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Comparative Analysis of Full-Length Genomic Sequences of 10 Dengue Serotype 1 Viruses Associated with Different Genotypes, Epidemics, and Disease Severity Isolated in Thailand over 22 Years

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Abstract. Comparative sequence analysis was performed on the full-length genomic sequences of 10 representative dengue virus serotype 1 (DENV-1) strains sampled from patients at Children's Hospital, Bangkok, Thailand over a 22-year period, which represented different epidemics, disease severity, and sampling time. The results showed remarkable inter-genotypic variation between predominant and non-predominant genotypes and genotype-specific amino acids and nucleotides throughout the entire viral genome except for the 5′-non-translated region. The frequency of intra-genotypic variation was correlated with dengue transmission rate and sampling time. The 5′-non-translated region of all 10 viruses was highly conserved for predominant and non-predominant genotypes and NS2B was the most conserved protein. Some intra-genotypic substitutions of amino acids and nucleotides in predominant genotype strains were fixed in the viral genome since 1994, which indicated that the evolution of predominant genotype strains *in situ* over time might contribute to increased virus fitness important for sustaining dengue epidemics in Thailand.

INTRODUCTION

Dengue is the most important arthropod-borne viral disease of humans in the tropics and subtropics.[1–3] It is caused by the dengue viruses (DENVs), which are single-stranded, positive-sense RNA viruses in the family *Flaviviridae*, genus *Flavivirus* and are transmitted to humans primarily by the peridomestic mosquito species *Aedes aegypti*. Dengue is associated with a diverse spectrum of diseases ranging from acute febrile dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). With increasing urbanization in tropical developing countries, international travel, lack of effective mosquito control, and globalization,[2,3] dengue is now rapidly spreading around the world. The global burden of dengue has increased as much as four-fold during the past three decades, and almost half the world's population is at risk for infection.[3] Annually, there are an estimated 50–100 million cases, with approximately 500,000 cases of DHF/DSS, and 25,000 deaths.[2–4]

The DENVs are comprised of four antigenically distinct but closely related serotypes, denoted DENV-1 to DENV-4, all of which can cause dengue disease. DENVs are further subdivided into subtypes or genotypes according to their genetic similarity/divergence. Three genotypes of the DENV-1 have been defined.[5,6] The approximately 11-kb DENV genome encodes three structural proteins: capsid (C), pre-membrane/membrane (prM/M), and envelope (E), and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, flanked by 5′- and 3′-non-translated regions (5′-NTR/3′-NTRs). The DENV genome is expected to rapidly accumulate mutations because of the error-prone nature of viral RNA polymerase.[7,8] Mutations that result in increased virus fitness then become fixed in the genome as the DENVs spread worldwide. Such genetic changes may have significant implications for the emergence of new genotype(s) and lead to viruses with altered antigenicity, virulence, or tissue tropism, and eventually influence disease patterns and transmission.[9]

Thailand has had some of the most severe dengue epidemics in the world. The dengue incidence in Thailand increased dramatically from 21/100,000 in 1973 to 211/100,000 in 1998, and was accompanied by an increase in disease severity.[10] Bangkok is at the epicenter of dengue transmission in Thailand with all four DENV serotypes co-circulating there since 1962.[11–13] Dengue has been hyperendemic in Bangkok since 1979. The predominant DENV serotype varies by year: DEN-1 during 1990–1992 and 2001; DEN-2, responsible for causing more severe dengue diseases during 1973–1986 and 1988–1989; DEN-3, the only serotype associated with severe outbreaks in 1987 and 1995–1999; and DEN-4 in 1993–1994.

According to records of the Queen Sirikit National Institute of Child Health (QSNICH) in Bangkok, the serotype associated with the highest incidence of disease is DENV-3, which is responsible for 46% of DF and 50% of DHF after primary infection and 34% of DF and 28% of DHF after secondary infection. This serotype is followed by DENV-1, which is responsible for 46% of DF and 44% of DHF cases after primary infection and 29% of DF and 20% of DHF after secondary infection, DENV-2, which is responsible for 6% of DF and 4% of DHF after primary infection and 24% of DF and 40% of DHF after secondary infection, and DENV-4 which is responsible for 2% of DF and DHF after primary infection and 13% of DF and 12% DHF after secondary infection.[10]

The causes of the increased incidence of DENV-1 infection and its association with more severe forms of disease are not fully understood but it is believed that host and viral factors are involved. Because of lack of suitable *in vivo* and *in vitro* disease models, it is difficult to directly identify those factors responsible for the increased incidence and severity of dengue. Comparative analyses of complete viral genomic sequences from samples with well-defined epidemiologic information and clinical records may be an alternative approach to define genetic markers associated with genotype, infectivity, and disease severity.

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The Armed Force Research Institute of Medical Sciences (AFRIMS) maintains a DENV bank containing thousands of DENV-positive samples sequentially collected from patients at Children's Hospital, QSNICH in Bangkok since 1973. This rich resource provides valuable samples for studies of molecular epidemiology, virus evolution, and viral pathogenesis. We have investigated molecular epidemiology of Thai DENVs.[6,14,15] Results showed that multiple serotypes, genotypes, and clades have been co-circulating but the predominant genotype for each serotype remained unchanged in the Bangkok area over the past three decades, suggesting that the observed increase in DEN incidence was not caused by introduction of new genotypes, but by existing strains.

This observation led us to hypothesize that the viruses currently circulating might be genetically different from those that circulated in the past because of mutations in the viral genome that enable the virus to counter the antiviral responses of the host. Therefore, in this study, we performed a comparative analysis of complete genomic sequences for 10 representative DENV-1 isolates sampled from the QSNICH in Bangkok during different dengue epidemic periods. These periods represented different disease severities, sampling time, and genotypes over a 22-year time span for which we characterized virus genetic variations among genotypes, clades, and strains with different isolation time and epidemics and defined possible virulence determinants in the viral genome associated with genotype, transmission, and disease severity.

