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Co-existence of major and minor viral populations from two different origins in patients secondarily infected with dengue virus serotype 2 in Bangkok

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ABSTRACT

Generally, RNA viruses exhibit significant genetic diversity that sometimes effect viral fitness in infected hosts and probably also pathogenesis. Dengue viruses (DENVs) consist of four antigenically distinct serotypes. All the serotypes of DENV can cause mild to severe dengue illnesses. In this study, we examined the sequence variation of DENV in plasma obtained from four patients living in Bangkok who had been secondarily infected with serotype 2 (DENV-2) in 2010. The plasma-derived RNA was directly subjected to reverse transcriptase (RT)–polymerase chain reaction (PCR) at a region including most of domain III of the envelope (E) protein gene, and the PCR products obtained were subjected to clonal sequencing. Using 19–20 clones sequenced from each patient (78 total) plus 601 corresponding sequences from a public database, phylogenetic analysis revealed that the nucleic acid sequences fell into two clusters with clearly different origins. Interestingly, all patients gave sequences indicating that they carried viral populations containing 2, 3 or 5 genetic variants that consisted of one major variant plus one or more minor variants. Three patients showed a major variant from one cluster plus one or more minor components from the other while one showed major and minor variants from a single cluster. Thus, it can be concluded that DENV belonging to two different genetic lineages were co-circulated in Bangkok in 2010. For these two genotype clusters there was also a clear difference in H or Y at the deduced amino acid position 346 (i.e. H346Y) that was consistent for our sequences and 601 sequences from the public database. Thus, one among the mixed viral genotypes introduced into human individuals seems to be variably selected as the predominant component of the carried viral population, and it is possible that the dynamics of this process could influence virus evolution and disease severity.

1. Introduction

Dengue virus (DENV) (family *Flaviviridae*, genus *Flavivirus*) consists of four antigenically related but genetically distinct serotypes named DENV-1 to DENV-4, each of which can infect humans via mosquito vectors. Infection with DENV is a major public health concern throughout the tropics [1]. Primary infection with any serotype of DENV confers life-long protection against the homologous serotype, but no protection against secondary infection with a heterologous serotype that may induce severe dengue disease with more enhanced viral replication due to the anti-DENV antibodies primed by the primary infection with a different serotype [2].

DENV has a positive-sense, single-stranded RNA genome of approximately 11 kb contained in an enveloped virion with a capsid protein, a membrane protein (M), and an envelope glycoprotein (E) [3]. Serotypic and genotypic differences among viral populations are likely to be associated with the severity of dengue illness [4–6]. There is 60–70% identity in nucleotide sequences [7] and approximately 70% [8] in deduced amino acid sequence identity among the four serotypes. This is especially true for genetic diversity of the E protein, the major target gene for variations that could affect viral virulence [9]. The E protein has multi-functions, i.e., binding with cell surface receptors and therefore affecting host cell tropism, and induction of immune responses that lead to the generation of escape mutants. The E protein ectodomain consists of

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three structural domains referred to as E protein domains I–III [10]. The phylogenetic analysis by Maximum Likelihood program supported the contention that the domain III, the most heterogeneous region, was under the influence of positive selection [11].

Sequence variation of DENV-2 has been studied well. Six genotypes of DENV-2 have been reported [4,12]. However, only DENV-2 assigned to Asian genotype 1 has been sampled since 1991, even though Asian genotype 2 was the major genotype studied during the 1980's [13]. Here, we focused on the E gene of DENV-2 in plasma samples from four patients to carry out clonal sequencing and study variation in DENV-2 circulating in Bangkok, Thailand in 2010. Phylogenetic analysis of the nucleic acid sequences of these clonal sequences together with 601 corresponding sequences from a public database revealed that the clones from the four patients fell into two phylogenetic clusters. All patients showed one predominant but variable genetic component from one of these clusters in the viral population they carried, but this was always accompanied by one or more minor components of variable sequence. Three among the four patients gave nucleic acid sequences indicating that they carried DENV-2 populations from both of the genetic clusters. Interestingly, sequences in the two clusters were also separable by the amino acid substitution H346Y.

