Journal of General Virology (2009), **90**, 678–686 **DOI** 10.1099/vir.0.005306-0

Genetic characterization of dengue virus type 1 isolated in Brunei in 2005–2006

Osmali Osman,[1] Mun Yik Fong[2] and Shamala Devi Sekaran[1]

[1]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia

[2]Department of Parasitology, Faculty of Medicine, University of Malaya, Malaysia

Correspondence
Shamala Devi Sekaran
shamalamy@yahoo.com

Received 3 July 2008
Accepted 30 October 2008

The full-length genomes of two DENV-1 viruses isolated during the 2005–2006 dengue incidents in Brunei were sequenced. Twenty five primer sets were designed to amplify contiguous overlapping fragments of approximately 500–600 base pairs spanning the entire sequence of the genome. The amplified PCR products were sent to a commercial laboratory for sequencing and the nucleotides and the deduced amino acids were determined. Sequence analysis of the envelope gene at the nucleotide and amino acid levels between the two isolates showed 92 and 96 % identity, respectively. Comparison of the envelope gene sequences with 68 other DENV-1 viruses of known genotypes placed the two isolates into two different genotypic groups. Isolate DS06/210505 belongs to genotype V together with some of the recent isolates from India (2003) and older isolates from Singapore (1990) and Burma (1976), while isolate DS212/110306 was clustered in genotype IV with the prototype Nauru strain (1974) and with some of the recent isolates from Indonesia (2004) and the Philippines (2002, 2001). In the full-length genome analysis at the nucleotide level, isolate DS06/210505 showed 94 % identity to the French Guyana strain (1989) in genotype V while isolate DS212/110306 had 96 % identity to the Nauru Island strain (1974) in genotype IV. This work constitutes the first complete genetic characterization of not only Brunei DENV-1 virus isolates, but also the first strain from Borneo Island. This study was the first to report the isolation of dengue virus in the country.

INTRODUCTION

Globally, dengue infection has caused many countries, especially in the tropics and subtropical regions, considerable economic burden from loss of working hours due to hospitalization. The common mild infection, dengue fever (DF), is an ancient disease with a history that dates back 200 years. The first global distribution of the severe form, dengue haemorrhagic fever (DHF), occurred after the Second World War, causing epidemics affecting millions of people. The causative agent of dengue is a virus belonging to the flavivirus family. It has been for many decades responsible for the most important mosquito-borne viral disease in humans in terms of morbidity and mortality. The main vectors responsible for human to human transmission are mosquitoes of the *Aedes* family. Currently, *Aedes aegypti* and *Aedes albopictus* have been implicated in causing transmission in urban and suburban areas. To date, the dengue toll has exceeded 100 million new DF cases annually, culminating in the high risk regions of the world, particularly in south-east Asia, the western Pacific and the Americas with population reaching 3 billion in more than 100 countries. From this figure, 500 000 have progressed to DHF, causing up to 25 000 deaths (Gubler & Clark, 1995; Monath & Heinz, 1996; WHO, 1997; Gibbons & Vaughn, 2002). The explosive growth of the world's population, failed urban infrastructure, inadequate mosquito control measures during the late 20th century, increased air travel and global warming have contributed significantly to the current increase of both deaths and hospitalization from dengue infection (Monath, 1994; Gubler, 1998a, b; WHO, 2002). Dengue virus comprises four genetically and antigenically distinct serotypes (DENV-1 to DENV-4). Infection with any of the four serotypes induces life-long protective immunity to the homologous serotype, but confers only transient or no cross-reactive immunity to subsequent infections by the other three serotypes. Clinical manifestations range from a mild, self-limiting illness DF to the more severe form DHF and dengue shock syndrome (DSS). The factors that determine the disease progression to the more severe form remain elusive as they involve multi-facet interactions between the virus and the host. The manifestations of DHF and DSS are more frequently seen in patients experiencing subsequent dengue infection, suggesting that sub-neutralizing heterotypic dengue virus antibodies are a risk factor

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are EU179860 and EU179861.

Sequence of the primers used in this study is available with the online version of this paper.

for progressing to DHF and DSS in secondary infections (Halstead, 1988; Kurane & Ennis, 1997). Studies conducted among children with dengue in Thailand reported that bleeding, secondary infection and haemoconcentration of >22 % from baseline haematocrit are the risk factors for developing to DSS (Tantracheewathorn & Tantracheewathorn, 2007), while other studies have shown the association of distinct genotypes with differences in virus virulence following reports of primary infections resulting in DHF/DSS (Rosen, 1977). It has been reported that certain genotypes such as Asian DENV-2 have been more often associated with DHF/DSS (Rico-Hesse *et al.*, 1997; Gubler, 1998a; Leitmeyer *et al.*, 1999).

The dengue viral genome is a ~11 kb, single-stranded (+) RNA, coding for a polyprotein of ~3400 aa which is co- and post-translationally cleaved at the rough endoplasmic reticulum by a combination of cellular and viral proteases into three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The single open reading frame is preceded by ~100 nt of 5′ untranslated region (UTR) and tailed by ~400 nt of the 3′ UTR. The 5′ end of the viral genome is capped with type I 7-methyl guanosine structure, while the 3′ end is not polyadenylated (Lindenbach & Rice, 2001; Bartenschlager & Miller, 2008). Many investigators have made comparative analyses of nucleotide and amino acid sequences of short segments of specific gene regions to study molecular epidemiology and evolution of the dengue virus strains by characterizing them into genotypes (Rico-Hesse, 1990; Blok *et al.*, 1991; Deubel *et al.*, 1993; Lewis *et al.*, 1993; Lanciotti *et al.*, 1994; Chungue *et al.*, 1995). Consequently, genotypic characterization has been useful in monitoring the eventual appearance of genetic changes in dengue viruses, identifying the circulating serotype in an area and detecting the introduction of new genotypes. In earlier studies, molecular analyses of DENV-1 were based on either a fragment in the 5′ end of the envelope (E) gene (Chungue *et al.*, 1995) or a 240 bp region of the E–NS1 gene junction (Rico-Hesse, 1990), generating three to five different genotypes. In the Americas, it was found that one or two genotypes of DENV-1 were maintained at an endemic level causing the classical DF and occasional sporadic DHF. Later, whole envelope gene analysis of 44 DENV-1 strains from all regions of the world further revealed the existence of five genotypes (Goncalvez *et al.*, 2002). Other E gene analysis also supported these genotypic groupings (Laille & Rochie, 2004). Neutralizing epitopes of the E gene have been localized at two sites, Asn-67 and Asn-153, with *N*-linked glycosylation potential. Changes to these sites may affect virus-mediated membrane fusion and neurovirulence (Anderson *et al.*, 1992; Chen *et al.*, 1996)