MATERIALS AND METHODS

Viruses. Ten DENV-1 strains representing two co-circulating genotypes (predominant genotype I and non-predominant genotype III), two clades (1980–1994 clade and 1990–2001 clade), two forms of disease outcome (DF/DHF), and different epidemic periods (high, intermediate, and low) were selected from a sample pool of 129 DENV-1 isolates that were used for investigation of molecular epidemiology and virus evolution processes of Thai DENVs in our previous study. The pool of 129 DENV-1 samples was sequentially selected from a large bank of DENV-positive serum specimens collected from pediatric patients admitted to the QSNICH in Bangkok during 1973–2001. Grading of disease severity was conducted by QSNICH physicians using World Health Organization classification guidelines. Further testing at AFRIMS characterized the specimens according to their DENV serotype and nature of the DENV infection. Primary versus secondary DENV infection was determined solely by hemagglutination assay inhibition before 1990 and by immunoglobulin M antigen-capture enzyme-linked immunosorbent assay supported by hemagglutination inhibition assay subsequently. Viral collections prior to 1980 had been isolated after 2–3 serial passages in LLC-MK2 cells; subsequently, they were isolated after amplification in *Toxorhynchites splendens* mosquitoes and 1–3 passages in C6/36 cells. Because of insufficient quantities of viruses for sequencing of isolates collected before 2000, these viruses were amplified from viremic serum samples in C6/36 cells (three passages) for this study.

Ten samples were grouped into five pairs with each pair containing one DF and one DHF virus isolated in the same year. Because this study is a continuation of previous studies, the background data pertaining to virus isolation and serologic, molecular, and epidemiologic characterization is described elsewhere.[6,10,14,15] The complete genomic sequences of the 10 DENVs are available in GenBank. A summary of these isolates, including genotypes, sampling years, patient demographic information, disease severity, transmission ability, and the accession numbers of viral complete genomic sequences in GenBank, is shown in Table 1.

Viral RNA extraction, reverse transcription–polymerase chain reaction, and sequencing of virus genome. Virus RNA was extracted from cell culture supernatant by using Trizol LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The extracted viral RNA was used for reverse transcription–polymerase chain reaction amplification. Oligonucleotide primers for reverse transcription–polymerase chain reaction amplification and sequencing were designed by using the primer design program (www.hgmp.mrc.ac.uk/GenomeWeb/nuc-primer.html) and have been described.[6]

Comparative analyses of full-length genomic sequences. The full-length genomic sequences obtained from sequencing and deduced amino acid sequences of 10 Thai DENV-1 strains were aligned using the ClustalW in DNAStar Lasergene V8.0 (DNASTAR, Inc., Madison, WI). ThD1-0008-81 sampled in 1981, the earliest isolate of the predominant genotype I strains in this sample set, was used as a reference strain for the later isolates to compare individual amino acid and nucleotide sequences. The total number and percentage of deduced amino acid and nucleotide substitutions in each gene and both 5′- and 3′- NTRs of these viruses were recorded and organized into groups on the basis of the protein or non-coding region represented.

Table 1

Sample information of 10 dengue virus serotype 1 strains isolated from patients admitted to the Queen Sirikit National Institute of Child Health, Bangkok, Thailand*

Sample name†	Transmission rate‡	Diseases severity§	Dengue infection§	Patient sex	Patient age (years)	GenBank accession no.
ThD1-0442-80	High	DHF I	Primary	M	7	AY732476
ThD1-0673-80	High	DF	Primary	M	3	AY732474
ThD1-0008-81	Low	DHF II	Primary	F	0.5	AY732483
ThD1-0081-82	Low	DF	Primary	F	12	AY732481
ThD1-0323-91	Intermediate	DHF III	Primary	M	4	AY732478
ThD1-0336-91	Intermediate	DF	Secondary	F	7	AY732477
ThD1-0097-94	Low	DHF II	Secondary	M	10	AY732480
ThD1-0488-94	Low	DF	Primary	M	4	AY732475
ThD1-0102-01	High	DHF II	Secondary	M	7	AY732479
ThD1-0049-01	High	DF	Secondary	M	11	AY732482

* DHF = dengue hemorrhagic fever; DF = dengue fever.
† The last two numbers of the sample name indicate the year of isolation.
‡ Transmission rate is determined according to the annual case rate at the Queen Sirikit National Institute of Child Health.
§ Defined on the basis of World Health Organization criteria.

Analyses of RNA secondary structures of 5′- and 3′-termini. The secondary – structures of 5′-NTR/3′-NTR and the 5′–3′ end cyclization of each virus strain, were folded by using online software MFOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) according to a previous protocol.[16] Briefly, 94 nucleotides of 5′-NTR and 462 nucleotides of 3′-NTR of each DENV-1 strain were used to generate RNA secondary structures with a minimum free energy (ΔG) and other default parameters in MFOLD. For the folding of the 5′–3′end cyclization, the first 136 nucleotides, which contain the 5′-NTR (94 nucleotides) and the first 42 nucleotides of the C gene, including the AUG start codon, were juxtaposed with the last 120 nucleotides of the 3′-NTR with an insertion of a 50 adenine sequence between the 5′- and the 3′- end to represent the rest of the viral genome. Each folded RNA secondary structure of the virus was then analyzed manually.