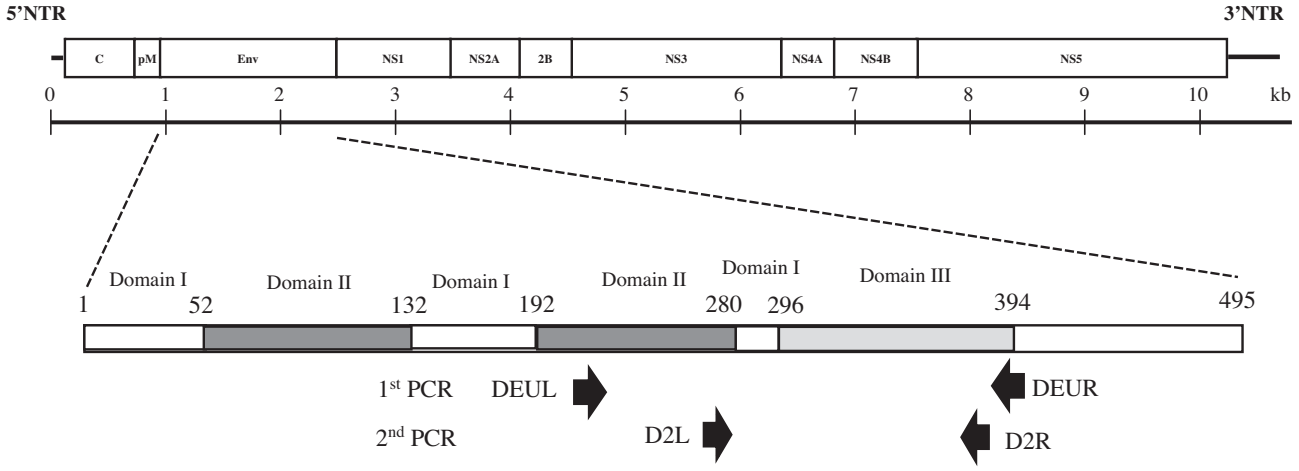
2. Materials and methods

2.1. Patients

Plasma samples were obtained from four patients with dengue illness who were living in Bangkok, Thailand. These patients had been admitted to the Tropical Medicine hospital at Mahidol University due to acute febrile illness with fever from July to August 2010. Their plasma samples were tested for dengue nonstructural (NS)1 antigen and anti-dengue IgM and IgG antibodies by immunochromatography (SD BIOLINE, Kyonggido, Korea). The plasma samples were obtained 3–7 days after disease onset. This study was approved by the ethical committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok. Written informed consent was obtained from each participant before this study.

2.2. Reverse transcriptase (RT)--polymerase chain reaction (PCR) and cloning

The plasma samples were ultracentrifuged at 50,000 rpm (77,000*g*) with a TLA 100.3 rotor in a Beckman Coulter model Optima TLX-Ultracentrifuge (Beckman Coulter, Brea, CA) at 4 °C for 30 min to collect the virus particle fraction in the precipitate. Total RNA was extracted from the precipitate using a QIAamp Viral RNA kit (Qiagen, Hilden, Germany) by following the manufacturer's protocol. The RNA was used as the template for reverse transcription by using specific anti-sense primer (DEUR) and SuperScript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). The oligonucleotide primer pairs previously reported for serotyping [14] were used for amplification of DENV E gene including most of domain III (Fig. 1): the 1st PCR primers DEUL/DEUR were designed to give amplicons from all serotypes of DENV while the 2nd PCR primer pairs D1L/D1R, D2L/D2R, D3L/D3R, and D4L/D4R were designed for specific amplification of the DENV-1, -2, -3, and -4, respectively. Both of the 1st and 2nd PCR reactions were performed using PrimeStar GXL DNA polymerase (TAKARA, Shiga, Japan) to create PCR products with blunt ends using a Thermal Cycler model GeneAmp PCR System 9700 (Perkin Elmer ABI). The 1st PCR was per-



DEUL (1552-1574)*	5'-TGGCTGGTGCACAGACAATGGTT-3'
DEUR (2170-2192)	5'-GCTGTGTCACCCAGAATGGCCAT-3'
D2L (1744-1764)	5'-ATCCAGATGTCATCAGGAAAC-3'
D2R (2070-2089)	5'-CCGGCTCTACTCCTATGATG-3'

*Nucleotide position in relation to that of the DENV-2 Jamaica strain [14].

Fig. 1. DENV gene map with location of the primers used for RT–PCR targeting the E gene region. The plasma samples from four patients with dengue illness were subjected to RT–PCR with primers DEUL and DEUR followed by nested PCR with primers D2L and D2R using cDNA template from the initial RT–PCR reaction.

formed under the following conditions: thirty five cycles of amplification consisting of denaturation at 98 °C for 10 s, annealing at 53 °C for 1 min, and primer extension at 72 °C for 1 min. The 1st PCR products were diluted 10,000-fold and 2.5 μl was used as template for the 2nd amplification. The 2nd PCR was performed with the primers described above using the following protocol: thirty five cycles of amplification consisting of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min, primer extension at 72 °C for 1 min, and additional primer extension at 72 °C for 7 min. The PCR products were then electrophoresed in 1% agarose gels and the bands were visualized using ethidium bromide staining. The expected 346-bp PCR products were extracted and subjected to cloning into pCR4Blunt–TOPO vector (Invitrogen), according to the manufacturer's instructions. *Escherichia coli* DH5α was transformed with the resulting recombinant plasmids. A total of 19–20 white colonies were randomly selected. Plasmid DNAs were extracted with QIAprep Spin Miniprep kit (Qiagen) and subjected to sequencing using M13 universal primers (Macrogen Inc., Seoul, Korea).