Although surrounded by countries known to be endemic or hyperendemic for dengue, the disease is not a major public health problem in Brunei, as there has been no report of deaths due to dengue virus infections. However, serological data from the Public Health Department, Ministry of Health, Brunei, indicated that from 1992 to 2006 a considerable number of patients suspected of dengue infection with dengue classical symptoms had IgM antibodies. This indicates that the virus had infected the patients and was in fact circulating in the country. Brunei is situated on the island of Borneo in south-east Asia with her close neighbours, the Malaysian states of Sabah in the east and Sarawak in the west. With a total area of a mere 5765 km^2, half of the country's population of 383 000 lives in the capital, Bandar Seri Begawan. Three quarters of the country is still covered with thick tropical rainforest and has an annual rainfall of 2540 mm. These humid and wet weather conditions together with poor infrastructure make it a favourable breeding ground for *Ae. aegypti* and *Ae. albopictus*. Furthermore, the yearly reports of deaths due to dengue in the neighbouring Malaysian states of Sabah and Sarawak have intensified the importance of this infection as movement of humans between Brunei and these countries could increase the risk of infection with more virulent strains. The lack of tissue culture and molecular laboratory facilities in the country has hampered the effort of isolating and typing the virus. In 2006, Sabah reported 630 DF and 14 DHF cases, while Sarawak had 1484 DF and 6 DHF cases [Annual Report 2006 WHO Collaborating Centre for Arbovirus Reference and Research – Dengue and Dengue Haemorrhagic Fever – Malaysia (WHO, 2006)]. Taking into account the current increase in air and land human travel between these countries, it is anticipated that outbreaks may be on the rise, which may increase the risk of DHF. It is well known that infected human travellers can introduce new serotypes or variants in a country or region. Our previous study showed that the predominant serotype infecting the population in Brunei in 2005–2006 was DENV-2, followed by DENV-1 (Osman *et al.*, 2007). This study aimed to characterize at the molecular level the DENV-1 virus isolated during the 2005–2006 dengue incidents and determine any significant differences between the isolates.

METHODS

Sample recruitment. A total of 719 serum samples were collected from patients suspected of dengue infection over a period of one year from April 2005 until April 2006. The samples were collected from clinics and hospitals in Brunei and hence covered the whole populated area of the country. Earlier, the samples were tested for dengue IgM and IgG antibodies using the commercial rapid test Panbio strip kit in the Department of Laboratory Services, Ministry of Health, Brunei, where they were stored at −20 °C. The samples were then transported to the Department of Medical Microbiology, University of Malaya, Kuala Lumpur, Malaysia, where further dengue confirmatory tests were carried out. Dengue RT-PCR (Kong *et al.*, 2006), in-house IgM capture ELISA (Lam *et al.*, 1987), NS1 antigen detection (Young *et al.*, 2000; Alcon *et al.*, 2002; Libraty *et al.*, 2002) and virus culture in the C6/36 mosquito cell line were employed.

Virus isolation and serotyping. The DENV-1 strains used were isolated in 2005–2006 from suspected dengue cases in the country. The viruses were cultured in the *Ae. albopictus* clone C6/36 mosquito cell line (Igarashi *et al.*, 1982). The C6/36 monolayer was grown in a