RESULTS

Inter-genotype comparison. Comparative analysis of 10 complete viral genomic sequences showed remarkable inter-genotype variations between predominant and non-predominant genotype strains, with changes throughout the entire genome except for the 5′-NTR. For example, non-predominant genotype III strains (ThD1-0442-80 and ThD1-0673-80) sampled in 1980 had 80 and 78 amino acid substitutions, respectively, compared with the reference strain, ThD1-0008-81 (genotype I) sampled in 1981. The differences represented almost 2.4% of the total amino acid substitutions, which was higher than that of all other genotype I strains and indicated remarkable genetic variations between genotypes (Table 2). Genotype-specific amino acids and nucleotides were observed in each gene and region except for the 5′-NTR, which was highly conserved in predominant and non-predominant genotypes. Significant genetic variation was observed within the coding region, in particular, the NS2A gene of two non-predominant genotype III strains (ThD1-0442-80 and ThD1-0673-80), which had 12 amino acid differences (5.5%) compared with the reference genotype I strain (Table 2). Of 12 amino acid differences in the NS2A protein, nine were genotype III specific (Table 3) and might be considered genotype III determinants. When genotype III strains were compared with the reference strain, the NS2A protein had the highest percentage of amino acid substitutions, followed by prM/M > NS1 > E > C > NS4A > NS3 > NS4B > NS5 > NS2B, and the highest number of amino acid substitutions irrespective of protein size was in the E protein, followed by NS5 > NS2A > NS3 > NS1 > prM/M > NS4B > NS4A > C > NS2B proteins (Table 2).

Two non-predominant genotype III strains had 54 unique inter-genotypic amino acid changes distributed in each protein (Table 3). Notably, when compared with all predominant genotype I strains, 4 of 54 unique amino acid variations in the two genotype III strains were associated with a charge change, one at residue 37 (N37D, from neutral N to negative charge D) in the E protein, one at residue 139 (D139N, from negative D to neutral N) in the NS1 protein, one at residue 162 (D162H, from negative charge D to neutral/positive charge H) in the NS2A protein, and one at residue 649 (H649Y, from positive H to neutral Y) in the NS5 protein. The amino acid charge changes may cause changes in protein structure and function.

Table 2

Number and percentage of amino acid and nucleotide substitutions among each gene and 5′ and 3′ non-translated regions of 10 Thai dengue virus serotype 1 strains, Thailand*

Viruses	No. (%) aa substitutions	No. (%) aa substitutions in each gene										No. (%) nt differences in 3′NTR (462 nt)†
		C (114 aa)	prM/M (166 aa)	E (495 aa)	NS1 (352 aa)	NS2A (218 aa)	NS2B (130 aa)	NS3 (619 aa)	NS4A (150 aa)	NS4B (249 aa)	NS5 (899 aa)	
ThD1-0442-80‡	80 (2.4)	3 (2.63)	5 (3.01)	15 (3.03)	11 (3.13)	12 (5.50)	1 (0.77)	12 (1.94)	3 (2.00)	4 (1.61)	14 (1.56)	18 (3.90)
ThD1-0673-80‡	78 (2.3)	3 (2.63)	6 (3.61)	13 (2.63)	11 (3.13)	12 (5.50)	1 (0.77)	11 (1.78)	4 (2.67)	4 (1.61)	13 (1.45)	18 (3.90)
ThD1-0008-81§	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ThD1-0081-82§	12 (0.35)	0 (0)	1 (0.60)	1 (0.20)	0 (0)	2 (0.92)	0 (0)	6 (0.97)	0 (0)	1 (0.40)	1 (0.11)	1 (0.22)
ThD1-0323-91§	25 (0.74)	2 (1.75)	0 (0)	3 (0.61)	1 (0.28)	5 (2.29)	0 (0)	3 (0.48)	0 (0)	1 (0.40)	10 (1.11)	4 (0.87)
ThD1-0336-91§	23 (0.68)	2 (1.75)	0 (0)	4 (0.81)	1 (0.28)	6 (2.75)	0 (0)	3 (0.48)	0 (0)	1 (0.40)	6 (0.67)	1 (0.22)
ThD1-0097-94§	29 (0.85)	1 (0.88)	2 (1.20)	6 (1.21)	3 (0.85)	4 (1.83)	0 (0)	4 (0.65)	2 (1.33)	2 (0.80)	5 (0.56)	7 (1.52)
ThD1-0488-94§	32 (0.94)	3 (2.63)	3 (1.80)	6 (1.21)	3 (0.85)	4 (1.83)	1 (0.77)	4 (0.65)	2 (1.33)	1 (0.40)	5 (0.56)	7 (1.52)
ThD1-0049-01§	47 (1.39)	3 (2.63)	2 (1.20)	8 (1.62)	5 (1.42)	4 (1.83)	0 (0)	6 (0.97)	1 (0.67)	3 (1.20)	15 (1.67)	9 (1.95)
ThD1-0102-01§	45 (1.33)	2 (1.75)	2 (1.20)	7 (1.41)	5 (1.42)	4 (1.83)	0 (0)	6 (0.97)	1 (0.67)	3 (1.20)	15 (1.67)	9 (1.95)

* aa = amino acid; prM/M = premembrane/membrane; E = envelope; NS = nonstructural; nt = nucleotide; NTR = non-translated region.
† There were no nucleotide differences in the 5′NTR.
‡ Genotype III.
§ Genotype I.

