II. LITERATURE REVIEW

A. Dengue virus

1. Structure and composition of virion

Dengue viruses are members of the Family *Flaviviridae*. There are four distinct serotypes of dengue virus, namely, dengue virus serotype 1 (DEN-1), dengue virus serotype 2 (DEN-2), dengue virus serotype 3 (DEN-3), and dengue virus serotype 4 (DEN-4). Dengue virus virion consists of a single-stranded linear RNA molecule, which is capped at the 5' end but lacks a poly(A) tract at the 3' end (Putnak and Henchal, 1990). The RNA genome is about 11 kb in length, surrounded by an icosahedral nucleocapsid of about 30 nm in diameter and covered by a lipid envelope of about 10 nm in thickness. The complete virion is about 50 nm in diameter.

Dengue virus is transmitted between human hosts by infected mosquitoes and *Aedes aegypti* is the most important vector. In man, all of the four serotypes of dengue virus can cause classical dengue fever and dengue hemorrhagic fever (Putnak and Henchal, 1990) and there is no long term cross-protective immunity between the dengue serotypes, so persons may have as many as four dengue infections during their life.

2. Genome structure and viral proteins

Complete nucleotide sequences of genomic RNA of all four serotypes of dengue viruses have now been described (Blok et al., 1992; Fu et al., 1992; Hahn et al., 1988;

Mackow et al., 1987; Osatomi and Sumiyoshi, 1990; Zhao et al., 1986). The order of the coding sequences is 5' C - prM (M) - E - NS1 - NS2A - NS2B - NS3 - NS4A - NS4B - NS5 3'. Sequences on the 5' one-fourth of the genome encode the structural proteins whereas the non-structural proteins are encoded in the 3' three-fourths of the genome. The genomic RNA has a type I cap at its 5' end (m^7GpppAmp) followed by the conserved dinucleotide sequence AG and ends with the conserved dinucleotide CU (reviewed by Rice, 1996).

The dengue virus genome contains a single long open reading frame of more than 10,000 nucleotides. The protein derived from translation of the viral genomic RNA is a single, long, precursor polyprotein that is successively cleaved by both host and viral-encoded proteases to produce structural proteins and non-structural proteins (reviewed by Rice, 1996). The three structural proteins: C, the nucleocapsid or core protein; M, a membrane-associated protein; and E, the envelope protein, are located at the amino terminus whereas the seven non-structural proteins (NS) are located toward the carboxyl terminus of the polyprotein.

Flanking the open reading frame are the 5' and 3' nontranslated regions both of which contain conserved RNA sequences. At the 3' end of the genomic RNA, there is a secondary structure of about 90 bases which appears to be conserved both in conformation and stability (Brinton et al., 1986). There is a short conserved RNA sequence, CS1, which is about 26 nucleotides in length, located on the 5' side of the secondary structure at the 3' end. Part of this CS1 is complementary to a conserved sequence that is located in the region encoding the capsid protein (5' CS). These complementary regions may function in the cyclization of the viral genome; this, in turn, may be important in the regulation of translation, replication or packaging. On the 5' side of CS1, there is an additional conserved sequence, CS2, which is about 24

nucleotides in length. This sequence is duplicated in dengue type 2 and type 4 viruses, but the significance of this CS2 is not yet known (reviewed by Rice, 1996).

3. Envelope glycoprotein

The envelope (E) glycoprotein is the major envelope protein which is associated with the lipid envelope. Being the major virion surface protein, the E protein plays a role in several important biological functions. It is the receptor-binding protein and is also probably responsible for the membrane fusion activity after acid pH-induced conformational changes (Heinz et al., 1990). In addition, the E glycoprotein also plays a role in viral neutralization by interacting with neutralizing antibody. Administration of monoclonal antibodies against the dengue serotype 2 virus E glycoprotein protects mice against subsequent lethal virus challenge (Kaufman et al., 1987). Purified E glycoprotein can elicit neutralizing antibodies against dengue serotype 2 virus and partially protected mice from fatal infection (Feighny et al., 1992).