The sequences of the dengue viruses studied here have been submitted to GenBank, under accession numbers AB622002 to AB622079.

2.3. Sequence analysis

Sequence variants were analyzed at both the nucleotide and deduced amino acid levels. The obtained sequences were analyzed in comparison to previously reported DENV-2 sequences from the ftp site of Molecular and Genetic Bioinformatics Facility (ftp://ftp.genome.uab.edu/vbrc/FASTA/) on November 12, 2010.

2.4. Phylogenetic analysis

The eight unique DENV-2 E sequences obtained from the patients were aligned together with 601 corresponding DENV-2 E sequences from the database using MAFFT v6.705b [15]. A neighbor-joining phylogenetic tree was created using MEGA5 [16].

3. Results

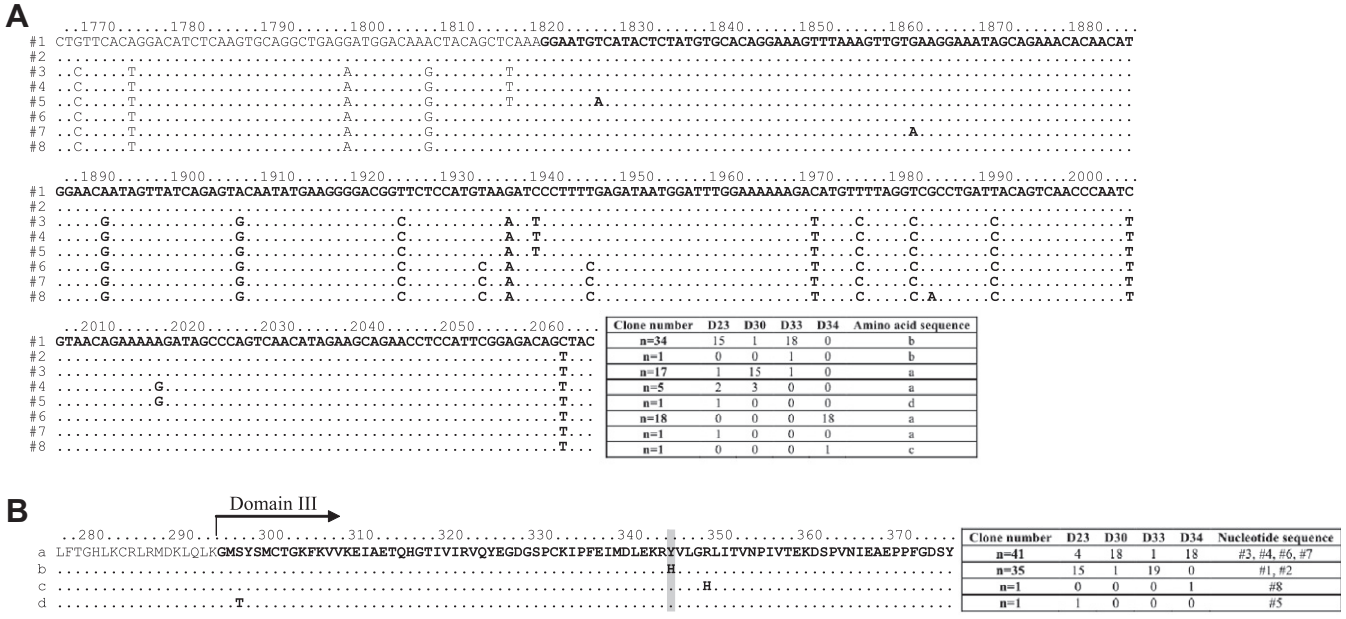
3.1. Diagnosis of four patients

Four patients living in Bangkok (D23, female, 33 years old; D30, female, 23 years old; D33, male, 31 years old; and D34, female, 20 years old), who were clinically diagnosed with dengue fever (D23, D33, and D34) or dengue hemorrhagic fever (D30) in 2010, were all positive by immunochromatography using anti-DENV IgM and IgG. NS1 antigen was positive from only patient D30 whose plasma was obtained earlier than that of the other patients, i.e., on days 6, 3, 5, and 7 after the onset of fever in D23, D30, D33, and D34, respectively. Thus, we concluded that these four patients were secondarily infected with DENV.