TABLE 3
Total amino acid/nucleotide substitutions among the coding regions and 3′ NTR of 10 Thai dengue virus subtype 1 strains, Thailand*

Viruses	ThD1-0442-80	ThD1-0673-80	ThD1-0008-81	ThD1-0081-82	ThD1-0323-91	ThD1-0336-91	ThD1-0097-94	ThD1-0488-94	ThD1-0102-01	ThD1-0049-01
Disease severity	DHF I	DF	DHF II	DF	DHF III	DF	DHF II	DF	DHF II	DF
Epidemic†	High	High	Low	Low	Intermediate	Intermediate	Low	Low	High	High
Genotypes	III	III	I	I	I	I	I	I	I	I
C (114 aa)										
(AA9)	G	G	G	G	G	G	**A**	**A**	**A**	**A**
(AA26)	(G)	(G)	V	V	V	V	V	V	V	V
(AA90)	(S)	(S)	N	N	N	N	N	N	N	N
(AA112)	A	A	A	A	[V]	[V]	A	A	A	A
prM/M (166 aa)										
(AA15)	S	S	T	T	T	T	**S**	**S**	**S**	**S**
(AA118)	(R)	(R)	K	K	K	K	K	K	K	K
(AA122)	(K)	(K)	R	R	R	R	R	R	R	R
(AA143)	(T)	(T)	A	A	A	A	A	A	A	A
E (495 aa)										
(AA8)	N	N	N	N	N	N	**S**	**S**	**S**	**S**
(AA37)	(N)‡	(N)‡	D	D	D	D	D	D	D	D
(AA155)	(T)	(T)	S	S	S	S	S	S	S	S
(AA161)	(I)	(I)	T	T	T	T	T	T	T	T
(AA225)	S	S	S	S	[T]	[T]	S	S	S	S
(AA272)	(M)	(M)	T	T	T	T	T	T	T	T
(AA297)	(V)	(V)	M	M	M	M	M	M	M	M
(AA324)	V	V	V	V	V	V	**I**	**I**	**I**	**I**
(AA343)	K	K	K	K	K	K	R	R	K	K
(AA351)	L	L	V	V	V	V	**L**	**L**	**L**	**L**
(AA369)	(T)	(T)	A	A	A	A	A	A	A	A
(AA380)	I	I	V	V	V	V	**I**	**I**	**I**	**I**
(AA397)	S	S	S	S	[T]	[T]	S	S	S	S
(AA439)	(V)	(V)	I	I	I	I	I	I	I	I
(AA461)	(I)	(I)	V	V	V	V	V	V	V	V
(AA484)	(M)	(M)	L	L	L	L	L	L	L	L
NS1 (352 aa)										
(AA93)	(A)	(A)	V	V	V	V	V	V	V	V
(AA94)	(N)	(N)	S	S	S	S	V	V	A	A
(AA98)	(T)	(T)	A	A	A	A	A	A	A	A
(AA128)	(I)	(I)	V	V	V	V	V	V	V	V
(AA139)	(D)‡	(D)‡	N	N	N	N	N	N	N	N
(AA178)	(M)	(M)	V	V	V	V	V	V	V	V
(AA224)	I	I	V	V	V	V	V	V	I	I
(AA227)	(R)	(R)	K	K	K	K	K	K	K	K
(AA286)	V	V	I	I	**V**	**V**	**V**	**V**	**V**	**V**
(AA347)	(R)	(R)	K	K	K	K	K	K	K	K
NS2A (218 aa)										
(AA2)	S	S	S	L	L	L	S	S	S	S
(AA15)	(V)	(V)	I	I	I	I	I	I	I	I
(AA41)	F	F	L	L	L	L	L	L	F	F
(AA64)	(V)	(V)	A	A	A	A	A	A	A	A
(AA133)	(M)	(M)	I	I	I	I	I	I	I	I
(AA147)	(T)	(T)	A	A	A	A	A	A	A	A
(AA148)	T	T	A	A	A	A	**T**	**T**	**T**	**T**
(AA155)	I	I	I	I	I	I	V	V	I	I
(AA159)	(L)	(L)	Y	Y	**F**	**F**	**F**	**F**	**F**	**F**
(AA162)	(D)‡	(D)‡	H	H	H	H	H	H	H	H
(AA168)	(I)	(I)	M	M	M	M	M	M	M	M
(AA182)	L	L	L	L	[M]	[M]	L	L	L	L
(AA199)	(F)	(F)	L	L	L	L	L	L	L	L
(AA210)	(T)	(T)	A	A	A	A	A	A	A	A
NS2B (130 aa)										
(AA112)	(M)	(M)	L	L	L	L	L	L	L	L
NS3 (619 aa)										
(AA26)	(L)	(L)	M	M	M	M	M	M	M	M
(AA43)	(D)	(D)	E	E	E	E	E	E	E	E
(AA44)	(G)	(G)	N	N	N	N	N	N	N	N
(AA85)	(F)	(F)	L	L	L	L	L	L	L	L
(AA112)	(T)	(T)	A	A	A	A	A	A	A	A
(AA185)	(K)	(K)	R	R	R	R	R	R	R	R
(AA255)	(R)	(R)	K	K	K	K	K	K	K	K
(AA293)	(A)	(A)	S	S	S	S	S	S	S	S
(AA323)	V	V	V	V	V	V	V	V	M	M
(AA350)	D	D	D	D	D	D	**E**	**E**	**E**	**E**
(AA368)	S	S	S	S	S	S	S	S	A	A
(AA466)	Q	Q	Q	Q	Q	Q	**H**	**H**	**H**	**H**

(Continued)