An X-ray crystallographic study done by Rey et al (1995) indicates that the sE dimer (dimeric, soluble fragment of E which is derived by treating the virion with trypsin) of tick-borne encephalitis virus takes an elongated shape and orientates parallel to the membrane. The polypeptide chain of the sE subunit folds into three distinct domains, namely, a central β-barrel (domain I), an elongated dimerization region (domain II) and a C-terminal, immunoglobulin-like module (domain III). The central domain I contains about 120 residues derived from three non-contiguous segments (residues 1-51, 137-189, 285-302) of the whole protein. The two loops between these segments form the dimerization domain II (residues 52-136, 190-284). The domain II contained a highly conserved sequence (residues 98-113) that is important for the

fusogenic activity of the viral envelope. The C terminal domain (domain III) is joined to the central domain through a connecting segment of about 15 residues and anchored to the end of that segment by a disulfide bridge. The domain III is likely to be involved in receptor binding.

Mapping of the antigenic epitopes by serological method indicates that there are three clusters of antigenic epitopes, designated A, B and C, on the E protein (Mandl et al., 1989). These antigenic clusters correspond well with the domains II, III and I, respectively, of the three dimensional structure. Flavivirus cross-reactive epitopes were found in the domain A, whereas type- or subtype specific epitopes were found in other two domains (Rey et al., 1995).

4. NS1, NS3 and NS5 proteins

NS1 is a nonstructural glycoprotein that can be found at the surface of cells infected with dengue and other flaviviruses (Cardiff and Lund, 1976) and also intracellular membranes (MacKenzie et al., 1996). Study by Winkler et al (1988) indicated that between 20 and 40 min after synthesized in the rough endoplasmic recticulum and before the protein passes the Golgi apparatus, NS1 converted to the high molecular weight form. Identification of the dissociation products of the high molecular weight form and comparison of the number of N-linked glycans in this high molecular weight form and the denatured NS1 revealed that the high molecular weight form is a dimer of NS1 (Winkler et al., 1988). In the next study, Winkler et al (1989) indicated that newly synthesized monomeric NS1 is a hydrophilic and water soluble protein which cannot be pelleted at 75,000 g. After dimerization, NS1 showed increased hydrophobicity and was completely pelletable at 75,000 g indicated that NS1

become membrane-associated (Winkler et al., 1989). Pryor and Wright (1993) employed the site-directed mutagenesis to determined the importance of the regions of the NS1 protein in the dimer formation and secretion. It was found that the carboxy terminal end of the protein is important in the dimer formation and only the protein which formed dimers were detected at the cell surface and in the extracellular medium, no monomers were secreted (Pryor and Wright, 1993). The role of NS1 in virus replication is not known. However, the study by Mackenzie et al (1996) who employed dual-labeling experiments with the anti-NS1 monoclonal antibody and anti-dsRNA serum revealed that NS1 co-localized with the viral dsRNA replication form in virus-induced intracellular vesicles within dengue virus-infected cells. This suggested the possible role of the NS1 glycoprotein in flavivirus genome replication as NS1 may constitute a structural component of the replication complex (Mackenzie et al., 1996).

NS3 and NS5 are large cytoplasmic proteins. Both proteins contain conserved sequences which are found in proteins with RNA polymerase activity. The NS3 protein contains protease, helicase and RNA triphosphatase activities (reviewed by Rice, 1996). To test the hypothesis that the NS3 protein is a viral protease that generates the termini of several nonstructural proteins, Preugschat et al (1990) employed an efficient in vitro expression system and monospecific antisera directed against the nonstructural proteins NS2B and NS3. Plasmid pT10 which contains the 5' untranslated region of the dengue virus genome and the nucleotides encoding the first 37 amino acids of the dengue virus capsid protein that is fused with the coding sequences for the amino acids 101-218 of NS2A, the whole length (amino acids 1 to 129) of NS2B, and the first 610 amino acids of NS3 was constructed. By employing the in vitro translation system, the RNA transcribed from this construct was translated into a polyprotein P2A2B3 that contained the first 37 amino acids of the capsid

protein, the C-terminal 118 amino acids of NS2A, all of NS2B, and most of NS3. SDS-PAGE analysis of protein patterns at different time points following RNA translation indicated that a protein with the molecular mass predicted for the slightly truncated NS3 was produced. This revealed that proteolytic processing had occurred. Furthermore, proteins with the molecular masses expected of the full-length (unprocessed) precursor P2A2B3 and the processing intermediate P2B3 were also observed. Detection of the processing intermediate P2B3 indicated that the cleavage between P2A and NS2B was preceded the cleavage between NS2B and NS3. With deletion analysis, the protease domain has been mapped within the first 184 amino acids at the N terminus of NS3; this region contains four subregions with strong homology with serine proteases. The first three subregions contain elements of the catalytic triad whereas the fourth subregion is thought to be involved in substrate binding (Preugschat et al., 1990).