The plasma samples were used for RT–PCR followed by clonal sequencing. The 1st PCR was performed with primers DEUL and DEUR that are common to all the serotypes (Fig. 1). The resulting products were then subjected to the 2nd PCR with primers specific to individual serotypes of DENV (see Section 2). Amplicons were produced only with 2nd PCR primers D2L and D2R and not with the other primer sets (data not shown), indicating that all four patients were secondarily infected with DENV-2. The final PCR products were cloned into pCR4Blunt vector. PCR amplicon clones (20 each from D23 and D33 and 19 each from D30 and D34) were arbitrarily selected from individual patients and subjected to sequence analysis.

3.2. Sequence variations of DENV-2 from four patients

Sequencing of a total of 78 clones from these patient samples identified eight discrete nucleotide variants named "#1"–"#8" (Fig. 2A). At the amino acid level, these nucleotide variants encoded four variables, deduced amino acid sequences named "a"–"d" (Fig. 2B). Patient D23 carried five variants (#1, #3, #4, #5, and #7) at the nucleotide level and three (a, b, and d) at the amino acid level; patient D30 carried three variants (#1, #3, and #4) at the nucleotide level and two (a and b) at the amino acid level; patient D33 carried three variants (#1, #2, and #3) at the nucleotide



Clone number	D23	D30	D33	D34	Amino acid sequence
n=34	15	1	18	0	b
n=1	0	0	1	0	b
n=17	1	15	1	0	a
n=5	2	3	0	0	a
n=1	1	0	0	0	d
n=18	0	0	0	18	a
n=1	1	0	0	0	a
n=1	0	0	0	1	c

Clone number	D23	D30	D33	D34	Nucleotide sequence
n=41	4	18	1	18	#3, #4, #6, #7
n=35	15	1	19	0	#1, #2
n=1	0	0	0	1	#8
n=1	1	0	0	0	#5

Fig. 2. Variation of the DENV-2 E sequences mostly including domain III among a total of 78 sequences derived from four patients. Domain III regions in the nucleotide (A) and amino acid (B) sequences are shown in bold typeface. The distribution of variant clone numbers for individual nucleotide and amino acid sequences derived from individual patients are shown in tables adjacent to the sequences in A and B.

level and two (a and b) at the amino acid level; and patient D34 carried two variants at both the nucleotide level (#6 and #8) and the amino acid level (a and c) (Fig. 2). D23 and D33 showed similar dominance of nucleotide sequence #1 corresponding to amino acid sequence b (15 clones and 18 clones, respectively) as their major population component. D30 and D34 showed dominant populations of nucleotide sequences #3 and #6, respectively (15 and 18 clones, respectively) that corresponded to amino acid sequence a. Interestingly, the patients also carried variable minor populations. For example, patient D23 (dominant #1 and b) also carried one minor clone of nucleotide sequence #3 (corresponding to amino acid sequence a), two of #4 (amino acid sequence a), one of #5 (amino acid sequence d), and one of #7 (amino acid sequence a), while D33 (also dominant #1 and b) showed one minor clone of #2 (amino acid sequence b) and one of #3 (amino acid sequence a). Patient D30 (dominant #3 and a) showed one minor clone of #1 (amino acid sequence b) and three of #4 (amino acid sequence a), while patient D34 (dominant #6 and a) showed one minor clone of #8 (amino acid sequence c).

3.3. Phylogenetic analysis of DENV-2 sequences from four patients, together with 601 sequences from public databases

Next, the nucleic acid sequences obtained in this study were used together with a total of 601 corresponding DENV-2 sequences from a database (Molecular and Genetic Bioinformatics Facility) to make a phylogenetic comparison (Fig. 3). The tree contains clusters named American (cluster A in this study), Cosmopolitan (clusters B and C-1), American/Asian (clusters C-2 and C-3), Asian 2 (cluster C-5), Asian 1 (cluster D-8), and other clusters including sub-clusters D-9-1 and D-9-2 (Table 1) [17]. The 78 sequences obtained in this study were located only in clusters C-5 (nucleotide sequences #1 and #2) and D-9 (nucleotide sequences #3–#5 in sub-cluster D-9-1 and #6–#8 in sub-cluster D-9-2). With respect to the phylogenetic tree in Fig. 3, the sequences from patients D23 (five nucleotide variants), D30 (three nucleotide variants) and D33 (three nucleotide variants) fell into clusters C-5 and D-9 (i.e. sub-clusters D-9-1/D-9-2), indicating that they carried virus populations that arose from two independent origins, whereas two nucleotide variants from D34 fell into the single sub-cluster D-9-2, indicating that the patient carried a viral population arising from a single origin. The major nucleic acid sequence for patients D23 and D33 was #1 from cluster C-5, but their minor sequences differed in number and type except for one shared minor sequence (#3). For patient D30, the dominant nucleic acid sequence was #3 from cluster D-9 (sub-cluster D-9-1). For this patient, the minor sequences #1 (the dominant sequence in D23 and D33) and #4 were from clusters C-5 and D-9 (sub-cluster D-9-1), respectively. For patient D34, the dominant sequence was #6 and the minor sequence #8, both absent from any other patient and both from cluster D-9 (sub-cluster D-9-2). In summary, the phylogenetic analysis of the nucleic acid sequences revealed that patients D23, D30, and D33 carried viral populations derived from two different origins while D34 carried a population derived from a single origin.