Table 3
Continued

Viruses	ThD1-0442-80	ThD1-0673-80	ThD1-0008-81	ThD1-0081-82	ThD1-0323-91	ThD1-0336-91	ThD1-0097-94	ThD1-0488-94	ThD1-0102-01	ThD1-0049-01
NS4A (150 aa)										
(AA19)	(L)	(L)	Q	Q	Q	Q	Q	Q	Q	Q
(AA39)	(K)	(K)	R	R	R	R	R	R	R	R
(AA76)	K	K	R	R	R	R	**K**	**K**	**K**	**K**
(AA93)	V	V	V	V	V	V	A	A	V	V
NS4B (249 aa)										
(AA19)	(V)	(V)	A	A	A	A	A	A	A	A
(AA20)	(A)	(A)	V	V	V	V	V	V	V	V
(AA27)	T	T	T	T	T	T	T	T	A	A
(AA31)	(I)	(I)	V	V	V	V	V	V	V	V
(AA148)	V	V	V	V	V	V	V	V	I	I
NS5 (899 aa)										
(AA34)	M	M	M	M	[I]	[I]	M	M	M	M
(AA108)	P	P	A	A	A	A	**P**	**P**	**P**	**P**
(AA127)	Y	Y	Y	Y	Y	Y	Y	Y	H	H
(AA135)	T	T	T	T	T	T	T	T	I	I
(AA270)	(I)	(I)	V	V	V	V	V	V	V	V
(AA285)	(N)	(N)	H	H	H	H	H	H	H	H
(AA325)	R	R	R	R	R	R	**K**	**K**	**K**	**K**
(AA365)	(P)	(P)	A	A	A	A	A	A	A	A
(AA367)	R	R	Q	Q	Q	Q	**R**	**R**	**R**	**R**
(AA372)	I	I	I	I	[V]	[V]	I	I	I	I
(AA378)	K	K	K	R	R	R	R	R	R	R
(AA428)	E	E	E	E	E	E	E	E	D	D
(AA513)	K	K	K	K	[R]	[R]	K	K	K	K
(AA565)	T	T	T	T	T	T	T	T	K	K
(AA585)	(S)	(S)	N	N	N	N	N	N	N	N
(AA616)	(V)	(V)	A	A	A	A	A	A	A	A
(AA631)	S	S	S	S	S	S	S	S	N	N
(AA635)	(N)	(N)	T	T	T	T	T	T	T	T
(AA636)	(S)	(S)	P	P	P	P	P	P	P	P
(AA649)	(H)‡	(H)‡	Y	Y	Y	Y	Y	Y	Y	Y
(AA669)	(T)	(T)	I	I	I	I	I	I	I	I
(AA746)	S	S	S	S	S	S	S	S	G	G
(AA807)	D	D	N	N	N	N	**D**	**D**	**D**	**D**
(AA891)	E	E	E	E	[D]	[D]	E	E	E	E
3′ NTR (462 nt)										
(nt2)	C	C	C	C	C	C	**T**	**T**	**T**	**T**
(nt3)	C	C	T	T	T	T	**C**	**C**	**C**	**C**
(nt10)	C	C	T	T	T	T	**C**	**C**	**C**	**C**
(nt12)	(C)	(C)	T	T	T	T	T	T	T	T
(nt14)	T	T	C	C	T	T	C	C	C	C
(nt15)	(G)	(G)	A	A	A	A	A	A	A	A
(nt31)	(G)	(G)	A	A	A	A	A	A	A	A
(nt32)	(A)	(A)	G	G	G	G	G	G	G	G
(nt38)	A	A	T	T	T	T	**A**	**A**	**A**	**A**
(nt44)	(A)	(A)	G	G	G	G	G	G	G	G
(nt45)	(A)	(A)	G	G	G	G	G	G	G	G
(nt62)	(T)	(T)	A	A	A	A	A	A	A	A
(nt167)	(G)	(G)	A	A	A	A	A	A	A	A
(nt193)	(C)	(C)	T	T	T	T	T	T	T	T
(nt262)	A	A	A	A	A	A	A	A	G	G
(nt264)	(G)	(G)	A	A	A	A	A	A	A	A
(nt268)	C	C	C	C	C	C	**T**	**T**	**T**	**T**
(nt347)	(C)	(C)	T	T	T	T	T	T	T	T

* NTR = non-translated region; DHF = dengue hemorrhagic fever; DF = dengue fever; aa = amino acid; prM/M = premembrane/membrane; E = envelope; NS = nonstructural; NTR = nontranslated region; nt = nucleotide. **Bold** letters indicate amino acid/nucleotide evolution. Letters in parentheses indicate amino acid substitutions observed among viruses sampled only from genotype III strains. Letters in brackets indicate amino acid substitutions observed among viruses sampled only from the intermediate epidemic.

† High, low, and intermediate indicate different dengue epidemic periods based on the rate of total dengue cases sampled at the Queen Sirikit National Institute of Child Health, Bangkok, Thailand during 1973–2001.

‡ Indicates the charge change of the amino acids.

Intra-genotype comparison. Comparison of eight predominant genotype I strains showed that the total number and percentage of amino acid and nucleotide substitutions ranged from 12 to 47 or 0.35% to 1.39% of the genome, respectively, indicating a year-by-year increase (Table 2). The most conserved protein was NS2B, which had no amino acid substitutions in any of the isolates except for sample ThD1-0488-94 collected in 1994, which had one amino acid substitution (Table 2). The C protein had the highest percentage of amino acid substitutions (Table 2).

When we compared amino acid substitutions between two clades (the 1980–1994 clade versus the 1990–2001 clade) of the predominant genotype I strains with the reference strain (ThD1-0008-81), among four strains of the 1980–1994 clade (ThD1-0008-81 and ThD1-0081-82 sampled in 1981–1982 versus ThD1-0323-91 and ThD1-0336-91 sampled in 1991), eight exclusive amino acid substitutions were found in samples ThD1-0323-91 and ThD1-0336-91 (Table 3). None of these eight amino acid substitutions resulted in amino acid charge

changes. Because the 1980–1994 clade was soon supplanted by the 1990–2001 clade, these mutations might have been deleterious for virus survival and were therefore eliminated by a process of purifying selection.[6,17] All strains within the 1990–2001 clade shared 14 unique amino acids that were associated with the separation of two clades and the lineage replacement event in DENV-1 (Table 3).[6] These amino acid substitutions have been fixed in the virus genome since 1994 and are present in subsequent isolates, suggesting that mutations at these sites led to viruses that were well adapted to human hosts and mosquito vectors.

