections in various types of cell has been widely investigated, little is known about this response to DV infection.

Modulation of COX-2/PGE_2 synthesis in stimulated cells by anti-inflammatory molecules could be away to suppress viral replication and spread. Nonsteroidal anti-inflammatory drugs (NSAIDs), potent nonselective COX inhibitors and pain relievers like aspirin, indomethacin and ibuprofen, all have been shown to exert antiviral effects or attenuate disease severity during infection by HSV, cytomegalovirus, VZV, influenza virus, JEV, respiratory syncytial virus, and mouse hepatitis coronavirus. Selective COX-2 inhibitors, such as celecoxib and NS398, also have shown great

potential for reducing virus infection by inhibiting viral protein synthesis in rotavirus-infected intestinal Caco-2 cells, and by reducing viral yields of alphaherpes in rate embryonic fibroblasts.

Accordingly, Liou JT et.al. hypothesized that the two compounds triptolide and tetrandrine might possess antiviral effects via their anti-inflammatory and immunomodulatory properties. In the present study, the researcher explored the effect of triptolide and tetrandrine on the immune response of COX-2/PGE_2 to DV infection in human lung cells (A549 cells). They found that triptolide, but not tetrandrine, was a dose-dependent suppressor of the COX-2/PGE_2 activated by DV infection, probably though inhibition of activation of NF-κB and AP-1. However, tetrandrine, but not triptolide, blocked DV production in A549 cells [9].

Complement activation in patients with DHF

Activation of complement is another important clinical manifestation in DHF. It was reported that the levels of C3a and C5a, complement activation products, are correlated with the severity of DHF and the levels of C3a and C5a reached the peak at the time of defervescence when plasma leakage becomes most apparent. This is consistent with the assumption that complement activation is also responsible for the pathogenesis of DHF. The mechanism of complement activation in DHF is not completely understood. Circulating immune complexes are present in the DHF patients, and it has been assumed that the complement is activated by the immune complexes [151]. High levels of secreted NS1 and pre-existing cross-reactive antibody may also mediate complement activation. Further, it was reported that DV-infected monocytes and endothelial cells activate complement via classical and alternative pathways. It is likely that complement is activated by various mechanisms in DHF patients, and that complement activation also contribute to the pathogenesis of various clinical features, especially plasma leakage [20].

Virulence of viruses as a cause of DHF

Although DHF occurs more frequently in secondary infection than in primary infection, DHF also occurs in primary infection. This suggests that virulence of the virus contributes to the development of DHF. It has been assumed that virulent DV

strains cause DHF, while avirulent DV strains cause DF. There are multiple genotypes in each of four DV. The introduction of the Southeast Asian genotype coincided with the appearance of DHF in different countries in the Americas, while the original American genotype was only associated with DF, but not with DHF. Other epidemiological studies demonstrated that there were no DHF cases reported in Peru where the Southeast Asian genotype of DV2 was not introduced [152]. Various groups have attempted to define the molecular determinants of DV virulence. It was reported that the determinants for virulence resided at the amino acid 390 of the E protein, in the 50 non-translated region and in the upstream 300 nucleotides of the 30 non-translated region [58]. The other group demonstrated non-synonymous amino acid replacements in the preM, NS1, NS2a, NS3, and NS5 by analyzing multiple strains of DV2 [153]. The studies done in Thailand demonstrated that viral loads in peripheral blood are higher in patients who develop DHF than in those with DF [154]. However, it is not determined why and how higher levels of viremia lead to the pathology and symptoms seen in DHF. The virulence of DV has been determined by the characteristics in vitro: plaque size, virus titers, etc. However, because of the absence of animal models, it is difficult to confirm that some ''virulent'' strains actually induce DHF in vivo. Further studies are needed to elucidate the molecular bases underlying these possible different phenotypes [20].