25 cm^3 angle-neck tissue culture flask and inoculated with 100 µl patient's serum or infected cell culture supernatant and kept at 37 °C for 1 h to allow for virus adsorption. The infected fluid was harvested after 10 days incubation at 28 °C. All isolates were cultured up to three passages before PCR amplification and sequencing. The growth was monitored by the indirect immunofluorescence antibody test (IFAT) using dengue-specific monoclonal antibodies [DEN 1 (MAB D2-1F1-3), DEN 2 (MAB 3H2-1-21), DEN 3 (MAB D6-8A1-12) and

```
Brunei2005      ATGCGATGCGTGGGAATAGGCAACAGAGACTTCGTTGAAGGACTGTCAGGAGCAACATGG 60
Brunei2006      ATGCGGTGCGTGGGAATAGGCAACAGAGACTTCGTGGAAGGACTCTCAGGAGCTACGTGG 60
                ***** ****************************** ******** ******** ** ***

Brunei2005      GTGGATGTGGTACTGGAGCATGGAAGCTGCGTCACCACCATGGCAAAAAATAAACCAACA 120
Brunei2006      GTGGATGTGGTGCTGGAGCATGGAAGTTGCGTCACTACCATGGCAAAAGACAAACCAACA 120
                ********** ************** ******** ************ * *********

Brunei2005      TTGGACATTGAACTCCTGAAGACGGAGGTCACGAACCCTGCCGTCTTGCGCAAACTGTGC 180
Brunei2006      CTGGACATTGAACTCCTGAAGACGGAGGTCACAAACCCTGCAGTCCTGCGCAAATTGTGC 180
                 ****************************** ******** *** ******** *****

Brunei2005      ATTGAAGCTAAAATATCCAACACTACTACCGATTCCAGATGTCCCACACCAGGAGAAGCT 240
Brunei2006      ATTGAAGCTAAAATATCAAACACCACCACCGATTCGAGATGTCCAACACAAGGAGAAGCC 240
                ****************** ***** ** ******** ******** **** *********

Brunei2005      ACCCTGGTGGAAGAACAAGACGCGAACTTTGTGTGTCGACGAACACTCGTGGACAGAGGC 300
Brunei2006      ACGCTGGTGGAAGAGCAGGACACGAACTTTGTGTGCCGACGAACGTTCGTGGACAGAGGC 300
                ** ************ ** *** ************** ********  ***************

Brunei2005      TGGGGTAATGGTTGTGGACTATTCGGGAAGGGAAGCTTACTGACGTGTGCTAAGTTCAAG 360
Brunei2006      TGGGGCAATGGTTGTGGGCTATTCGGAAAAGGTAGCTTAATAACGTGTGCTAAGTTTAAG 360
                ***** ************ ******** ** ** ****** * ************** ***

Brunei2005      TGTGTGACAAAACTTGAAGGAAAGATAGTTCAATATGAAAACTTAAAATATTCAGTGATA 420
Brunei2006      TGTGTGACAAAACTGGAAGGAAACATAGTCCAATATGAAAACTTAAAATATTCAGTCATA 420
                ************** ******** ***** ************************** ***

Brunei2005      GTCACTGTCCACACTGGGGACCAGCACCAGGTAGGAAATGAGACTACAGAGCATGGAACA 480
Brunei2006      GTCACCGTACACACTGGAGACCAACACCAAGTTGGAAATGAGACCACAGAACATGGAACA 480
                ***** ** ******** ***** ***** ** ************ ***** *********

Brunei2005      ATTGCAACCATAACACCTCAGGCTCCCACGTCGGAAATACAGCTGACTGACTACGGAGCC 540
Brunei2006      ACTGCAACCATAACACCTCAAGCTCCCACGTCGGAAATACAGCTGACAGACTACGGAGCT 540
                * ****************** ************************** ***********

Brunei2005      CTTACATTAGACTGCTCACCTAGAACTGGGCTGGACTTTAATGAGATGGTGCTGTTGACA 600
Brunei2006      CTAACATTGGACTGCTCACCTAGAACTGGGCTGGACTTTAATGAGATGGTGCTGTTGACA 600
                ** ***** ************************************************

Brunei2005      ATGAAAGAAAAATCATGGCTTGTCCACAAACAATGGTTTCTAGACTTACCACTGCCTTGG 660
Brunei2006      ATGAAAGAAAAATCATGGCTTGTCCACAAACAATGGTTTCTAGACTTACCACTGCCTTGG 660
                ************************************************************

Brunei2005      ACCTCGGGGGCTTCAACATCTCAAGAGACTTGGAACAGACAAGATCTGCTGGTCACATTT 720
Brunei2006      ACCTCGGGGGCTTCAACATCTCAAGAGACTTGGAACAGACAAGATCTGCTGGTCACATTT 720
                ************************************************************

Brunei2005      AAGACAGCTCATGCAAAGAAGCAGGAAGTAGTCGTACTGGGGTCACAAGAAGGAGCAATG 780
Brunei2006      AAGACAGCTCATGCAAAGAAGCAGGAAGTAGTCGTACTGGGGTCACAAGAAGGAGCAATG 780
                ************************************************************

Brunei2005      CACACTGCGTTGACTGGGGCGACAGAAATCCAGACGTCAGGAACGACGACAATCTTCGCA 840
Brunei2006      CACACTGCGTTGACTGGGGCGACAGAAATCCAGACGTCAGGAACGACGACAATCTTCGCA 840
                ************************************************************

Brunei2005      GGACACCTGAAATGTAGACTAAAAATGGATAAACTGACTTTAAAAGGGGTGTCATATGTG 900
Brunei2006      GGACACCTGAAATGCAGACTAAAAATGGATAAACTGACCTTAAAAGGGGTGTCATATGTA 900
                ************** ********************** ********************

Brunei2005      ATGTGCACGGGCTCATTTAAGCTAGAGAAGGAAATGGCTGAGACCCAGCATGGAACTGTC 960
Brunei2006      ATGTGCACAGGGTCATTCAAGCTAGAGAAGGAAGTGGCTGAGACTCAGCATGGAACTGTT 960
                ******** ** ***** *************** ********** **************

Brunei2005      CTAGTGCAGGTTAAATATGAAGGAACAGATGCACCATGCAAGATTCCCTTTTCGACCCAA 1020
Brunei2006      CTAGTGCAGGTCAAATACGAAGGAACAGATGCACCATGCAAGATCCCCTTTTCGACCAAA 1020
                *********** ***** ************************** ************ **

Brunei2005      GATGAGAAAGGAGTGACCCAGAAT---AGATTGATAACAGCCAATCCCATAGTTACTGAC 1077
Brunei2006      GATGAGAAGGGAGTGACCCAGAAT---AGATTGATAACAGCCAACCCCATAGTCATTGAC 1077
                ******** ***************   ****************** ******** * ****

Brunei2005      AAGGAAAAATCAGTCAACATTGAAACAGAACCACCTTTTGGTGAGAGCTACATCGTGATA 1137
Brunei2006      AAAGAAAAACCAGTTAACATTGAAGCGGAGCCACCTTTTGGTGAGAGCTACCTTGTGGTA 1137
                ** ****** **** ********* * ** ********************** * *** **

Brunei2005      GGGGCGGGTGAAAAAGCTTTGAAACTAAGCTGGTTCAAGAAAGGAAGCAGCATAGGGAAA 1197
Brunei2006      GGAGCAGGTGAAAAAGCTTTGAAACTAAGCTGGTTCAAGAAGGGAAGCAGTATAGGAAAG 1197
                ** ** ************************************ ******** ***** **

Brunei2005      ATGTTCGAAGCTACCGCCCGAGGAGCACGAAGGATGGCTATCCTGGGAGACACAGCATGG 1257
Brunei2006      ATGTTTGAAGCAACTGCTCGTGGAGCACGAAGGATGGCCATCCTGGGAGACACCGCATGG 1257
                ***** ***** ** ** ** ***************** *************** ******

Brunei2005      GACTTCGGCTCCATAGGAGGGGTGTTCACATCTGTGGGAAAATTGGTACACCAGGTTTTT 1317
Brunei2006      GACTTCGGTTCTATAGGAGGGGTGTTTACGTCTGTGGGAAAATTGATACACCAGATTTTT 1317
                ******** ** *************** ** *************** ******** *****

Brunei2005      GGAACCGCATATGGGGTCTTGTTCAGCGGTGTTTCTTGGACCATGAAAATAGGAATAGGG 1377
Brunei2006      GGGACTGCGTATGGAGTTTTGTTCAGCGGGGTTTCTTGGACTATGAAAATAGGAATAGGG 1377
                ** ** ** ***** ** *********** *********** ******************

Brunei2005      ATTCTGCTGACATGGCTAGGATTAAATTCAAGGAGCACGTCACTCTCGATGACGTGCATT 1437
Brunei2006      ATTCTGCTGACATGGCTAGGATTAAATTCAAGGAGCACGTCCCTTTCAATGACGTGTATC 1437
                ****************************************** ** ** ******** **

Brunei2005      GCAGTTGGCATGGTCACACTGTACCTAGGAGTCATGGTTCAAGCG 1482
Brunei2006      GCAGTTGGCATGGTCACACTGTACCTAGGAGTCATGGTTCAGGCG 1482
                ******************************************* ***
```

Fig. 1. Pairwise nucleotide alignment of the E gene of Brunei isolates DS06/210505 (Brunei2005) and DS212/110306 (Brunei2006). * Indicates identity in both sequences in the alignment.