Nucleotide comparison and secondary structure prediction of 5′- and 3′- RNA termini. The 5′-NTR of all strains, including two non-predominant genotype III and eight predominant genotype I strains, was extremely conserved with no nucleotide substitutions over the 22-year sampling period. This finding suggested that strict conservation of this region is critical for virus survival. In contrast, nucleotides in the 3′-NTR of all viral genomes of all Thai DENV-1 analyzed varied significantly. All substitutions occurred within the first 347 nucleotides, whereas, the last 115 nucleotides were highly conserved in predominant and non-predominant genotype strains (Table 3). This observation is consistent with a previous report that the last 100 nucleotides in the 3′-NTR are conserved in all four DENV serotypes.[18] Similar to what was seen for the coding region, the more recent isolates of the genotype I strains had more nucleotide substitutions than earlier isolates; the percentage of nucleotide substitutions ranged from 0.22% to 1.95% (Table 2). Genotype III had 3.90% nucleotide substitutions in the 3′-NTR, which was approximately 60% more than the genotype I strain ThD1-0081-82 (2.2%) isolated two years later (Table 2).

We extended our studies to RNA secondary structure analysis of the 3′-NTR of the 10 DENV-1 strains. However, all secondary structures of 3′-NTR analyzed by MFOLD exhibited a similar topology and none of these nucleotide substitutions were predicted to disrupt the RNA secondary structures. Because DENV replication requires circularization of the 5′-3′termini involving the entire 5′-NTR, the first 43 nucleotides of the C protein and the last 120 nucleotides of the 3′-NTR, we performed the secondary structure analysis for the circularized RNA to determine whether mutations in these regions affected virus RNA secondary structure. The results showed that the 5′-3′end cyclization of all genotype III and genotype I virus strains also displayed similar image patterns.

Comparison of virus strains sampled from different dengue epidemic periods. The comparison of DENVs sampled from different dengue epidemic periods was performed only for two clades representing the predominant genotype I strains. Within the 1980–1994 clade, samples (ThD1-0323-91 and ThD1-0336-91) collected during the dengue epidemic of 1991, a period of moderate DEN-1 activity, showed almost double the number and percentage of total amino acid and nucleotide substitutions compared with samples (ThD1-0008-81 and ThD1-0081-82) collected during 1981–1982, a period of lower DEN-1 activity (Table 2). Likewise, within the 1990–2001 clade, samples (ThD1-0102-01 and ThD1-0049-01) collected during 2001, a period of high DEN-1 activity, had 45–47 (1.33–1.39%) amino acid substitutions compared with samples (ThD1-0097-94 and ThD1-0488-94) collected in 1994 during a period of low DEN-1 activity, which had 29–32 amino acid substitutions in the virus genome (Table 2). The data suggest that the rate of genetic variation among viral genomes correlated directly with dengue epidemic activity, with more amino acid/nucleotide substitutions occurring during periods of high dengue epidemic activity. Comparison of amino acid and nucleotide substitutions in each gene of all predominant genotype I strains showed a higher percentage of substitutions in the E, NS1, NS3, NS4B, and NS5 proteins and the 3′-NTR for those viruses sampled during periods of high DEN-1 epidemic activity (Table 2), which indicated a positive correlation between amino acid/nucleotide changes in the genes and/or the 3′-NTR of viral genome and increased dengue epidemic activity.

Comparison of early isolates versus late isolates. Comparative analysis of virus strains isolated early versus late in the 22-year sampling period was performed only for eight predominant genotype I strains, which were grouped into four sample pairs based on the year of isolation. The sampling time spanned 20 years from the pair 1 strains sampled in 1981–1982 to the pair 4 strains sampled in 2001. The virus strains sampled more recently had more amino acid/nucleotide substitutions than those sampled earlier (Table 2). For instance, there were 13 amino acid/nucleotide substitutions in the 1982 isolate (ThD1-0081-82), 24–29 in the 1991 isolates, 36–39 in the 1994 isolates, and 54–56 in the 2001 isolates (Table 2). This observation demonstrated that the virus had significantly mutated and the DENV-1 currently circulating in Thailand differs from the earliest isolates.

Microevolution of the amino acid and nucleotide sequences occurred in the coding region and the 3′-NTR. Two amino acid substitutions in NS1-286 and NS2A-159 were fixed in virus genome and were present in subsequent isolates since 1991 (Table 3). Similarly, 17 amino acid substitutions across the cording region and five nucleotide substitutions in the 3′-NTR were fixed in the virus genome of all strains sampled after 1994 (Table 3), which suggested that these mutations did not adversely affect virus fitness. The mutations at these sites were not deleterious and were perhaps even favorable for virus survival.

Comparison of virus strains associated with different disease severities. Comparative analysis of complete genomic sequences for viruses associated with DF versus DHF was performed for each pair of samples collected from the same year in the Bangkok area. The percentage amino acid sequence identities for each pair of samples were 98.8% for sample pair 1: two non-predominant strains [ThD1-0442-80 (DHF)/ThD1-0673-80 (DF)] collected in 1980; 99.6% for sample pair 2: ThD1-0323-91 (DHF)/ThD1-0336-91 (DF) collected in 1991; 99.9% for sample pair 3: ThD1-0097-94 (DHF)/ThD1-0488-94 (DF) collected in 1994, and 99.9% for sample pair 4: ThD1-0102-01 (DHF)/ThD1-0049-01 (DF) collected in 2001. The percent identity of the nucleotide and amino acid sequences of these viruses were similar (Table 2), and no specific genetic markers or patterns in the viral genome associated with disease severity were found in any sample pairs, which is consistent with results of a previous report.[19]