To demonstrate the RNA helicase and RNA triphosphatase activities of NS3 protein, Wengler and Wengler (1991) isolated the carboxy-terminal segment of the NS3 protein by digesting the West Nile (WN) virus-infected BHK cells with subtilisin. The soluble protein of about 50 kDa, which was a fragment of NS3 protein, was released from the membrane. The 50 kDa protein was purified by gel filtration chromatography and the fractions were subjected to automated amino acid sequence determination. Comparison with the published sequence of WN NS3 protein indicated that the 50 kDa protein contained a subregion of the NS3 protein which had been predicted to represent RNA helicase. No functional study of the RNA helicase was performed. However, the fractions were tested for ATPase activity and revealed that an ATPase activity comigrated with the 50 kDa protein when analyzed by DEAE paper electrophoresis. The nucleoside triphosphatase activity of the 50 kDa protein was greatly stimulated by

the presence of ssRNA molecules. From these data, Wengler and Wengler concluded that this 50 kDa segment of the NS3 protein has the RNA helicase and nucleoside triphosphatase activity (Wengler and Wengler, 1991).

To demonstrate that the dengue virus NS5 protein has the RNA-dependent RNA polymerase activity, Tan et al (1996) transformed bacteria with a plasmid construct containing the whole NS5 coding sequence and purified recombinant NS5 protein from the cell lysate. Polymerase activity of the recombinant NS5 protein was demonstrated by incorporation of [^{32}P]UMP into newly made RNA strands when RNA templates whose 3' terminal sequence were identical to that of the viral genome were incubated with NS5 protein. The synthesis of new RNA strands was specifically inhibited by an immune serum specific for NS5 protein, but not by the preimmune serum, indicating that RNA synthesis was dependent on the recombinant NS5 protein. Hybridization analysis with M13mp18NP4(+) RNA probe which contained ssDNAs corresponding to the positive strand revealed that the RNA product was of negative polarity and complementary to the RNA template (Tan et al., 1996).

5. Heterogeneity of dengue viruses

Several methods have been used for the demonstration of structural and genetic differences of dengue viruses. These include serological methods such as the plaque reduction neutralization assay, the indirect immunofluorescence assay, and the antigen signature analysis, and molecular biological assays, such as the RNase T1 oligonucleotide fingerprinting assay, cDNA-RNA hybridization analysis and nucleotide sequence analysis. These methods reveal distinctive differences between all four serotypes of dengue viruses.

5.1. Serologic study

In 1967, Russell and Nisalak employed the plaque reduction neutralization assay for distinguishing dengue viruses into distinct serological types. By using monkey antisera which were generated against each serotype, Russell and Nisalak found that dengue serotype 1- and serotype 2-antiserum reacted only within the corresponding serotype with no detectable reactivity to dengue serotype 3 and serotype 4 viruses. Antisera to serotype 3 and serotype 4 viruses exhibited some degree of cross reaction with heterologous serotypes, but there were large differences between the titers against the heterologous and the homologous viruses. For example, one notable cross reaction was detected when an antiserum against dengue serotype 3 virus neutralized dengue serotype 1 with the titer of 1 : 90 whereas the titer against the homologous virus was 1 : 350. The cross reactivity indicated that there were some antigenic determinants that were shared between dengue serotype 1 and dengue serotype 3 viruses (Russell and Nisalak, 1967). This result was confirmed by Henchal et al (1982) who employed an indirect immunofluorescence assay to differentiate dengue viruses. By using monoclonal antibodies against all four dengue virus serotypes, four patterns of reactivities were identified: a) flavivirus group reactivity which reacted with cells infected with all flaviviruses tested, b) dengue complex-specific reactivity which stained cells infected with only dengue viruses, c) dengue subcomplex-specific reactivity that showed positive staining only with dengue serotype 1 and dengue serotype 3 virus-infected cells, and 4) dengue type-specific reactivity which reacted only with the homologous dengue virus serotype. Data from the dengue subcomplex-specific reactivity pattern indicated that there were epitopes that were common between dengue serotype 1 and dengue serotype 3 viruses (Henchal et al., 1982).