DEN 4 (MAB 1410-6-7)] (kindly provided by the Centers for Disease Control and Prevention, Fort Collins, CO, USA) to detect the antigen in fixed infected cells. The presence and typing of virus in tissue culture supernatants were further confirmed by a one-step real-time RT-PCR amplification protocol (Kong *et al.*, 2006) which was carried out using the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen). The amplification process was carried out in a Bio-Rad iCycler system. Five microlitres of the extracted RNA was assayed in a 25 µl reaction mixture. The thermal profile consisted of a reverse transcription step at 50 °C for 30 min, *Taq* polymerase activation at 95 °C for 10 min, followed by 35 cycles at 94 °C for 30 s (denaturation), 60 °C for 40 s (annealing) and 72 °C for 50 s (extension). The two Brunei DENV-1 isolates used for the study were designated DS06/210505, isolated in 2005, and DS212/110306, isolated in 2006.

Primer design. A total of 25 synthetic oligonucleotide primer pairs were designed to amplify overlapping fragments of sizes between 500 and 600 bp spanning the whole genome of DENV-1 viral RNA. The primer sequences were based on the full-length sequence of Singapore DENV-1 strain S275/90 (GenBank accession no. M87512). The sequences of the oligonucleotide primer pairs used in the study are available in Supplementary Table S1 (available in JGV Online).

RNA extraction and cDNA synthesis for amplification and sequencing. Viral RNA was extracted from the supernatant of infected cells using a QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis and PCR of the target sequence were carried out using a commercial kit, AccessQuick RT-PCR System (Promega). The amplification process was carried out using the in-house protocol parameters as follows in the PTC-200 Peltier thermal cycler: 40 cycles of denaturation at 94 °C (30 s), primer annealing at 55 °C (45 s), primer extension at 72 °C (1 min) and final extension at 72 °C (5 min). Gel electrophoresis was used to confirm the size of the target PCR product. The PCR product was purified using a QIAquick PCR purification kit (Qiagen). The risk of error incurred as a result of using *Taq* DNA polymerase is significantly minimized as the PCR products generated by using 25 sets of primers were of 500–600 bp. Purified PCR products of less than 1 kbp are known to be stable and therefore can be used for direct sequencing.

DNA sequencing of PCR products. DNA sequencing of the PCR products was carried out by a commercial laboratory (Macrogen) using the Sanger dideoxy sequencing method (Sanger *et al.*, 1977).

Nucleotide sequences used in the study. For the whole envelope gene analysis, 70 DENV-1 E gene sequences were used, retrieved from the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/), with the accession numbers listed in Table 2. For the whole genome analysis, 20 DENV-1 full genomes were used. Sequences of DENV-2, DENV-3 and DENV-4 were used for rooting the phylogenetic tree.

Sequence and phylogenetic analysis. Multiple sequence alignments were performed using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses were conducted using the MEGA4 program (Tamura *et al.*, 2007). Phylogenetic trees were constructed from aligned nucleotide sequences using neighbour-joining and maximum-parsimony methods using maximum composite likelihood for DNA substitution test. Fig. 2 shows the phylogeny tree based on the DENV-1 envelope gene sequence. The tree that represents comparison between DENV-1 whole genome sequences is presented in Fig. 3. Branch topology was verified by generating 1000 bootstraps.

RESULTS

Of the 719 serum samples studied, 71 (9.87 %) were dengue capture IgM ELISA positive and 16 (2.22 %) had viral RNA detected in their blood. Dengue RT-PCR and IFAT identified 13 DENV-2 and 3 DENV-1. Two of the Brunei DEN-1 viruses were full-length sequenced, the third isolate failed to grow in cell culture. The sequences were deposited in GenBank with accession nos EU179860 and EU179861 for isolates DS06/210505 and DS212/110306, respectively. Pair-wise nucleotide sequence alignment spanning the envelope (E) gene of the two Brunei strains showed an identity of 92 % and a divergence of 7.6 %, reflecting a total of 113 nt changes between them (Fig. 1). The changes were randomly scattered throughout the gene without a particular region of hypervariability. Between the two isolates, 95 changes were located at the third position of the codon, therefore rendering them silent mutations at the amino acid level. The remaining 18 nt changes, of which 15 were located in the first position and 3 in the second position of the codon, resulted in 16 aa changes (Table 1) at sites 37 (N↔K), 77 (P↔Q), 88 (A↔T), 96 (L↔F), 114 (L↔I), 128 (K↔N), 161 (I↔T), 312 (M↔V), 340 (Q↔K), 359 (T↔I), 364 (S↔P), 369 (T↔A), 377 (I↔L), 379 (I↔V), 436 (V↔I) and 439 (V↔I). Multiple sequence alignment of the E gene incorporating 70 DENV-1 strains showed that isolate DS06/210505 had a 96 and 98 % identity at the nucleotide and amino acid levels, respectively, with Burma strain PRS 228686 (isolated in 1976) in the genotypic group V (Table 2). The identity with the Singapore strain was 94 and 98 %, while with the Indian strains isolated in 2003, the identity was 94 and 97 %, respectively (Table 2). However, isolate DS212/110306 had an identity of 97 and 98 % at the nucleotide and amino acid level, respectively, with the Indonesia strain SC01 (isolated in 2004), placing it in the genotype IV (Table 2) of the DENV-1 classification (Goncalvez *et al.*, 2002; Ong *et al.*, 2008). The identity with the Philippines strains isolated in 2002, 2001 and 1999 in the same

Table 1. The location of the amino acid changes in the E protein compared between the two Brunei DENV-1 strains, DS06/210505 and DS212/110306