Analysis of deduced amino acid sequences derived from our eight distinct nucleic acid sequences (#1–#8), revealed four different sequences called "a"–"d" (i.e., a = #3, #4, #6 and #7 from nucleic acid tree cluster D-9; b = #1 and #2 from cluster C-5; c = #8 from cluster D-9; and d = #5 from cluster D-9) (see table in Fig. 2). Of particular interest was amino acid residue 346 (Fig. 2) that was tyrosine in amino acid sequences a, c, and d (all from cluster D-9) but histidine in b (from cluster C-5) (i.e., Y346H outlined in gray background in the sequence alignment in Fig. 2B). Thus, there was a clear difference between amino acid sequences in cluster C-5 (all histidine) and D-9-1/D-9-2 (all tyrosine). Analysis of the deduced amino acid sequences from the database revealed that 406 out of 601 DENV-2 viruses gave histidine at this location, while the other 195 gave tyrosine. Interestingly, all the records in clusters A, B, and C gave histidine for this residue, except for 10 sequences derived from Puerto Rico in cluster C-2 that carried tyrosine (Table 1). In contrast, all of the cluster D series carried tyrosine, except for several ancestral clusters, such as D-1 and D-2, that carried histidine (Table 1). In cluster D-3, one (DQ181804) had tyrosine, while the other (DQ181805) had histidine. With respect to variation in other amino acid, the substitution R350H in amino acid sequence c and S298T in amino acid sequence d were observed only within cluster D-9 (Figs. 2B and 3) and not in any of the 601 sequences derived from the database.

4. Discussion

Sequence variations of DENV-2 in the plasma derived from four patients with dengue illness were examined by RT–PCR directly from plasma samples to prevent the possibility of virus population selection that might occur with virus isolation in culture. Phylogenetic analysis of nucleotide sequences revealed that two patients carried a virus population with a major component belonging to phylogenetic cluster Asian 2 (C-5) and that the other two patients carried a major component in cluster D-9 within sub-clusters D-9-1 and D-9-2. Thus, the phylogenetic tree suggested that clusters C-5 and D-9 arose from different origins. Interestingly, three out of four patients carried sequences representative of both of these clusters, one as a major and the other as a minor component of their DENV-2 populations, indicating that at least two DENV-2 populations were co-circulating in Bangkok in 2010.

The two populations of DENV-2 identified in this study might be carried by mosquitoes, as previously suggested for the sequence variation in this vector [18]. It is unknown whether the two viral populations were introduced to the patient who was bitten by a single mosquito or multiple mosquitoes carrying different genotype clusters. After bitten, a certain virus could be selected and propagate more efficiently to become the major component of the host population, i.e., cluster C-5 in two patients and cluster D-9 in two patients. This phenomenon might arise due to differences in the DENV serotype that was the primary DENV infection experienced by these patients, since the anti-dengue virus IgG antibodies derived from the primary infection could have a significant influence on active viral replication by antibody-dependent enhancement, as previously reported [19,20]. Another possible explanation may be fitness differences in DENV-2 populations in humans who are led from an asymptomatic condition [21] to a wide spectrum of clinical manifestations [22,23]. Consequently, as seen in these four patients, concurrent infection of DENV-2 of at least two different origins might occur often in Bangkok. In fact, previous reports showed similar evidence for concurrent infections of DENV in several regions [24,25]. Although detailed data on clinical aspects of the four patients in this study were not available, there was no apparent association between clinical severity and virus clusters among the four patients in this study, and this was similar to observations noted previously [13,26–28].

Variation in the amino acid residue at position 346 (i.e., histidine or tyrosine) may constitute a good marker for the cluster C and D series: 346H for all sequences in the cluster C series (except for 10 sequences with 346Y in cluster C-2), versus 346Y for all sequences in cluster D series (except for clusters D-1 and D-2 and one sequence of cluster D-3 (DQ181805), all of which were ancient clusters within the cluster D series that carried 346H). This suggests that the viral population with 346Y evolved after the appearance of a cluster D-3 lineage from a DENV-2 population with 346H. We could not detect any other amino acid variant at this position among our sequences or among the 601 sequences from the data-