DISCUSSION

The findings that the 5′-NTR and NS2B protein were highly conserved suggest that strict conservation of these regions is critical for the DENV life cycle. It is known that the 5′-NTR plays essential regulatory roles during translation, RNA

replication and assembly of progeny DENV viral particles, and the NS2B protein is a critical cofactor for the viral serine protease encoded by the NS3 gene. NS2B not only enhances the structural stability of the domains of NS3 protein but also contributes to proper conformation of the domains that comprise the enzymatically active site, facilitating substrate accessibility and promoting catalytic activity.[20] Thus, mutations in these regions are likely to be deleterious to virus survival. The frequency of mutations in the E, NS1, NS3, NS4B, and NS5 proteins and the 3′-NTR of the predominant genotype I strains increased gradually over the sampling period (Table 2), during which there was also an increased incidence of DENV-1–associated disease in the Bangkok area.[10] These proteins and regions of the genome are responsible for many important steps in the virus life cycle, and mutation in key parts of these regions can lead to changes in protein function or RNA secondary/tertiary structure resulting in changes in virus infectivity or transmission.

The genotype I and III strains were defined by 54 amino acid changes across the coding region. Of 54 amino acid substitutions observed, four (at E-37, NS1-139, NS2A-162, and NS5-649) (Table 3) resulted in amino acid charge changes. The amino acid at E-37 for all nonpredominant genotype III strains was Asn (N), which has a neutral charge, and the same residue in all predominant genotype I strains was Asp (D), which has a negative charge. This residue fell into domain I, which functions as a molecular hinge for low pH–catalyzed E protein reorganization and contains many important antigenic sites. Amino acid charge changes from neutral to negative can cause structural and functional changes in the E protein. Most of the neutralization-resistant variants of flaviviruses obtained have amino acid substitutions that cause a change in charge.[21–23] Charged residues are important in the interaction of antigenic sites with antibodies.[24] The charge change at this residue of the E protein may result in neutralization resistance, and thus enable predominant genotype I strains to escape host immune responses. The amino acid at NS5-649 (RNA-dependent RNA polymerase [RdRp] domain) for all predominant genotype I strains was Tyr (Y), which has a neutral charge, and the same residue in two non-predominant strains was His (H), which has a positive charge. This amino acid charge change in the RdRp domain could lead to a functional change in NS5 protein with increased replication rate in predominant genotype I strains. It may be a contributing factor for the occurrence of non-predominant and predominant genotypes of DENV-1.

Fourteen amino acid changes across the DENV-1 genome of predominant genotype I strains associated with a major clade replacement event were fixed at those substitution sites in the viral genome, which might change the degree of virus fitness or other viral functions. Selection analyses performed in our previous study throughout the entire viral genomes of these 10 strains showed no evidence to support a process of positive selection acting at the gene, codon, or lineage levels.[6] However, the presence of a striking inverse correlation between the prevalence of DENV-1 and DENV-4 (i.e., when the DENV-1 infection rate is high DENV-4 is low and visa versa) in patients admitted to QSNICH in Bangkok, Thailand over the past three decades[6] led us to hypothesize that the selection pressures imposed on different serotypes co-circulating in the population, which can shape patterns of cross-immunity, might have played a role in the major clade replacement event.[6,9]

Although we have demonstrated that amino acid changes that distinguish clades within DENV-1 are fixed by chance at a time of relative serotype abundance, their effect on viral fitness is not realized until the wider serologic landscape changes and selection pressure becomes stronger.[6] It appears that positive selection acting on these amino acid substitution sites strongly favors virus survival and transmission between humans and mosquitoes. Among 14 amino acid changes observed here, one that was fixed at C-9 in the 1990-2001 clade fell into nuclear-localization signal motif I, which might affect the nuclear localization of C protein and thereby alter virus infection events and play a role in disease outcome. Although DENV C protein is responsible for encapsidation of viral RNA, which takes place in the cytoplasm of host cells, it also has been detected in the cytoplasm and nucleus[25,26] and perhaps in the nucleolus of infected cells.[27] Three putative nuclear-localization signal motifs of DENV have been predicted at residues 6–9 (motif I), 73–76 (motif II), and 85–100 (motif III) of C protein.[28] The function of the C protein in the nucleus remains to be investigated, but it has been hypothesized that the DENV C protein services as a transcription modulator similar to the core protein of hepatitis C virus,[9] which is involved in regulating the life cycle of DENV and is a building block of the virus.[30] The hepatitis C virus core protein has been shown to interact with the transcription factor hnRNP K, which is located in the nucleus[31] and is known to enhance c-Myc to promote apoptosis.[32] It is possible that DENV C protein also modulates apoptosis of host cells, leading to the development of disease.

Four of 14 changed amino acids were fixed at E-8, E-324, E-351, and E-380 of the predominant genotype I strains. Of these four changes, one fell into domain I (spanning residues 1–52, 133–192, and 275–299), which is the central domain and functions as an organizer of the protein structure and contains important antigenic sites. The other three changes fell into domain III (residues 300–495), which is an immunoglobulin C-like module and responsible for receptor binding and contains type-specific virus neutralizing epitopes.[33,34] Because these mutations might have an effect on virus binding to host cells or neutralization, mutations present in currently circulating strains of the predominant genotype I could lead to viruses that bind to host cell receptors more efficiently and thus are more highly infectious and are also partially able to escape host neutralizing antibodies. Additionally, mutations in DENV-1 at E-8, E-324, and E-380 occurred at positions that are invariant in the other DENV serotypes, which suggests that they might have major effects on virus fitness.[6] In particular, it has been reported that a change at E-380 is associated with positive selection in DENV-3.[35]