E gene position	37	77	88	96	114	128	161	312	340	359	364	369	377	379	436	439
DS06/210505	N	P	A	L	L	K	I	M	Q	T	S	T	I	I	V	V
DS212/110306	K	Q	T	F	I	N	T	V	K	I	P	A	L	V	I	I

Table 2. Percentage identity of Brunei DENV-1 isolates DS06/210505 and DS212/110306 at the nucleotide and amino acid levels compared with DENV-1 strains from neighbouring countries and other regions of the world representing all five genotypes of DENV-1

Genotype	Strains	GenBank accession no.	Year	Country	DS06/210505		DS212/110306	
					nt	aa	nt	aa
I	D1/H/IMTSSA-CAMB/98/658	AF309641	1998	Cambodia	91	96	91	96
	GZ/80	AF350498	1980	China	90	95	91	96
	PUO 359	AF425630	1980	Thailand	90	96	91	96
	LAO CH 323	AB003090	1996	Laos	90	95	90	96
	D1/H/IMTSSA/98/606	AF298808	1998	Djibouti	91	96	90	95
	765101	AF425628	1987	Taiwan	90	95	90	96
	Mochizuki	AB074760	1943	Japan	91	95	92	95
	Hawaii	AF425619	1945	Hawaii	92	96	92	96
II	TH-SMAN	D10513	1954	Thailand	91	95	92	96
	16007	AF180817	1964	Thailand	92	96	92	96
	2543-63	AF425629	1963	Thailand	92	96	92	96
III	P72-1244	AF425622	1972	Malaysia	91	95	92	96
IV	A88	AB074761	1988	Indonesia	90	95	95	98
	AUS HCS1	AF425611	1983	Australia	91	96	95	98
	West Pac 74	U88535	1974	Nauru Island	91	96	96	98
	PRS 228682	AF425627	1974	Philippines	91	96	95	98
	SC01	AY858983	2004	Indonesia	91	96	97	98
	98901530 DF DV-1	AB189121	1998	Indonesia	90	97	94	98
	71/02GZ	EF025110	1971	China	90	97	94	97
	AUS H TI 7	AF425612	1983	Australia	91	96	94	97
	98901518 DHF DV-1	AB189120	1998	Indonesia	90	97	94	97
	D1/hu/Yap/NIID27/2004	AB204803	2004	Japan	89	96	93	97
	GZ01/95	EF032590	1995	China	91	96	94	97
	1480/04	DQ285561	2004	Seychelles	90	96	93	97
	D1/hu/Seychelles/NIID41/2003	AB195673	2003	Seychelles	90	96	93	97
	185/04	DQ285558	2004	Reunion	90	96	93	97
	257/04	DQ285560	2004	Reunion	90	96	93	97
	FP/01/192206	AY630407	2001	French Polynesia	90	97	93	98
	02SA079	AY422783	2002	Philippines	91	96	96	98
	02SA073	AY422782	2002	Philippines	91	96	96	98
	02SA047	AY422780	2002	Philippines	91	96	96	98
	02SA029	AY422779	2002	Philippines	91	96	96	98
	02RBD008	AY422778	2002	Philippines	91	97	96	98
	02DA071	AY422781	2002	Philippines	90	96	96	98
	01St219	AY422777	2001	Philippines	91	97	96	99
	99SA236	AY422785	1999	Philippines	91	97	96	99
	99SA660	AY422786	1999	Philippines	91	96	96	98
V	IBH 28328	AF425625	1968	Nigeria	94	98	92	96
	D1/H/IMTSSA-ABID/99/1056	AF298807	1999	Ivory Coast	94	98	91	96
	PRS 228686	AF425615	1976	Burma	96	98	92	96
	S275/90	M87512	1990	Singapore	94	98	91	96
	INS 371869	AF425617	1996	Colombia	94	98	91	96
	DEI 0151	AF425626	1991	Peru	94	98	91	96
	1378	AF425624	1983	Mexico	95	98	92	97
	BeH 584526	AF425614	1997	Brazil	94	98	91	96
	BR-90	S64849	1990	Brazil	94	98	91	96
	28164	AF425634	1997	Venezuela	95	98	92	97
	CAREC 780572	AF425631	1978	Trinidad and Tobago	95	98	92	96
	Rio H 36589	AF425610	1988	Angola	94	98	92	97
	CAREC 778156	AF425618	1977	Grenada	95	98	92	97
	PRS 228690	AF425621	1977	Jamaica	95	98	92	97
	495-1	AF425609	1985	Aruba	95	98	92	97
	FGA/89	AF226687	1989	French Guyana	95	98	91	96
	Dakar A-1520	AF425620	1985	Ivory Coast	94	97	91	96

Table 2. cont.

Genotype	Strains	GenBank accession no.	Year	Country	DS06/210505		DS212/110306	
					nt	aa	nt	aa
	125239	AF425637	1994	Venezuela	95	98	91	97
	INS 347869	AF425616	1985	Colombia	95	98	91	96
	BE AR 404147	AF425613	1982	Brazil	95	98	91	97
	1298/TVP 951	AF425623	1980	Mexico	95	98	92	97
	CAREC 86471	AF425639	1986	Trinidad and Tobago	95	98	92	97
	6222	AF425632	1995	Venezuela	94	98	91	96
	5736	AF425638	1995	Venezuela	95	98	91	97
	5345	AF425635	1995	Venezuela	95	98	91	97
	150172	AF425633	1995	Venezuela	94	98	91	97
	141602	AF425636	1994	Venezuela	94	98	91	97
	FP/89/5103	AY630408	1989	French Polynesia	94	98	91	96
	WI52VI04	DQ016653	2003	India	94	97	92	96
	SK1216VI04	DQ016654	2003	India	94	97	92	96
	SK1222VI04	DQ016655	2003	India	94	97	91	96

genotype was 96 and 98 % at the nucleotide and amino acid level, respectively (Table 2). The E gene of the Brunei DENV-1 isolates has a deletion of three nucleotides from positions 1045 to 1047. All the other 68 DENV-1 strains used had E gene sequences of 1485 nt. The percentage divergence at the nucleotide level between all the DENV-1 envelope gene sequences used ranged from 0.2 to 10.3 %. The phylogenetic analysis of the whole genome sequence has also classified the two Brunei DENV-1 isolates into the same genotypic groupings. The sequences were compared with 20 other DENV-1 full-length genomes (Ong *et al.*, 2008). In previous studies, five genotypes were observed using the 6 % cut-off value as the maximum genetic distance within a given genotypic group or subgroup when the whole envelope gene of 31 DENV-1 strains worldwide was analysed (Rico-Hesse, 1990). Other studies that follow also confirmed this genotypic grouping (Goncalvez *et al.*, 2002; Laille & Rochie, 2004; Domingo *et al.*, 2006; Ong *et al.*, 2008).