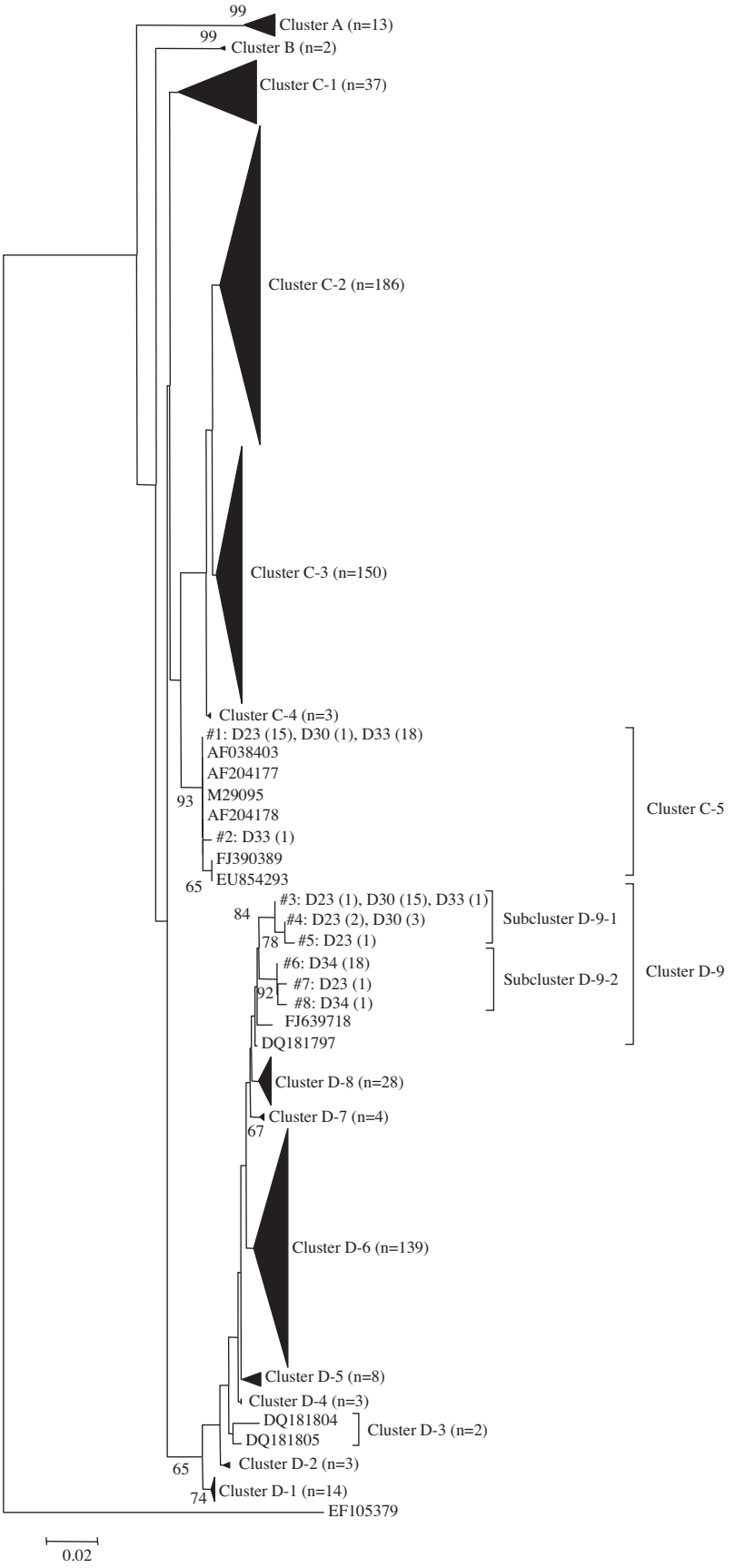


Fig. 3. Neighbor-joining phylogenetic tree created using a total of 78 nucleotide sequences from the DENV-2 E region in this study, together with a total of 601 sequences from the corresponding region from a public database. Bootstrap values less than 65 are not shown in figure. The nucleotide sequences #1–#8 in this study are located in clusters C-5, D-9-1 and/or D-9-2. Clone numbers (parenthesis) from the individual patients are indicated in the tree. The sequences from database are indicated by their accession numbers or totally shown by tentatively named clusters.

Table 1
Amino acid substitution H346Y in DENV-2 sequences at clusters C-5 and D-9 (D-9-1 and D-9-2) derived from four patients in this study as well as a total of 601 DENV-2 sequences derived from database.