The other method that can be used to determine the antigenic variation of dengue viruses is the antigen signature analysis (Monath et al., 1986). This technique involved the use of monoclonal antibodies that recognized distinct dengue serotype 2 type-specific and flavivirus cross-reactive epitopes and performing the reaction over a range of antigen concentrations. The study of dengue serotype 2 viruses that were isolated from different locations by antigen signature analysis revealed a high degree of antigenic similarity among viruses from the same geographic region (Monath et al., 1986). Variation between the viruses occurred in both type-specific and group-reactive epitopes; the majority of antigenic differences occurred at flavivirus group-reactive epitopes and the minority occurred at type-specific epitopes (Monath et al., 1986).

5.2. RNA fingerprinting study

Analysis of the genomic RNA of prototype strains of dengue virus by using comparative oligonucleotide fingerprinting procedures revealed that the RNase T1 oligonucleotide fingerprints of each of dengue virus serotypes were distinct (Vezza et al., 1980). This technique identifies differences in only about 10 to 15% of the dengue viral genome (Repik et al., 1983; Monath et al., 1986).

5.3. cDNA-RNA hybridization study

Genetic relationship between dengue viruses serotype was first determined by employing the cDNA-RNA hybridization using dengue-3 and dengue-4 specific cDNA probes (Blok, 1985). It was found that the serotype 1 and serotype 4 were genetically very closely related as were the serotype 3 and serotype 4. On the other hand, the

serotype 2 viruses did not seem to be very closely related to other dengue serotypes by this cDNA-RNA hybridization analysis (Blok, 1985).

5.4 Nucleotide sequence comparison

Haishi et al (1990) analyzed the amino acid sequences deduced from the nucleotide sequences of genomic RNA of 4 dengue virus serotypes to determine the homology between dengue viruses at the molecular level. Dengue viruses serotype 1 and serotype 3 showed close sequence relationships, while dengue virus serotype 4 was relatively distinct from the other types (Haishi et al., 1990).

6. Clinical features

Dengue virus infection in human may be asymptomatic or may lead to undifferentiated fever, dengue fever, or dengue hemorrhagic fever (WHO, 1986).

6.1. Undifferentiated fever

Some infants and children who are infected with dengue virus for the first time develop simple fever indistinguishable from other viral infections. Maculopapular rashes may accompany the fever (Nimmannitya, 1993).

6.2. Dengue fever

Dengue fever is most common in adults and older children. It is an acute biphasic fever with headache, myalgia, arthralgia, rashes and leucopenia (Nimmannitya, 1993; WHO, 1986).

6.3. Dengue hemorrhagic fever

Dengue hemorrhagic fever is most common in children less than 15 years old; it is characterized by acute fever associated with hemorrhagic diathesis and hepatomegaly. Abnormal hemostasis and plasma leakage are the main pathophysiological changes. Thrombocytopenia and signs of hemoconcentration are important findings (Nimmannitya, 1993; WHO, 1986).

In most children and adults with dengue hemorrhagic fever, serologic studies always revealed secondary antibody response against flavivirus antigens (Burke et al., 1988; Kliks et al., 1989). However, the mechanism by which the secondary infection leads to dengue hemorrhagic fever is still unknown. It has been proposed that circulating antibodies resulted from primary infection can bind to the secondarily infecting, heterologous viruses and enhance viruses uptake into mononuclear cells (Halstead, 1988). The enhanced infection may probably result in higher viral load and severe disease (Vaughn et al., 1996). In contrast, differences in viral virulence has been thought to be the underlying cause of dengue hemorrhagic fever by other (Rosen, 1977; Pang, 1987).

Recently, hyperendemicity of dengue infection has been suggested to be a prerequisite for the occurrence of DHF at the population level (Gubler and Trent, 1994). Although this hypothesis does not provide any mechanism to explain the presence of DHF at the individual level, it is compatible with both the immune enhancement and the virus virulence hypothesis. Supposedly, simultaneous circulation of multiple virus serotypes in an area increases the probability of secondary dengue infection and of immune enhancement. At the same time, hyperendemicity and increased movement of dengue viruses between geographic regions also increase the

probability that new and possibly more virulent strains of dengue virus will occur in the same area (Gubler and Trent, 1994).