The 16 aa changes (Table 1) in the envelope gene between the two Brunei strains include the non-conservative substitutions at positions 37 (domain I), 379 (domain III) and 439 (transmembrane region) which was also shown in the E gene analysis of two DENV-1 isolates from French Polynesia (Laille & Rochie, 2004). The other non-conservative changes found among Brunei strains were at positions 128 (domain I) and 436 (transmembrane region). They also share conservative changes at positions 88, 114 (domain I) and 369 (domain III) reported in the study (Laille & Rochie, 2004). In addition, other conservative changes recognized were at positions 77, 96, 161 (domain I) and 312, 340, 359, 364, 377 (domain III) (Table 1). As indicated from previous studies, predominant changes of the amino acids in the E protein may be due to virus adaptation in tissue culture (Lee *et al.*, 1997)

Phylogenetic analyses based on the E gene and the full-length genomes were conducted using the MEGA4 software. For the E gene, 70 DENV-1 strains were uploaded in the program together with 3 other dengue virus serotypes (DENV-2, DENV-3 and DENV-4) as outgroups, while 20 DENV-1 strains were used in the full-length genome analysis. Full genome sequences of DENV-2, DENV-3 and DENV-4 were used to root the tree. Phylogenetic trees were built using neighbour-joining (NJ) and maximum-parsimony (MP) methodologies, but only MP trees of higher bootstrap values and desired topology were used. The phylogeny was constructed using the maximum composite likelihood as the parameter for the DNA substitution and bootstrapped with 1000 replicates. Five genotypes were observed for DENV-1 from the E gene analysis (Fig. 2) but, due to the lack of full-length genome sequences representing genotype III in the GenBank database, the tree for the full-length sequence analysis only showed four genotypic groupings (Fig. 3). All trees were rooted using DENV-2, DENV-3 and DENV-4 as outgroups.

DISCUSSION

The serological data have indicated that dengue virus is circulating in the country; furthermore, since Brunei is surrounded by countries experiencing an increasing incidence of the severe form of the disease, the importance of this study becomes more prominent, as it will provide useful information regarding the distribution of dengue virus in the country and in the region so appropriate measures by the public health authority can be put in place. Phylogenetic analyses group viruses into their respective subtype based on the percentage identity and divergence between paired nucleotide and deduced amino acid sequences. The main objective of the study is to characterize DENV-1 viruses isolated in Brunei. So far, Brunei has experienced dengue infection throughout the year, peaking during the rainy season; however, there have not been reports of deaths from dengue.

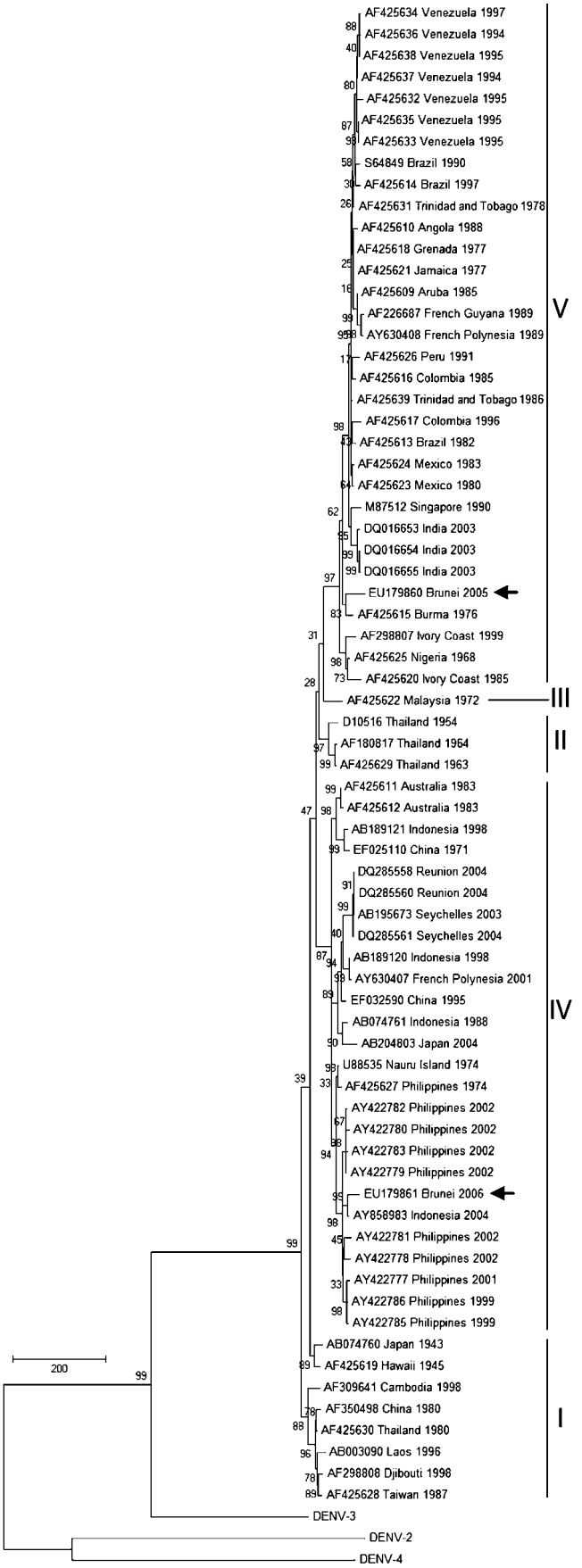

Fig. 2. Phylogenetic tree showing the E gene sequence analysis of two Brunei DENV-1 isolates and 68 other DENV-1 strains. Each strain is indicated by GenBank accession no. and place of origin followed by the year of isolation. Arrows show positions of the Brunei DENV-1 isolates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is rooted using representative strains of DENV-2, DENV-3 and DENV-4.