Cluster	Amino acid residue at 346		Sequence source (sequence number)	Genotype
	Histidine	Tyrosine		
A	13	0	Tonga (4), Peru (3), Venezuela (1), Mexico (1), Puerto Rico (1), Cambodia (1), Unknown (2)	American
B	2	0	China (1), Unknown (1)	Cosmopolitan
C-1	37	0	Taiwan (19), Indonesia (5), Singapore (4), Brunei (3), China (2), Vietnam (2), Australia (1), Burkina Faso (1)	Cosmopolitan
C-2	176	10	Puerto Rico (127)[a], Vietnam (46), Cambodia (7), Thailand (1), China (1), Venezuela (1), Unknown (3)	American/ Asian
C-3	150	0	Nicaragua (110), Venezuela (13), Puerto Rico (11), Colombia (6), Cuba (6), Dominican Republic (3), Martinique (1)	American/ Asian
C-4	3	0	Puerto Rico (3)	
C-5	41	0	Thailand (35)[b], New Guinea (2), China (2), Colombia (2)	Asian 2
D-9-1	0	23	Thailand (23)[b]	
D-9-2	0	20	Thailand (20)[b]	
FJ639718 DQ181797	0	2	Cambodia (1), Thailand (1)	
D-8	0	28	Thailand (27), Unknown (1)	Asian 1
D-7	0	4	Cambodia (4)	
D-6	0	139	Vietnam (136), Cambodia (2), Thailand (1)	
D-5	0	8	Thailand (8)	
D-4	0	3	Cambodia (3)	
D-3	1	1	Thailand (2)	
D-2	3	0	Thailand (2), Unknown (1)	
D-1	14	0	Unknown (14)	
EF105379[c]	1	0	Malaysia (1)	

[a] Ten sequences for 346Y are derived from Puerto Rico.
[b] Sequences obtained in this study.
[c] Accession number of a virus derived from sentinel monkey is shown.

base, indicating that these two amino acids are highly conserved in the cluster C and D series. Thus, it is not likely that the substitution was derived from escape mutation by immune pressure, although this amino acid residue was included in the epitope region in a previous report [29]. Further study is needed to determine any functional association between virus evolution and disease severity resulting from the difference between the two viral populations, one carrying histidine and the other tyrosine.