B. Epidemiology

1. Global level

Geographical distribution of dengue viruses is worldwide, involving nearly all tropical and subtropical countries (reviewed by Thongcharoen and Jatanasen, 1993). At the end of the XVIIIth century, the first pandemic of dengue fever was observed in Philadelphia in 1780. The virus and transmitting mosquito were introduced by ships engaged in the sugar, rum and slave trade between Africa, colonial American and Caribbean ports (Halstead, 1992). This first pandemic produced reports of sporadic dengue outbreaks in the United States of America, Caribbean and South American coastal cities. A second known epidemic of dengue infection was found in semi-tropical northern Queensland where the human population was growing rapidly from gold and sugar trade. The storage of water from the roof runoff rain resulted in the increase of *Aedes aegypti* populations that supported continuous dengue activity in Australia from the 1870s until the World War I (Halstead, 1992).

Prior to the 1950, the epidemic of dengue infection was rare and occurred at 10 to 40 year intervals. Because both the viruses and the mosquito vectors relied on sea-going vessels for transport between countries of the tropics, epidemic would begin at inport cities and subsequently moved inland after the introduction of a new virus strain (Gubler and Trent, 1994). Early in the World War II, dengue viruses were carried by combatants from South-East Asia to Japan and the Pacific Islands, including Hawaii.

The consequence of the war such as temporary housing for war refugees, the explosive post-war growth of populations through high fertility and rural-to-urban migration, and the deterioration of urban environments, have led to the growth in density and the area occupied by *Aedes aegypti*. These factors have resulted in the endemic transmission of all four dengue serotypes in most of the Asian tropics (Halstead, 1992). Because of the decrease in the eradication activities of *A. aegypti* in the tropical American countries, the introduction and spread of dengue viruses beginning in the 1960s. Where once they were absent, dengue viruses have invaded Cuba, many of the Caribbean Islands, Mexico, the United States, most of Central America, Columbia, Ecuador, Peru, Paraguay, Bolivia, Argentina and Brazil (Gubler and Trent, 1994). In the 1990s, dengue had spread north to China, including the province of Taiwan, and south to Queensland (Australia) and repeatedly eastward to nearly all of the Pacific Islands. In Africa and the Middle East, areas of epidemic activity include Kenya, Mozambique, Somalia and Yemen (Halstead, 1992).

2. Asia

The first outbreak of dengue hemorrhagic fever in Asia was recognized in Manila, the Philippines (Quintos et al., 1954). In Thailand, the first outbreak occurred in 1958 in Bangkok-Thonburi and nearby areas. Almost 2,500 cases and a 10% case fatality rate were recorded. Most of the affected people were children under 10 years old (reviewed by Thongcharoen and Jatanasen, 1993). In 1962, the disease started to spread to other big cities and thereafter to throughout the country (Nimmannitya, 1987). After the first severe outbreak of dengue fever in countries in Asia between 1954 and 1958, epidemics have occurred every year and all four dengue serotypes have

been isolated (reviewed by Thongcharoen and Jatanasen, 1993). The highest record of DHF in Thailand was reported in 1987 (174,285 cases with 1,007 deaths) (Ungchusak, 1987; Ungchusak and Kunasol, 1988; Thongcharoen and Jatanasen, 1993). DHF/DSS in Thailand is associated with a secondary infection with dengue serotype 2 virus whereas in Malaysia and Indonesia dengue serotype 3 virus appears to be more important (Pang, 1987).

To determine what dengue serotype caused severe illness over time in Thailand, acute sera from children admitted to the Bangkok Children's Hospital with clinically suspected dengue infection from 1973 to 1989 were cultured for virus. There were at least three serotypes of dengue viruses that was recovered from this group of patients. This revealed that multiple dengue serotypes were simultaneously transmitted in Bangkok. During this period, dengue serotype 2 viruses were most consistently isolated from hospitalized children (Nisalak et al., 1990). The study of dengue infection in Bangkok between June 1980 to January 1981 also indicated that dengue serotype 2 viruses accounted for most of the cases of DHF observed in Bangkok (Burke et al., 1988).