6. Clinical manifestations of DV infection

DV cause a range of well-described clinical illnesses [155, 156]. Infection ranges from an asymptomatic infection to a self-limiting febrile illness, DF, to severe dengue, to severe and potentially life-theatenning disease, a clinical syndrome that typically presents with capillary permeability and can lead to DSS and DHF. Less common features of severe dengue are encephalitis, hepatitis, and renal dysfunction. The WHO classifi cation scheme for dengue was developed in the 1970s and is at present under review because of concerns that it might lead to under-reporting of severe dengue [157]. What defi nes the clinical outcome of dengue infection is not completely understood, although numerous hypotheses exist. Epidemiological observations from studies in Thailand in the 1960s showed that greater than 85% of cases of severe dengue happened in people with pre-existing heterotypic DV

antibodies, and this has led to research into dengue pathogenesis focusing on the immune response [158]. Additional evidence from infant studies with maternal antibodies lends weight to the hypothesis of immune involvement in the development of severe dengue [123]. However, severe dengue can also happen in primary dengue infection and only a small proportion of secondary infections progress to severe disease, which suggests roles for other factors. There is evidence for links between disease severity and nutritional status [159], ethnicity [160], virulence of infecting viral strain [111, 161, 162] and host genetic background [163, 164].

Each of the four DV serotypes is capable of causing a spectrum of diseases ranging from mild infection to a potentially deadly disease. Infection with one serotype leads to life-long immunity to that serotype but only partial and temporary immunity to the others [8]. As outlined above, circulation of more than one serotype upon infection can increase the risk of serious and complicated infections, i.e., DHF and DSS [165]. Infection with any serotype can be asymptomatic or lead to one of the four clinical scenarios of increasing severity: undifferentiated fever, DF, DHF, and DSS [9, 130, 166].

Undifferentiated fever

Usually strat after 2-7 day after mosquito bit an average incubation period of 4-6 days (range 3-14 days), various non-specific, undifferentiated prodromes, such as headache, backache and general malaise may develop [8].

Dengue fever

DF follows the bite of a mosquito carrying infectious DV. DF is an acute and a self-limited dengue disease, and is a syndrome that is associated with the occurrence of fever that lasts from 2 to 7 days, headache, myalgia, bone/joint pain and rash, often accompanied by leucopenia. Occasionally variable degrees of thrombocytopenia and cutaneous hemorrhage are observed. More severe cases with incapacitating bone/joint pain (''break-bone-fever'') are common among adults. Infrequently, DF may be accompanied by unusual bleeding complications that may cause death [8].

Dengue hemorrhagic fever

The clinical features of DHF usually starts by third day in many aspects are very similar to that of DF during the early febrile phase the hemorrhage. The prominent feature of DHF is epistaxis, hematemesis, skin/mucosal bleed, melana. Thrombocytopenia (less than 100,000 per cm^2) and evidence of plasma leak are characteristic of DHF. DHF its potential to develop into fatal DSS [8].

Dengue shock syndrome

DSS is a severe case of the DHF which is accompanied by circulatory failure and shock cause by plasma leakage that can lead to hypovolemic shock in patients with DSS, if detection and management of shock is delayed, the morbidity and mortality from prolonged shock or massive bleeding is high. DSS includes all above and signs of circulatory failure manifested by rapid and weak pulse, narrow pulse pressure, hypotension for age restlessness. The severity of the disease can be modified by early diagnosis and adequate replacement of plasma loss [167]. The major pathophysiologic hallmarks that determine disease severity and distinguish DHF from DF are plasma leakage as a result of increased vascular permeability and abnormal hemostasis, occurring in a select group of patients during the course of dengue infection [8].

7. Treatment

Because DHF is caused by a virus for which there is no known cure or vaccine, the only treatment is to treat the symptoms.

1. Rehydration with intravenous fluids is often necessary to treat dehydration and electrolyte replacement therapy [168].
2. Antipyretics, good diet, drinking plenty of fluid and bed rest are to be taken care. Parpcetamolmis preferred antipyretic [168].
3. Treatment of DHF usually requires hospitalization because this form of infection can be fatal if left untreated.
4. Take commercially available anti-viral drug, ribavirin [169].