The two Brunei DENV-1 isolates appear to have originated from two different genotypes but, since data about the circulation of DENV-1 in the previous year in Brunei are unavailable, the possibility of multiple introduction of dengue virus into the country in 2005–2006 could not be confirmed. Furthermore, due to an insufficient number of Brunei DENV-1 isolates for strain recombination study, the analysis could not be performed satisfactorily. The divergence of 7.6 % between these strains has classified them into two different genotypes, IV and V. The position of isolate DS06/210505 in the MP tree comparing the E gene suggests that it might have a common ancestor with the Burmese strain (isolated in 1976) with mutations occurring over the years in its nucleotides causing it to

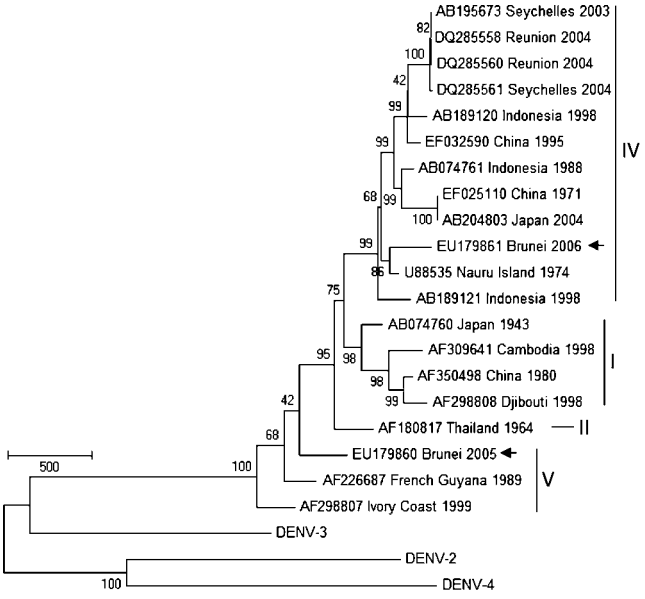

Fig. 3. Maximum-parsimony tree showing the phylogenetic relationships between two Brunei DENV-1 isolates and 18 other DENV-1 strains based on the nucleotide sequences of the whole genome. Each strain is indicated by GenBank accession no. and place of origin followed by the year of isolation. Arrows show positions of the Brunei DENV-1 isolates. The percentage support, based on 1000 replicates in which the associated taxa clustered together in the bootstrap test, is shown next to the branches. The tree is drawn to scale, with branch lengths calculated using the average pathway method, in units of the number of changes over the whole sequence. The tree is rooted using representative strains of DENV-2, DENV-3 and DENV-4.

diverge by 4.1 %. Isolate DS212/110306 also diverged by 2.3 % from its common ancestor shared with the Indonesian strain (isolated in 2004). However, the inclusion of more sequences from recent years from nearby neighbouring countries would give further information to narrow the source of the virus origin. At the envelope protein level, for isolate DS06/210505, the substitutions at sites 54 (S→A) and 77 (Q→P) and the deletion at location 349 would make that protein region hydrophobic. Changes at sites 96 (F→L), 297 (M→V), 312 (V→M) and 349 (deletion) have no effect on the protein, while amino acid substitutions at positions 364 (P→S) and 442 (A→T) would cause it to be hydrophilic. In the case of isolate DS212/110306, changes at the sites 52 (D→N), 128 (K→N), 339 (S→T) and 340 (Q→K) have no effect on the hydrophobicity, but only some charge reversal properties. The substitution of threonine to isoleucine at position 359 (T→I) and the deletion at position 349 have made that part of the envelope hydrophobic. It is not known what effect these changes have on the function of this protein. The important biological functions of viral attachment to a specific cell-surface receptor and membrane fusion are performed by the E glycoprotein, which is also a target and a modulator of the host immune response, and is located on the surface of dengue viruses (Heinz & Roehrig, 1990; Anderson *et al.*, 1992; Chen *et al.*, 1996).

Currently we are working to investigate the biological characteristics of these isolates and to correlate the changes to the biology of the Brunei isolates. Due to the lack of nucleotide sequences in the GenBank database representing either the E gene region or the whole genome of the dengue virus from the nearest neighbouring countries, the precise origin of the virus infecting the population of Brunei cannot be determined; however, this study has generated some valuable data at the genetic level which can be used as a preliminary source for current and future molecular epidemiology of dengue virus in Brunei, as well as for monitoring the introduction of potential virulent strains.

ACKNOWLEDGEMENTS

We would like to thank Dr Hj. Mohammad bin Hj. Kassim, Director of Laboratory Services, and Dr Hjh. Jaliha bte Hj. Md. Yussof, Head of Virology Laboratory, RIPAS Hospital, Ministry of Health, Brunei for allowing the use of serum samples collected and stored temporarily at the Virology Laboratory in Brunei. The study is part of Mr Osmali Osman's PhD project supported by the Brunei Government PhD-Scholarship fund under Bursary A/C: 211611414231395. All the monoclonal antibodies used in the IFAT were provided by Centers for Disease Control and Prevention, Fort Collins, CO, USA.

REFERENCES

Alcon, S., Talarmin, A., Debruyne, M., Falconar, A., Deubel, V. & Flammand, M. (2002). Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. *J Clin Microbiol* **40**, 376–381.

Anderson, R., King, A. D. & Innis, B. L. (1992). Correlation of the E protein binding with cell susceptibility to dengue 4 virus infection. *J Gen Virol* **73**, 2155–2159.

Bartenschlager, R. & Miller, S. (2008). Molecular aspects of Dengue virus replication. *Future Microbiol* **3**, 155–165.

Blok, J., Gibbs, A. J., McWilliam, S. M. & Vitarana, U. T. (1991). NS1 gene sequences from eight dengue-2 viruses and their evolutionary relationships with other dengue-2 viruses. *Arch Virol* **118**, 209–223.

Chen, Y., Maguire, T. & Marks, R. M. (1996). Demonstration of binding of dengue virus envelope protein to target cells. *J Virol* **70**, 8765–8772.

Chungue, E., Cassar, O. & Drouet, M. T. (1995). Molecular epidemiology of dengue-1 and dengue-4 viruses. *J Gen Virol* **76**, 1877–1884.

Deubel, V., Nogueira, R. M., Drouet, M. T., Zeller, H., Reynes, J. M. & Ha, D. Q. (1993). Direct sequencing of genomic cDNA fragments amplified by the polymerase chain reaction for molecular epidemiology of dengue-2 viruses. *Arch Virol* **129**, 197–210.