Dengue infection was also the major public health problem in other countries beyond Thailand. A dengue-like illness has been reported in Yangon, Myanmar since 1954. In 1975, the viruses spreaded to other parts of the country with the highest incidence occurring in Mandalay. The results of virus isolation studies in 1976 indicated that dengue serotype 2 and 3 viruses were most commonly isolated (reviewed by Jatanasen and Thongcharoen, 1993). In Indonesia, the first outbreak of DHF occurred in Jakarta and Surabaya in 1968. The following year, DHF spreaded to other parts of the country (reviewed by Jatanasen and Thongcharoen, 1993). All four dengue serotypes were present but dengue serotype 3 virus was most commonly associated

with DHF/DSS in Indonesia (Pang, 1987). In India, the first recorded outbreak of dengue fever was in 1812 and all four serotypes could be detected. In Sri Lanka, DHF was reported in 1965. After the first episode, small outbreaks with only one to four cases per year were reported (reviewed by Jatanasen and Thongcharoen, 1993).

3. Molecular epidemiology

Several molecular biological techniques have been employed in order to study fine differences of dengue viruses isolated from diverse geographic areas and also at different time periods. These techniques include RNase T1 oligonucleotide fingerprinting, restriction enzyme analysis, and nucleotide sequence analysis. From these analyses, evolutionary relationship can be determined by employing phylogenetic methods. Available data indicate independent evolution and spread of dengue virus serotypes throughout the world and they are considered separately.

3.1. Dengue serotype 1 virus

Fine differences in the RNA genome of dengue viruses were first studied by RNase T1 oligonucleotide fingerprinting of 12 dengue serotype 1 viruses isolated from various parts of the world (Repik et al., 1983). The fingerprints of isolates derived from the same geographic area were very similar but different from those of the other areas. From this limited set of viruses, dengue serotype 1 viruses were subdivided into three genetic groups; those with high (45-100 %) percentage of shared oligonucleotides were included in the same group. The three genetic subgroups of dengue serotype 1 were: the Caribbean subgroup (Jamaica and Bahamas), the African subgroup (Nigeria),

and the Pacific/Southeast Asian subgroup (Hawaii, Nauru Island, Bangkok and Sri Lanka) strains (Repik et al., 1983).

3.2. Dengue serotype 2 virus

Similar to dengue serotype 1 viruses, a study of dengue serotype 2 virus isolates from various geographic areas by oligonucleotide fingerprinting also revealed that the fingerprints of the viruses isolated from each of the geographic region were unique and different from the other regions (Trent et al., 1983). This result was confirmed by Monath et al, in 1986, in the comparison of dengue serotype 2 viruses that isolated from different locations by using the antigen signature analysis. They found that a high degree of antigenic similarity was found among strains from the same geographic region (Monath et al., 1986).

To determine the changes through time of dengue serotype 2 viruses, Trent et al (1989) analyzed dengue serotype 2 viruses collected for more than 25 years (1962-1986) from DHF patients and *A. aegypti* mosquitoes in Thailand, Burma and Vietnam by computer assisted T1-RNase-resistant oligonucleotide fingerprinting. Individual dengue serotype 2 isolates have a constant number of large oligonucleotide fingerprints. The consensus fingerprints were constructed from the conserved oligonucleotides that occurred in the majority (> 50%) of the fingerprints. When fingerprints of dengue serotype 2 viruses collected from the 1962-1986 were compared with the consensus fingerprints, they found that dengue serotype 2 viruses that were isolated from the same decade had a constant set of oligonucleotides that differed from the other decades. Therefore, the consensus fingerprints of dengue serotype 2 viruses analyzed in this study could be segregated into three subgroups (1960s, 1970s, and 1980s)

according to the decades in which the viral were isolated. The changes in the oligonucleotide fingerprint patterns from one decade to the next revealed that RNA genome of dengue serotype 2 virus gradually changed through time (Trent et al., 1989).

Dengue serotype 2 viruses have been classified into distinct subtypes by employing several methods. Employing the oligonucleotide fingerprinting technique, Trent et al (1983) classified dengue serotype 2 viruses isolated from various geographic regions into five subtypes (genotype) (Trent et al., 1983):

Genotype I contained viruses from the South Pacific and Puerto Rico;

Genotype II contained isolates from Jamaica and West Africa;

Genotype III included viruses from Burma and Thailand;

Genotype IV included isolates from the Philippines;

Genotype V contained isolates from Seychelle Islands.

