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Complete genome sequence analysis of dengue virus type 2 isolated in Brunei

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ABSTRACT

In a previous study, we have reported the detection and isolation of dengue virus in Brunei (Osman, O., Fong, M.Y., Devi, S., 2007. A preliminary study of dengue infection in Brunei. JJID 60 (4), 205–208). DEN-2 was the predominant serotype followed by DEN-1. The full genomic sequences of 3 DEN-2 viruses isolated during the 2005–2006 dengue incident in Brunei were determined. Twenty-five primer sets were designed to amplify contiguous overlapping fragments of approximately 500–600 base pairs spanning the entire sequence of the viral genome. The amplified PCR products were sent for sequencing and their nucleotides and the deduced amino acids were determined. All three DEN-2 virus isolated were clustered in the Cosmopolitan genotype of the DEN-2 classification by Twiddy et al. This work constitutes the first complete genetic characterization of three Brunei DEN-2 virus strains.

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1. Introduction

Dengue viruses (DENV) have been for many decades, responsible for the most important mosquito-borne viral disease in humans in terms of morbidity and mortality. For countries in the tropical and sub-tropical belt, it has become a major emerging problem causing considerable economic burden. This resulted from global population growth, inadequate infrastructure to support increased urbanization which leads to the increase in breeding sites causing spread of vectors and the virus, insufficient mosquito control measures and increased air travel (Monath, 1994; Gubler, 1998a, 1998b; WHO, 2002). Currently more than 100 countries worldwide are affected with estimates of 50–100 million cases of dengue fever (DF) annually, up to 500,000 progressed to dengue hemorraghic fever (DHF) causing 25,000 deaths and posing high risk to some 3 billion people (Gubler and Clark, 1995; Monath and Heinz, 1996; WHO, 1997; Gibbons and Vaughn, 2002). Dengue virus comprises of four genetically and antigenically distinct serotypes (DEN-1 to DEN-4). Infection with any of the four serotypes induces life-long protective immunity to the homologous strain, but confers only transient or no cross-reactive immunity to subsequent infections by the other three serotypes. Clinical manifestations ranged from a mild, self-limiting illness (DF) to a more severe form (DHF), and dengue shock syndrome (DSS).

The evolutionary and epidemiological relationships between many isolates of the same virus become apparent only by comparing nucleotide sequences that share a common ancestry, from which data can be generated for phylogenetic trees to be inferred quantitatively (Zuckerkandl and Pauling, 1965). 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 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.

Brunei is situated on the island of Borneo in Southeast Asia (Fig. 1) where dengue is endemic and epidemics occur throughout the year in many parts of this region. With an area of 2226 sq. miles, the country is divided into four districts; Brunei-Muara, Tutong, Belait and Temburong (Fig. 1). Bandar Seri Begawan, the capital, lies in the Brunei-Muara district where more than half of the total population of 383,000 live, 75% of the country is covered with tropical rainforest and with the annual rainfall of 100 in, making it a favourable breeding ground for the Aedes mosquitoes, the vector for dengue virus. The statistical report published by the Public Health Department, Ministry of Health, Brunei, indicated that from the year 1992–2006 dengue was present in the country and due to lack of molecular epidemiological studies, the circulating serotype was not identified. Brunei shares boundaries with the Malaysian States of Sarawak in the west and Sabah in the east. In 2006, Sabah

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Fig. 1. Map showing the location of Brunei.

had 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). To date, no fatality due to DF and the severe form of dengue manifestation has ever been reported in Brunei but with the current increase in air and land human travel, it is anticipated that outbreaks may be on the rise which may increase the risk of having DHF. It is well known that human travelers infected and incubating dengue virus can act as reservoirs and thus introduce new serotypes or variants in a country or region. Our previous study had shown that the predominant serotype infecting the population in Brunei in 2005–2006 is DEN-2 followed by DEN-1 (Osman et al., 2007). The limited sequence analysis of the E/NS1 gene junction of Brunei DEN-2 virus had shown that the isolates were closely related to strains from Indonesia and Malaysia and well clustered in the genotype IV of the Rico-Hesse DEN-2 classification. This study was designed to determine the relatedness of Brunei DEN-2 isolates with that of its neighbours in the Borneo Island, countries in the Southeast Asia region and other parts of the world and localize the source of transmission of dengue infection in the country. This constitutes the first complete sequencing and molecular characterization of Brunei DEN-2 virus strains.