Two of 14 amino acid mutations at NS3-350 and NS3-466 were fixed in the 1990–2001 clade of predominant genotype I strains. Both mutations fell into the C-terminal domain spanning residues 180–618. The NS3 protein is a multifunctional protein, with protease, helicase, and nucleoside 5′-triphosphatase (NTPs) activities. The N-terminal 180 amino acids comprise a serine protease domain with the protein NS2B acting as a membrane anchor.[36–39] The C-terminal domain of NS3, which spans amino acid residues 171–618, is involved in viral RNA replication.[40,41] It comprises three structural domains of approximately 150 amino acids each. Domain I (residues 181–326) contains two motifs known as Walker A (motif I, ATP phosphate-binding loop), which interacts with the NTP substrate, and Walker B (motif II), which interacts

with Mg^{2+}.[42,43] Domain II (residues 327–481) contains an arginine finger (Arg458 and Arg461) in motif IV, which is involved in ATPase and RNA triphosphatase activities[44] and in the structure rearrangement that results in RNA unwinding after ATP hydrolysis. Domain III (residues 482–618) influences NTPase and helicase activities, as demonstrated by the mutation of a single Arg residue within helix (Arg513Ala), which slightly decreases NTPase activity and produces a defective helicase.[41] Thus, mutations of residues 350 and 466, and in particular, G466H, which resulted in an amino acid charge change (from neutral to positive) in domain II of the NS3 protein for all four strains in 1990–2001 clade of predominant genotype I may increase the activities of ATPase, RNA triphosphatase, and RNA unwinding, and enable the virus to replicate more efficiently.

Four of 14 fixed amino acid mutations fell into important functional areas[45] of NS5-108, NS5-325, NS5-367, and NS5-807 for all of the 1990–2001 clade of predominant genotype I strains. One mutation in NS5-108 fell into the N-terminal S-adenosyl methionine, methyltransferase (MTase) domain that spans residues 1–296 and is responsible for both guanine N-7 and ribose 2′-O methylations. The other three mutations fell into the C-terminal residues 273–899 that form the RdRp catalytic domain responsible for viral replication initiating with the synthesis of minus-strand RNA from the DENV positive-strand RNA genome, which is subsequently used as a template for synthesizing progeny plus-strand RNA genome.

In addition to two major functional domains (MTase and RdRp), the NS5 protein contains two nuclear localization sequences (NLS) between residues 320 and 405 within the RdRp domain. The first NLS (βNLS) is located between residues 320 and 368 and is responsible for binding cellular factor β1-importin and facilitating protein transport to the nucleus.[46,47] The second NLS (α/βNLS) spans residues 369–405 and is responsible for binding cellular factor α/β-importin.[48] The specific role of nuclear-localized NS5 in the viral life cycle appears to be, in part, to antagonize the antiviral response, including up-regulated modulation of interleukin-8 production by infected cells.[49] Interleukin-8 has been speculated to enhance viral production by antagonizing the effects of interferon.[50]

We observed that two amino acid mutations in all four strains of the 1990–2001 clade occurred in these critical regions at NS5-325 (R325K) β1-NLS and NS5-367 (G367R, from a neutral charge to a positive charge) within α/β-NTS. Although the mutation of R325K did not cause an amino acid charge change in the protein, R was present in all strains of non-predominant genotype III and the 1980–1994 clade of predominant genotype I, and K was present only in all strains of the 1990–2001 clade of predominant genotype I. These mutations might be expected to lead to a change in protein function and perhaps give rise to viruses able to evade host antiviral responses. It has also been reported that mutations in the α/β-NLS decrease NS5 nuclear accumulation and reduce virus production.[51] These mutations may have contributed to the non-predominance of genotype III and the extinction of the 1980–1994 clade that occurred in 1994.

The amino acid substitution rate in the NS5 protein of the predominant genotype I strains was directly proportional to the degree of dengue epidemic activity in the Bangkok area. During the period of high dengue epidemic activity, there were more amino acid substitutions (Table 2). The highest DENV-1 epidemic year in Thailand was 2001; two representative samples collected from that year had the greatest number of amino acid substitutions in the NS5 protein (Table 2). However, most of the amino acid substitutions fell into the RdRp domain and only two fell into the MTase domain (Table 3). Notably, two amino acid substitutions that led to charge changes (both from neutral to positive) were located at NS5-127 (Y127H) of the MTase domain and at NS5-565 (T565K) of the RdRp domain, respectively. These mutations might alter the function of RdRp and MTase, which can increase the virus replication rate and, although highly speculative, could have contributed to the high DENV-1 epidemic activity.

Two of 14 amino acid mutations associated with clade replacement were at NS2A-148 and NS4A-76. Although the functions of NS2A, NS4A, and NS4B have not yet been fully elucidated, several studies suggest that they may anchor the viral replicase proteins to cellular membranes,[52] assist in virion assembly,[53,54] and lead to inhibition of the interferon a/β response.[55–58] It has been reported that NS4A may play a role in induction of membrane alterations, assumed to serve as a scaffold for the formation of the viral replicase complex,[59] and the interaction between NS1 and NS4A, which is critical for viral RNA replication.[59,60] Mutations in the NS2A protein that diminish its ability to inhibit the host interferon response could contribute to the non-predominant status of genotype III viruses.

Although we did not find any determinants associated with disease severity, this study addresses the possible relationships between pathogenesis and virus genetic sequence based on systematic analysis of the complete genome of DENV-1 strains sampled over a 22-year period within the same community. The outcome of a DENV infection is determined by complex interactions between host and viral factors. Thus, further efforts to understand these interactions should use the systematic analysis of larger numbers of clinical samples, including asymptomatic ones collected globally and longitudinally, along with phenotypic characterization by reverse genetics, site-directed mutagenesis, and other technologies.

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