8. Vaccine

There is an urgent need for a safe and effective vaccine for dengue and the features of an ideal dengue vaccine are outlined in the panel. The association of increased disease severity in people with heterotypic immunity necessitates a vaccine that will confer long-term protection against all four serotypes. All vaccines in development are multivalent and aimed at producing immunity against all four serotypes. A further challenge for vaccinologists has been the lack of a suitable animal model with the same pathological features of human infection. Replication and immunogenicity can be studied in rhesus macaques, acknowledged as the best available primate model, although levels of viraemia are substantially lower than in human beings and the clinical syndrome is different, with little or no shock, making extrapolation of research findings difficult.

Vaccines in development include live vaccines (classically attenuated vaccines, site-directed mutagenesis vaccines, dengue and dengue–yellow fever chimeras), inactivated vaccines (recombinant E protein subunit and purified inactivated virus), and DNA vaccines [170]. The front-runners are entering phase 3 trials. Because pre-existing heterotypic immunity is a risk factor for DHF, a successful vaccine must protect against all four serotypes. Vaccine-induced enhancement has been a major stumbling block in the development of flavivirus, coronavirus, paramyxovirus and lentivirus vaccines [171]. There is evidence that ADE played a role in the failure of an inactivated experimental RSV vaccine in the 1960s [172-174]. Immunological interference has been documented for several multitypic vaccines dating back to the 1950s [175]. Analogous to the dengue situation is that of the oral polio vaccine, which contains three different serotypes of polio virus. It was found that seroconversion rates, which had exceeded 90% for monovalent polio vaccines, were significantly reduced whenthe serotypes were combined in trivalent formulations. Adjusting the dose of each serotype tended to increase seroconversion rates to some degree, but in an unpredictable manner. The imbalances in seroconversion wasfinally overcome by the administration of three doses of the multivalent vaccine. Similar difficulties have been encountered in the development of dengue vaccines. The monovalent vaccines had higher neutralizing antibody responses than when they were combined in various multivalent formulations [176, 177]. As for the oral polio

vaccine, it has been anticipated that multiple doses of the vaccine will be necessary to achieve seroconversion to three or four DV serotypes.

DNA vaccines

DNA shuffling and screening technologies have been used to construct DNA expression vectors encoding the epitopes of the four dengue serotypes. Shuffled DNA vaccines have shown immunogenicity, and phase 1 monovalent dengue vaccine studies are currently underway [178]. DNA vaccines afford advantages in terms of ease of production, stability, and transport at room temperature, decreased likelihood of replication interference, and the possibility to vaccinate against multiple pathogens in a single vaccination [179]. However, DNA vaccines necessitate multiple dosing and experimental adjuvants, and they are unlikely economical [180]

Inactivated vaccines

Whole-virus inactivated vaccines have two major advantages over live attenuated virus vaccines. First, it is not possible for inactivated vaccines to revert to a more pathogenic phenotype; second, induction of a balanced antibody response is easier to attain [179]. The requirement for multiple dosing and the relatively shorter term of immunity make this approach less attractive compared to the others, although both limitations could be overcome through the development of novel adjuvants. However, such developments are likely to be expensive and would not meet the needs of dengue in endemic countries, which mostly have developing economies.

Live attenuated vaccines

Live attenuated vaccines (LAVs) can induce durable humoral and cellular immune responses because they most closely mimic a natural infection. Several parameters are crucial for LAVs: the viruses must be sufficiently attenuated and must have low viremia, low reactogenicity, and high immunogenicity. The viremia level must also be low so that no transmission of the viruses by mosquitoes can occur. The mutations that confer the attenuation phenotype should be stable and not revert to wild type or other virulent forms. Managing viral interference and balancing attenuation to produce acceptable tetravalent immunogenicity with minimal

reactogenicity is another challenge [181]. Each of the four components of the vaccine must induce a balanced neutralizing antibody response.