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References

[1] S.B. Halstead, Pathogenesis of dengue: challenges to molecular biology, Science 239 (1988) 476–481.
[2] S.B. Halstead, Dengue, Lancet 370 (2007) 1644–1652.
[3] R.J. Kuhn, W. Zhang, M.G. Rossmann, S.V. Platnev, J. Corver, E. Lenches, C.T. Jones, S. Mukhopadhyay, P.R. Chipman, E.G. Strause, T.S. Baker, J.H. Strauss, Structure of dengue virus: implications for *flavivirus* organization, maturation, and fusion, Cell 108 (2002) 717–725.
[4] R. Rico-Hasse, L.M. Harrison, R.A. Salas, D. Tovar, A. Nisalak, C. Ramos, J. Boshell, M.T. de Mesa, R.M. Nogueira, A.T. de Rosa, Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas, Virology 230 (1997) 244–251.
[5] D.W. Vaughn, S. Green, S. Kalayanarooj, B.L. Innis, S. Nimmannitya, S. Suntayakorn, T.P. Endy, B. Raengsakulrach, A.L. Rothman, F.A. Ennis, A. Nisalak, Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity, J. Infect. Dis. 181 (2000) 2–9.
[6] D.M. Watts, K.R. Porter, P. Putvatana, B. Vasquez, C. Calampa, C.C. Hayes, S.B. Halstead, Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever, Lancet 354 (1999) 1431–1434.
[7] M. Parida, K. Horioke, H. Ishida, P.K. Dash, P. Saxena, A.M. Jana, M.A. Islam, S. Inoue, N. Hosoka, K. Morita, Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay, J. Clin. Virol. 43 (2005) 2895–2903.
[8] R.M. Kinney, C.Y.-H. Huang, B.C. Rose, A.D. Kroeker, T.W. Dreher, P.L. Iverson, D.A. Stein, Inhibition of dengue virus serotypes 1 to 4 in vero cell cultures with morpholino oligomers, J. Virol. 79 (2005) 5116–5128.
[9] E.C. Holmes, S.S. Burch, The causes and consequences of genetic variation in dengue virus, Trends Microbiol. 8 (2000) 74–77.
[10] Y. Modis, S. Ogata, D. Clements, S.C. Harrison, A ligand-binding pocket in the dengue virus envelope glycoprotein, Proc. Natl. Acad. Sci. USA 100 (2003) 6986–6991.
[11] D.-Y. Chao, C.-C. King, W.-K. Wang, W.-J. Chen, H.-L. Wu, G.-J. Chang, Strategically examining the full-genome of dengue virus type 3 in clinical isolates reveals its mutation spectra, Virol. J. 2 (2005) 72.
[12] R. Rico-Hesse, Molecular evolution and distribution of dengue viruses type 1 and 2 in nature, Virology 174 (1990) 479–493.
[13] C. Zhang, M.P. Mammen Jr, P. Chinnawirotpisan, C. Klungthong, P. Rodpradit, A. Nisalak, D.W. Vaughn, S. Nimmannitya, S. Kalayanarooj, E.C. Holmes, Structure and age of genetic diversity of dengue virus type 2 in Thailand, J. Gen. Virol. 87 (2006) 873–883.
[14] P. Yenchitsomanus, P. Srichareon, I. Jaruthasana, S. Pattanakitsakul, S. Nitayaphaa, J. Mungkolsapaya, P. Malasit, Rapid detection and identification of dengue viruses by polymerase chain reaction (PCR), Southeast Asian J. Trop. Med. Public Health 27 (1996) 228–236.
[15] K. Katoh, G. Asimenos, H. Toh, Multiple alignment of DNA sequences with MAFFT, Method Mol. Biol. 537 (2009) 39–64.
[16] K. Tamura, J. Dudley, M. Nei, S. Kumar, MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.
[17] O. Osman, M.Y. Fong, S. Devi, Complete genome sequence analysis of dengue virus type 2 isolated in Brunei, Virus Res. 135 (2008) 48–52.
[18] S.-R. Lin, S.-C. Hsieh, Y.-Y. Yueh, T.-H. Lin, D.-Y. Chao, W.-J. Chen, C.-C. King, W.-K. Wang, Study of sequence variation of dengue type 3 virus in naturally infected mosquitoes and human hosts: implications for transmission and evolution, J. Virol. 78 (2004) 12717–12721.
[19] S.B. Halstead, N.T. Lan, T.T. Myint, T.N. Shwe, A. Nisalak, S. Kalyanarooj, S. Nimmannitya, S. Soegijanto, D.W. Vaughn, T.P. Endy, Dengue hemorrhagic fever in infants: research opportunities ignored, Emerg. Infect. Dis. 8 (2002) 1474–1479.
[20] T.H. Nguyen, H.Y. Lei, T.L. Nguyen, Y.S. Lin, K.J. Huang, B.L. Le, C.F. Lin, T.M. Yeh, Q.H. Do, T.Q. Vu, L.C. Chen, J.H. Huang, T.M. Lam, C.C. Liu, S.B. Halstead, Dengue hemorrhagic fever in infants: a study of clinical and cytokine profiles, J. Infect. Dis. 189 (2004) 221–232.
[21] E. Harris, E. Videa, L. Perez, E. Sandoval, Y. Tellez, M.L. Perez, R. Cuadra, J. Rocha, W. Idiaquaz, R.E. Alonso, M.A. Delgado, L.A. Campo, F. Acevedo, A. Gonzalez, J.J. Amador, A. Balmaseda, Clinical, epidemiologic, and virologic features of dengue in the 1998 epidemic in Nicaragua, Am. J. Trop. Med. Hyg. 63 (2000) 5–11.
[22] D.J. Gubler, Dengue and dengue hemorrhagic fever, Clin. Microbiol. Rev. 11 (1998) 480–496.
[23] G. Kuno, Emergence of the severe syndrome and mortality associated with dengue and dengue-like illness: historical records (1890 to 1950) and their compatibility with current hypotheses on the shift of disease manifestation, Clin. Microbiol. Rev. 22 (2009) 186–201.
[24] H. Kinoshita, E.G.M. Mathenge, N.T. Hung, V.T.Q. Huong, A. Kumatori, F. Yu, M.D.C. Parquet, S. Inoue, R.R. Matias, F.F. Natividad, K. Morita, F. Hasebe, Isolation and characterization of two phenotypically distinct dengue type-2 virus isolates from the same dengue hemorrhagic fever patient, Jpn. J. Infect. Dis. 62 (2009) 343–350.

[25] W.K. Wang, D.Y. Chao, S.R. Lin, C.C. King, S.C. Chang, Concurrent infections by two dengue virus serotypes among dengue patients in Taiwan, J. Microbiol. Immunol. Infect. 36 (2003) 89–95.
[26] C. Klungthong, C. Zhang, M.P. Mammen Jr, S. Ubol, E.C. Holmes, The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand, Virology 329 (2004) 168–179.
[27] R. Rico-Hesse, L.M. Harrison, A. Nisalak, D.W. Vaughn, S. Kalayanarooj, S. Green, A.L. Rothman, F.A. Ennis, Molecular evolution of dengue type 2 virus in Thailand, Am. J. Trop. Med. Hyg. 58 (1998) 96–101.
[28] A.C. Shurtleff, D.W. Beasley, J.J. Chen, H. Ni, M.T. Suderman, H. Wang, R. Xu, E. Wang, S.C. Weaver, D.M. Watts, K.L. Russell, A.D. Barrett, Genetic variation I the 3′ non-coding region of dengue viruses, Virology 281 (2001) 75–87.
[29] B.L. Innis, V. Thirawuth, C. Hemachudha, Identification of continuous epitopes of the envelope glycoprotein of dengue type 2 virus, Am. J. Trop. Med. Hyg. 40 (1989) 676–687.