Quite similar results were obtained by Rico-Hesse (1990) who studied 40 strains of dengue serotype 2 viruses, that were isolated over the period of 45 years from humans and mosquitoes in 36 countries, by primer-extension sequencing and pairwise comparison of the nucleotide sequence at the E-NS1 junction. When a genotype was defined as a group of dengue viruses with no more than 6% sequence divergence at the E/NS1 gene junction, 5 distinct genotypic groups were detected (Rico-Hesse, 1990):

Genotype I contained viruses from the Caribbean and South Pacific;

Genotype II contained isolates from Taiwan, the Philippines, the New Guinea prototype virus and the 1964 Thai strains;

Genotype III included Vietnamese, Jamaica and Thai strains;

Genotype IV contained isolates from Indonesia, the Seychelles, Burkina Faso and Sri Lanka;

Genotype V included isolates from rural Africa.

Similarly, Thant et al (1995) also compared the E/NS1 gene junction of 56 dengue serotype 2 viruses and classified them into five genotypic groups which corresponded to that of Rico-Hesse (Thant et al., 1995).

These results were confirmed by the sequence analysis of the entire envelope glycoprotein gene. Lewis et al (1993) compared the nucleotide sequence of the E glycoprotein gene of 33 dengue serotype 2 viruses by the maximum parsimony analysis. Again, five genetic subtypes were identified (Lewis et al., 1993):

Genotype I contained viruses from Sri Lanka and the New Guinea prototype viruses;

Genotype II included the Philippines and Taiwan strains;

Genotype III contained isolates from Jamaica, Thai, Malaysia and Brazil strains;

Genotype IV included isolates from Indonesia, Sri Lanka, the Seychelles, Burkina Faso and Somalia;

Genotype V contained isolates from Puerto Rico, Trinidad, Tonga and India.

In a subsequent study of dengue viruses in Thailand, Sittisombut et al (1997) analyzed the envelope gene sequences of 20 additional dengue serotype 2 viruses by the maximum likelihood analysis. The five distinct genotypes that they found were identical to the subtypes reported previously by Lewis et al (Sittisombut et al., 1997).

The classification of dengue serotype 2 viruses into five distinct genotypes based on the comparison of the nucleotide sequence of the entire envelope gene or at the E/NS1 junction is summarized in Table 1. Group of viruses that were classified by the pairwise sequence comparison and by the maximum parsimony analysis corresponded

Table 1. The genotypic comparison of dengue serotype 2 viruses from various studies.

Rico-Hesse (1990)[1]	Thant et al (1995)[1]	Lewis et al (1993)[2]	Sittisombut et al[2] (1997)
Genotype I: Caribbean, South Pacific	I	V	V
Genotype II: Taiwan, Philippine, New Guinea Prototype, older Thai strains	II	II, IIIa	II, IIIa
Genotype III: Vietnamese, Jamaica, Thai	III	IIIb	IIIb
Genotype IV: Indonesia, Seychelles, Burkina Faso, Sri Lanka	IV	IV	IV
Genotype V: rural Africa	V	-	-
-	-	Genotype I: Sri Lanka, New Guinea	I

1, analysis by pairwise comparison of the E/NS1 gene sequences. 2, analysis by maximum parsimony analysis of the E gene sequence.

well to each other. However, because the designation of each particular subtype was arbitrary, the exact identification number for the subtype may not be identical.