Chimeric live attenuated vaccines

The dengue vaccine with the most advanced development is the dengue/yellow fever chimeric vaccine. This vaccine uses the 17D yellow fever vaccine virus as its genetic backbone and replaces the yellow fever E and prM genes with those from each of the four dengue viruses. This vaccine was shown to be attenuated, efficacious, safe, and highly unlikely to be transmitted by arthropod vectors [182]. ChimeriVax-Dengue (Sanofi Pasteur, Lyon, France) elicits antibodies only to dengue [183]. Current requirements for the development of live viral vaccines (including yellow fever 17D) produced from potentially neurotropic wild-type viruses, include tests for neurovirulence in nonhuman primates [184]. Neurovirulence in mice and monkeys was reduced compared to the yellow fever 17D vaccine viruses [184]. These results suggest that rare neurotropic disease seen with the yellow fever vaccine [185] are less likely to occur with ChimeriVax-Dengue, although rare events can only be excluded with very large sample sizes. ChimeriVaxDengue has also been tested in subjects with preexisting yellow fever immunity. The results showed that preexisting immunity to YFV did not interfere with immunization, and long-lasting and cross-neutralizing antibody responses to all four dengue serotypes were documented [135]. Currently, a large phase 2b trial on safety and immunogenicity is taking place in Singapore.

Another chimeric live vaccine uses the PDK-53 DV-2 Mahidol vaccine candidate as a backbone. This virus was attenuated by passage in primary dog kidney cells. Similar to the concept of the yellow fever/dengue chimeric vaccine, the prM and E genes of DV2 are replaced with those of DV1, DV3, and DV4. Phase 1 safety trials are underway in the United States and Colombia. The three attenuating mutations of the PDK-53 DV-2 are located outside the structural protein genes of and appear to be quite stable. The tetravalent vaccine produced by combining the four chimeric DV is protective when administered to mice and monkeys [186, 187]. Phase 1 trials in humans are planned.

A third chimeric live vaccine uses a DV4 attenuated by a Δ30 deletion of the 3′-untranslated region as the backbone. The prM and E proteins are replaced as above.

Tetravalent formulations are being developed incorporating the into wild-type DV4, DV2, and DV3 viruses resulting in rDV1/4Δ30, rDV2/4Δ30, and rDV3/4Δ30[180].

Replication-incompetent vaccines

The new approach of creating replication-incompetent (encapsidation-defective) viruses has been taken to resolve the problem of increasing the immunogenicity of inactivated vaccines while maintaining their safety profile. These are capsid gene-deleted viruses, named RepliVAX, that undergo only a single cycle of infection in vaccinated hosts. A DENV2 vaccine has been generated by replacing the prM and E genes of RepliVAX WN (a WNV vaccine construct). After passage the vaccine developed further mutations enabling efficient growth in suitable cell lines (named RepliVAX DEN2.2) and has been shown to have good immunogenicity and efficacy in an established immunodefi cient mouse model of dengue. Similar vaccines have been produced for WNV and JEV, which are potent and efficacious in animals, showing potential use of this technology for the production of third-generation flavivirus vaccines that should be potent, economical to produce, and safe in immunocompromised people [3, 188].

Panel: characteristics of the ideal dengue vaccine

- Good safety profile
- Rapid immunisation regime requiring a single vaccine or two that fit in with established vaccine programmes
- Balance between reactogenicity and immunogenicity
- Suitable for use in target age groups
- Genetically stable
- Stimulates neutralising antibodies and T-helper-1 cell-mediated immunity
- Provide longlasting immunity
- Generates neutralising immunity to all four serotypes
- Does not contribute to immunopathogenesis (vaccine-induced enhancement)
- Easily stored and transported
- Affordable and cost effective