Domingo, C., Palacios, G., Jabado, O., Reyes, N., Niedrig, M., Gascón, J., Cabrerizo, M., Lipkin, W. I. & Tenorio, A. (2006). Use of short fragment of the C-terminal E gene for detection and characterization of two new lineages of dengue virus 1 in India. *J Clin Microbiol* **44**, 1519–1529.

Gibbons, R. V. & Vaughn, D. W. (2002). Dengue: an escalating problem. *BMJ* **324**, 1563–1566.

Goncalvez, A. P., Escalante, A. A., Pujol, F. H., Ludert, J. E., Tovar, D., Salas, R. A. & Liprandi, F. (2002). Diversity and evolution of the envelope gene of dengue virus type 1. *Virology* **303**, 110–119.

Gubler, D. J. (1998a). The global pandemic of dengue/dengue hemorrhagic fever: current status and prospects for the future. *Ann Acad Med Singapore* **27**, 227–234.

Gubler, D. J. (1998b). Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev* **11**, 480–496.

Gubler, D. J. & Clark, G. G. (1995). Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerg Infect Dis* **1**, 55–57.

Halstead, S. B. (1988). Pathogenesis of dengue: challenges to molecular biology. *Science* **239**, 476–481.

Heinz, F. X. & Roehrig, J. T. 1990. Flaviviruses. In *Immunochemistry of Viruses. Volume II. The Basis of Serodiagnosis and Vaccines*, pp. 289–305. Edited by M. H. V. Regenmortel & A. R. Neurath. Amsterdam: Elsevier Science Publishers BV (Biomedical Division).

Igarashi, A., Fujita, N. & Okuno, Y. (1982). Isolation of dengue viruses from patients with dengue haemorrhagic fever (DHF) and those with fever of unknown origin (FUO) in Jakarta, Indonesia, in the years of 1981 and 1982. *ICMR Annals Kobe University* **2**, 7–17. (in Japanese)

Kong, Y. Y., Thay, C. H., Tin, T. C. & Devi, S. (2006). Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. *J Virol Methods* **138**, 123–130.

Kurane, I. & Ennis, F. A. (1997). Immunopathogenesis of dengue virus infections. In *Dengue and Dengue Hemorrhagic Fever*, pp. 273–290. Edited by D. J. Gubler & G. Kuno. Wallingford: CAB International.

Laille, M. & Rochie, C. (2004). Comparison of dengue-1 virus envelope glycoprotein gene sequences from French Polynesia. *Am J Trop Med Hyg* **71**, 478–484.

Lam, S. K., Devi, S. & Pang, T. (1987). Detection of specific IgM in dengue infections. *Southeast Asian J Trop Med Public Health* **18**, 532–538.

Lanciotti, R. S., Lewis, J. G., Gubler, D. J. & Trent, D. W. (1994). Molecular evolution and epidemiology of dengue-3 viruses. *J Gen Virol* **75**, 65–75.

Lee, E., Weir, C. W. & Dalgarno, L. (1997). Changes in the dengue virus major envelope protein on passaging and their localization on the three-dimensional structure of the protein. *Virology* **232**, 281–290.

Leitmeyer, K. C., Vaughn, D. W., Watts, D. M., Salas, R., Villalobos de Chacon, I., Ramos, C. & Rico-Hesse, R. (1999). Dengue virus structural difference that correlate with pathogenesis. *J Virol* **73**, 4738–4747.

Lewis, J. A., Chang, G. J., Lanciotti, R. S., Kinney, R. M., Mayer, L. W. & Trent, D. W. (1993). Phylogenetic relationships of dengue-2 viruses. *Virology* **197**, 216–224.

Libraty, D. H., Young, P. R., Pickering, D., Endy, T. P., Kalayanarooj, S., Green, S., Vaughn, D. W., Nisalak, A., Ennis, F. A. & Rothman, A. L. (2002). High circulating levels of dengue virus non-structural protein NS1 early in dengue illness correlate with the development of dengue haemorrhagic fever. *J Infect Dis* **186**, 1165–1168.

Lindenbach, B. D. & Rice, C. M. (2001). *Flaviviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 991–1041. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Monath, T. P. (1994). Dengue: the risk to developed and developing countries. *Proc Natl Acad Sci U S A* **91**, 2395–2400.

Monath, T. P. & Heinz, F. X. (1996). Flaviviruses, In *Fields Virology*, 3rd edn, pp. 961–1034. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott-Raven Publishers.

Ong, S. H., Yip, J. T., Chen, Y. L., Liu, W., Harun, S., Lystiyaningsih, E., Heriyanto, B., Beckett, C. G., Mitchell, W. P. & other authors (2008). Periodic re-emergence of endemic strains with strong epidemic potential—a proposed explanation for the 2004 Indonesian dengue epidemic. *Infect Genet Evol* **8**, 191–204.

Osman, O., Fong, M. Y. & Devi, S. (2007). A preliminary study of dengue infection in Brunei. *Jpn J Infect Dis* **60**, 205–208.

Rico-Hesse, R. (1990). Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology* **174**, 479–493.

Rico-Hesse, R., Harrison, L. M. & Salas, R. A. (1997). Origin of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology* **230**, 244–251.

Rosen, L. (1977). The emperor's new clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. *Am J Trop Med Hyg* **26**, 337–343.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tantracheewathorn, T. & Tantracheewathorn, S. (2007). Risk factors of dengue shock syndrome in children. *J Med Assoc Thai* **90**, 272–277.

WHO (1997). *Dengue Hemorrhagic Fever: Diagnosis, Treatment, Prevention and Control*, 2nd edn. Geneva: World Health Organization.

WHO (2002). Fact sheet No. 117, April 2002. Geneva: World Health Organization.

WHO (2006). Annual Report. WHO Collaborating Centre For Arbovirus Reference and Research (Dengue and Dengue hemorrhagic Fever) – Malaysia. Geneva: World Health Organization.

Young, P. R., Hilditch, P. A., Bletchly, C. & Halloran, W. (2000). An antigen capture enzyme-linked immunosorbent assay reveals high levels of dengue virus protein NS1 in the sera of infected patients. *J Clin Microbiol* **38**, 1053–1057.