Molecular epidemiologic study of dengue serotype 2 viruses in Thailand was done by several workers. Walker and his colleague (1988) analyzed 10 dengue serotype 2 viruses isolated in Bangkok in 1980 to determine the extent of variation within a serotype. By employing the oligonucleotide fingerprinting technique and restriction enzyme mapping, they found that one dengue serotype 2 strain, D80-141, produced distinctly different T1 oligonucleotide fingerprint and restriction enzyme map when compared with other isolates. This isolate also lacked an epitope on the NS1 protein which was commonly present in other Bangkok isolates (Walker et al., 1988). This is the first indication of the presence of the minor subtype of dengue serotype 2 which co-circulated with the major subtype in Bangkok in 1980. This result was confirmed by Blok et al (1989) when they determined and compared the sequences of the envelope glycoprotein gene of five Thai and three Sri Lanka dengue serotype 2 viruses. They found that Thai isolates could be divided into two groups while the Sri Lanka isolates were distinct (Blok et al., 1989). The segregation of dengue serotype 2 into two groups was also evident when the NS1 sequences of this group of viruses were compared (Blok et al., 1991). Furthermore, phylogenetic studied by Lewis et al, in 1993, indicated that viruses from Thailand, Malaysia, Jamaica and Brazil formed a major branch in the phylogenetic tree that diverged into two distal branches, designated subtype IIIa and subtype IIIb.

To further delineate the genetic relationship and evolution of dengue serotype 2 viruses in Bangkok, Sittisombut et al (1997) determined the E gene sequences of nineteen dengue serotype 2 viruses isolated during the 1987 epidemic season. The comparison using the maximum likelihood method revealed that the subtype IIIa

viruses represented the majority (18/19) of dengue serotype 2 viruses in this year. Furthermore, a comparison of the E gene sequences of the 1987 strains with earlier isolates revealed three types of substitutions: 1) the substitutions that were unique to individual isolate or group of isolates; 2) the substitutions that were common to the subtype IIIa and subtype IIIb viruses of both the 1980 and 1987 epidemic seasons; and 3) the substitutions that were shared by all subtype IIIa or subtype IIIb viruses of the 1987 epidemic seasons but were absent from the corresponding viruses in 1980. For the subtype IIIa viruses, the third type of substitutions was the His346→Tyr substitution which had never been reported in any dengue serotype 2 viruses (Sittisombut et al., 1997). The third type of substitution was suggestive of the occurrence of population (genetic) bottleneck in Bangkok between 1980 and 1987.

There is scant information on dengue serotype 2 viruses from other parts of Thailand. A study of dengue serotype 2 viruses that circulated in Maha Sarakham by Duangchanda et al (1994) revealed that 3/3 viruses isolated in 1986-1987 were all subtype IIIb strains. In contrast, 1/1 dengue serotype 2 viruses isolated from Kanchanaburi in 1987 (Sittisombut et al., 1997), 3/3 that isolated from Chiang Mai in 1991 (Sistayanarain et al., 1996) and 4/4 that isolated from Nakhon Phanom in 1993 (Thant et al., 1995) were all subtype IIIa strains. These results indicated that there was an uneven distribution of dengue serotype 2 subtypes in various localities of Thailand.

In the American region, the subtype IIIb viruses were associated with the epidemic of dengue infection. Concurrent with the outbreak of DHF in Cuba in 1981, dengue serotype 2 virus subtype IIIb was first isolated in Jamaica. This virus is genetically related to the subtype IIIb dengue serotype 2 viruses isolated in Thailand in

1980. This subtype IIIb viruses spread rapidly throughout the Americas during the 1980s and is currently the dominant genotype in this region (Gubler and Trent, 1993).

3.3. Dengue serotype 3 virus

The relationship among dengue serotype 3 viruses from several regions and the worldwide distribution of dengue serotype 3 virus was studied by Lanciotti et al (1994) by analyzing the nucleotide sequences of the pre-M, M and E protein genes of 23 geographically and temporally distinct dengue serotype 3 viruses. The similarity among the viruses from similar geographical regions was greater than 90%. Phylogenetic analysis of these viruses indicated that dengue serotype 3 viruses could be separated into four distinct subtypes. Subtype I consisted of viruses from Indonesia, Malaysia, the Philippines and the South Pacific island; subtype II included isolates from Thailand; subtype III contained isolates from Sri Lanka, India, Africa and Samoa; and subtype IV included the Puerto Rico and the 1965 Tahiti viruses (Lanciotti et al., 1994). This separation of dengue serotype 3 viruses into four subtypes corresponded well to the study of Chungue et al. (1993).

3.4. Dengue serotype 4 virus

Phylogenetic analysis of dengue serotype 4 viruses based on the sequence of the E glycoprotein gene can divide this serotype into two subtypes. The first subtype contains viruses from the South Pacific islands, Indonesia, and Americas; and the second contains viruses from Thailand and Philippines (Gubler and Trent, 1993).