9. Anti-viral agents for dengue infection

Currently, no specific antiviral therapy is available for dengue, and treatment remains only supportive. With the emergence and rapid spread of 50–100 million infections per year caused by DV globally, the threat of dengue infection as re-emerging human pathogens has become a reality. To support the efforts that are currently being pursued to develop effective vaccines against these viruses, many researchers are also actively pursuing the development of small molecule compounds that target various aspects of the virus life cycle. Recent advances in the structural characterization of the DV have provided a strong foundation towards these efforts. Most importantly, these studies have highlighted specific structural insights into the DV life cycle and viral interactions with cellular molecules and antibodies provide great opportunities for identifying new inhibitors. The ability to obtain high-resolution structures of viral components and inhibitory compounds suggests that powerful structure-based approaches could rapidly focus the development of highly efficacious compounds. The same techniques could be used to design compounds that evade virus resistance and demonstrate broad anti-flaviviral activity [84]. In 2001, Rory et al. studied probing the interaction of DV E protein with heparan sulfate GAG is a putative receptor for DV E protein, soluble GAGs and other highly charged polyanions could be effective inhibitors of viral infectivity. In support of this hypothesis, they found that heparin, heparin-derived oligosaccharides, and the polysulfonated urea Suramin, inhibited DV E protein binding and viral infectivity in vitro [189]. Furthermore a member of anti-viral agent with a wide variety of putative targets have been reported, including those that act on viral membranes as attachment molecule or β-OG binding pocket on E of virus, translation, c-Src protein kinase, RNA polymerase, α-glucosidase, mRNA, cellular IMP dehydrogenase, RNA-dependent RNA polymerase. However, at the present time, there are no clinically useful anti-viral agents [190].

The rationale for dengue antivirals arises from clinical studies that have noted that the quantity of virus circulating in the blood of patients who develop severe dengue (eg, DHF and DSS) is higher by around 1–2 logs compared with patients suffering from mild dengue disease [154, 180]. Similar differences in viral load have been observed in animal models of ADE [119]. This observation suggests the

potential benefit of antiviral therapy such as safe small molecule compounds that can reduce viral load in the acute phase of the disease. An animal model—AG129 mice that are deficient for the IFN-α/β and IFN-γ receptors—is now being used for testing anti-dengue drugs. As a proof of concept, Schul et al. demonstrated that a clinical isolate of DV can be used to infect AG129 mice, and antiviral compounds that block viral replication can clear viremia in a dosedependent manner, even after delayed treatment, and suppress proinflammatory host responses [191]. The life cycle of DV readily shows that the steps involved in virus entry, membrane fusion, RNA genome replication, assembly, and ultimate release from the infected cell can be targeted by small molecules, [6, 192]. The entry of virus into host cells is mediated by the E protein and is the target for neutralizing antibodies that limit viral replication. Several small-molecular inhibitors have been shown to target viral entry [190, 193]. Currently, the most advanced targets are the NS2B/NS3 protease and the NS5 RdRp, and several in-silico and highthroughput screens yielding several lead compounds have been reported [194-196]. New targets—including E, NS3 helicase, and NS5 methyltransferase—are being explored [193, 197-199]. Further extensive characterization of the AG129 mouse model has demonstrated it to be one of the only models that permits infection by all four serotypes of DV and allows antibody-mediated protection and enhancement of DENV infection [200]. In summary, the efforts to find specific dengue inhibitors are intensifying and the tools to evaluate the efficacy of new drugs are in place for rapid translation to human patients [180].

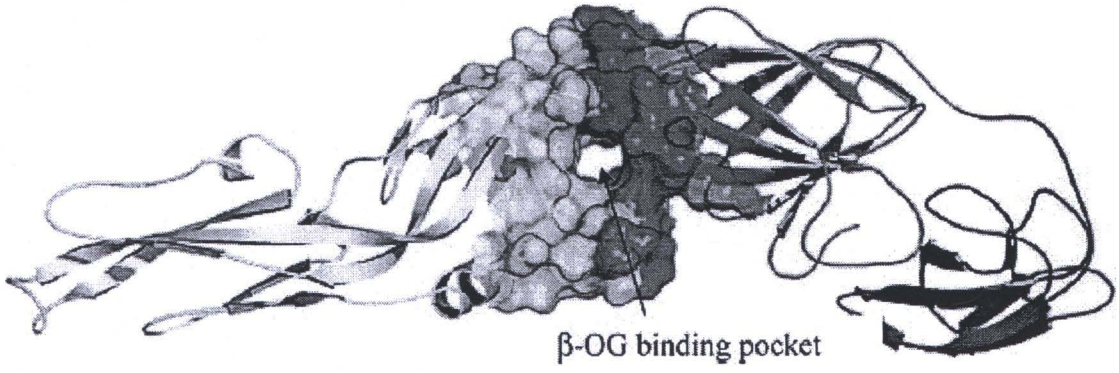

Figure 14 Structure of dengue 2 E protein. Domain I: red; domain II: yellow; domain III: blue. The *β*-OG binding pocket is located between domains I and II [84].

10. Medicinal plant

Symptomatic DV infection ranges from a self limited febrile illness, DF to a more severe disease, DHF and DSS. The anti-dengue treatment is severely hampered as no specific therapeutic agents are available. Even present treatment strategies for Dengue are more supportive than curative. There is no magic bullet or specific drug that kills off infection. Many researches indicate that plants as source of medicines and human sustenance have been in vogue since antiquity. In 1999 have been reported about, different flavonoids extracted from Mexican plants *Tephrosia madrensis, Tephrosia viridiflora* and *Tephrosia crassifolia.* The flavonoids glabranine and 7-O-methyl-glabranine presented 70% inhibition on the DV at a concentration of 25 microM, while methyl-hildgardtol-A, hildgardtol A and elongatine had no effect on viral growth. this results show that glabranine and 7-O-methyl-glabranine isolated from *Tephrosia s.p.* exert a dose-dependent inhibitory effect in vitro on the DV by plaque reduction assay [201]. In 2005 Lucy et al. discovery of anti-dengue activity *Mimosa scabrella* and *Leucaena leucocephala* against YF virus and DV1. In other studies, the anti-flaviviral activity of sulfated polysaccharides extracted from seaweeds was demonstrated protection from lethal DV by two methods: plaque reduction and virus yield inhibition assays [202]. In 2006 Tan Siewkiat and Richard Pippen studied about, the crude extracts and the methanol and hexane partitioned fractions from the rhizomes of six Zingiberaceae comprising five *Curcumas* and one

Zingiber were screened for DV2 NS2B/NS3 protease inhibition by DV2 NS2B/NS3 Protease Inhibition Bioassay using Fluorogenic Peptides. They found the methanol fractions of the extracts of *Curcuma longa* (L.), *Zingiber zerumbet* Smith and *Curcuma rubescen* Roxb.were much more inhibiting of DV2 NS2B/NS3 protease. In 2007 have reported about, Sulfated derivatives preparation of two a-D-glucans from *Gastrodia elata Bl* showed strong anti-DV bioactivities. Antiviral activity was evaluated by three methods: CPE assay in C6/36 cells, MTT colorimetric assay and quantitative analysis of dengue viral RNA in BHK cells [203]. For 2008 have present study anti-dengue activity of *Hippophae rhamnoides* (Seabuckthorn, SBT) leaf extract was evaluated in DV2 infected blood-derived human macrophages as macrophages are the primary target of DV infection. Antiviral activity was study by plaque reduction assay and evaluated level of cytokine by ELISA. They found infected cells were treated with SBT leaf extract and compared with commercially available anti-viral drug, Ribavirin. The extract was able to maintain the cell viability of Dengue-infected cells at par with Ribavirin along with the decrease and increase in TNF-α and IFN-γ respectively[169]. *Uncaria tomentosa* a large woody vine native to the Amazon and Central American rainforests has been used medicinally by indigenous peoples since ancient times and has scientifically proven immunomodulating, anti-inflammatory, cytotoxic and antioxidant activities. The antiviral activity from *U. tomentosa* was determined viral antigen (DV-Ag) detection in monocytes by flow cytometry results demonstrated that inhibitory activity of DV infection [204]. Jun-Ting Liou et.al report *Triptolide* and *tetrandrine*, compounds derived from two commonly used Chinese herbs, they study anti-viral activity by RT-PCR ans immunomodulation by Electrophoretic mobility shift assay (EMSA), both demonstrate anti-inflammatory and immunosuppressive effects partly through modulation of COX-2 expression and, hence, may have antiviral [9]. Furthermore Sulfated polysaccharide from a marine alga have effect anti-DV activity, here they found this compound potently inhibits DV2 infection by DV2 particles bound exclusively to fucoidan, indicating that fucoidan interacts directly with E glycoprotein on DV2 [205]. The leaves of tulsi are specific for many fevers as malaria and DF, tender tulsi leaves, act as preventive against these diseases.

Andrographis paniculata

Andrographis paniculata is an herbaceous plant in the family *Acanthaceae*, a plant native to India, Sri Lanka and Thailand. It is widely cultivated in southern Asia, where it is used to treat infections and some diseases, often being used before antibiotics were created. Mostly the leaves and roots were used for medicinal purposes. Scientists have studied this herb for nearly thirty years. Properties of *A. paniculata* has scientifically demonstrated that the plant extract exhibits antifungal activities, antibiotic, anti-malarial, anti-inflammatory, Immunostimulatory activity by increased proliferate ion of lymphocytes and production of interleukin 2, enhanced the tumor necrosis factor α production.

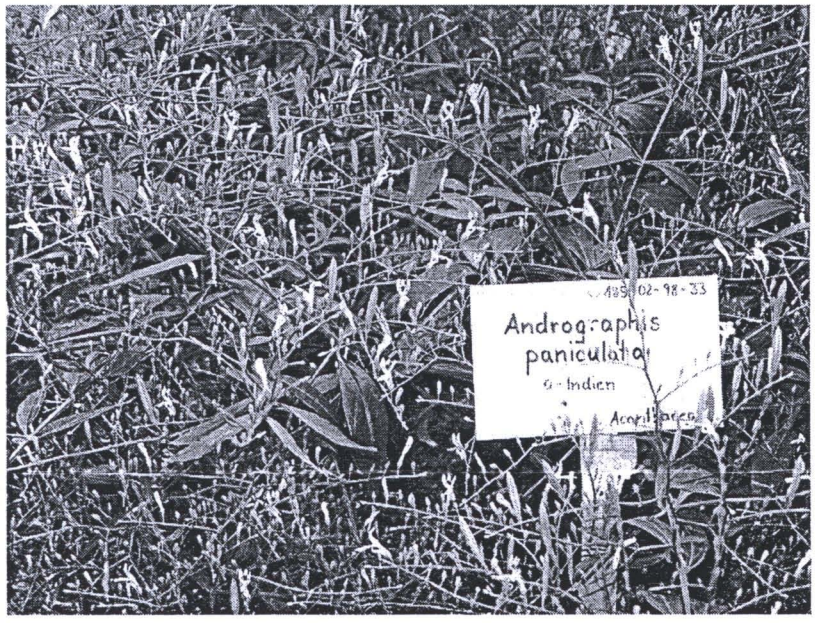

Figure 15 *Andrographis paniculata* (Burn) Wall

Clinacanthus nutans (Burm.f.) Lindau.

Clinacanthus nutans (Burm. f.) Lindau belongs to the family of *Acanthaceae.* This family includes many species, which are known to have medical properties. In Thailand, many Thai medicinal plants have been collected, including medicinal plants of the family *Acanthaceae* including: *A. paniculata* (Burm. f.).

In 1986 Chuakul W et al. and in 1996 Satayavivad J et al. demonstrated that the leaves of *C. nutans* have long been traditionally used in Thailand as Flavoniod of the plant extract effect to anti-inflammation drug for the treatment of insect bites. *C.*

nutans has been traditionally used in Thailand for the treatment of herpes infections. Laboratory investigations in Thailand have indicated that the extract of this herb exhibits anti-viral properties against the HSV and varicella zoster virus (VZV); ethanolic extracts of plat have ability to inhibit Newcastle disease virus infection [206].

Figure 16 *Clinacanthus nutans (Burm.f.) Lindau*