2. Materials and methods

2.1. Virus isolates

The DEN-2 strains used in this study were from patients suspected of having dengue fever infection in Brunei. They were isolated during the 2005–2006 dengue outbreak in the country. The viruses were cultured in *Aedes albopictus* clone C6/36 mosquito cell line (Igarashi et al., 1982). The C6/36 monolayer were grown in a 25-cm^3 angle-neck tissue culture flask and inoculated with 100 μl of 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 10 days after incubating at 28 °C. All isolates were cultured up to at least three passages for amplification and sequencing. The growth was monitored and identified by the indirect immunofluorescence antibody test (IFAT) using dengue specific monoclonal antibodies (DEN-1 (MAB D2-1F1-3), DEN-2 (MAB 3H3-1-21), DEN-3 (MAB D6-8A1-12) and DEN-4 (MAB 1410-6-7)) (gift from CDC, Atlanta, 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 RT-PCR amplification protocol (Kong et al., 2006) which was carried out using the QuantiTect® SYBR® Green RT-PCR reagent kit (Qiagen, Germany). Viral RNA was extracted from the supernatant of infected cells using QIAamp Viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The amplification process was done in a BioRad iCycler system. Five microliters of the extracted RNA were assayed in a 25 μl reaction mixture. The thermal profile consisted of reverse transcription step at 50 °C for 30 min., *Taq* polymerase activation at 95 °C for 10 min., followed by 35 cycles of PCR at 94 °C for 30 s, denaturation; 60 °C for 40 s, annealing; 72 °C for 50 s, extension. The strains used in this study were DS31/291005 (GenBank accession no. EU179857) and DS04/221205 (GenBank accession no. EU179858) isolated in Temburong district while isolate DS09/280106 (GenBank accession no. EU179859) was from Tutong district.

2.2. 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 DEN-2 viral RNA based on the alignment of the full-length sequences of the Indonesian DEN-2 strain TB16i (GenBank accession no. AY858036). The sequences of the oligonucleotide primer pairs used in the study are available upon request.

2.3. cDNA synthesis for amplification and sequencing

cDNA synthesis and PCR of the target sequence were carried out using a commercial kit, AccessQuick™ RT-PCR System (Madison, WI 53711-5399, USA). 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 QIAquick PCR purification kit (Qiagen, Germany). The risk of error incurred as a result of using Taq polymerase is significantly minimized as the PCR product generated by using 25 sets of primers were of 500–600 bp. Purified PCR products of less than 1 kbp is known to be stable and therefore can be sent for direct sequencing.

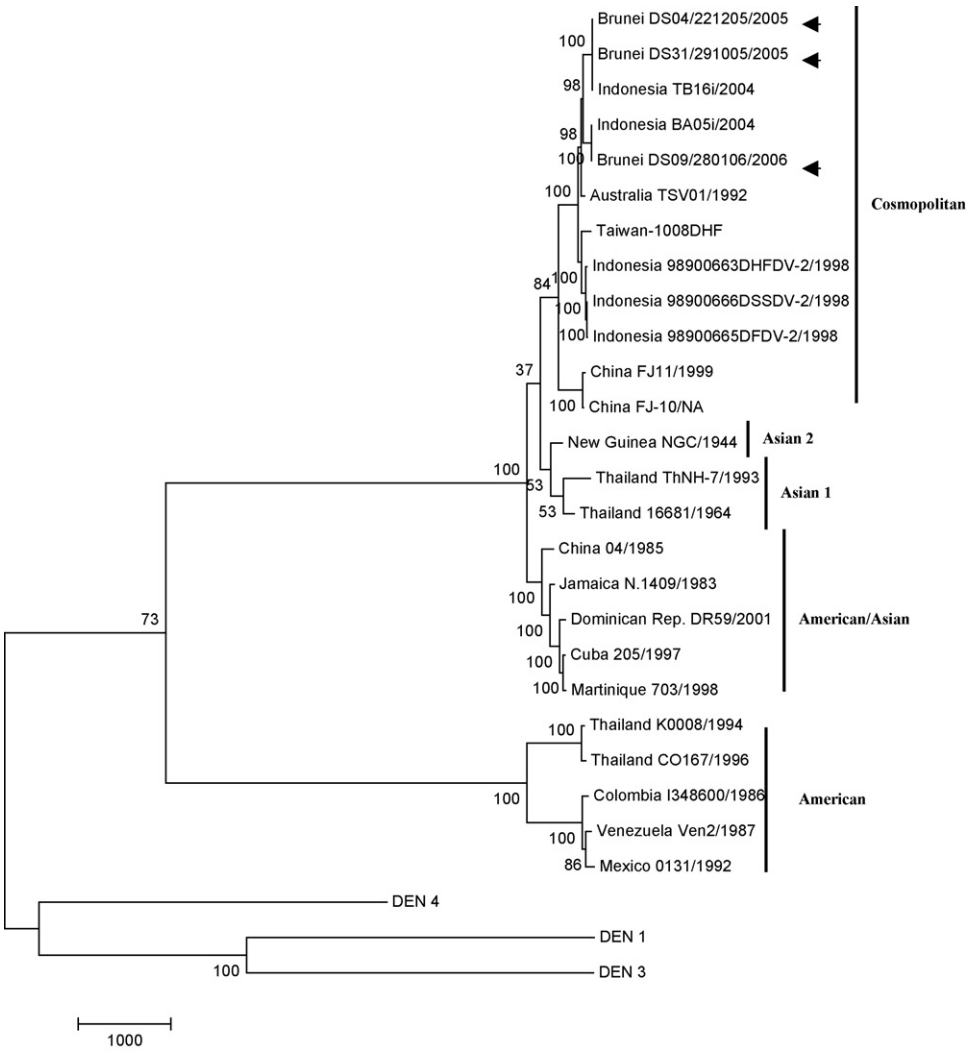

Fig. 2. A phylogenetic tree showing the complete full-length sequence analysis of Brunei DEN-2 and 22 other DEN-2 strains retrieved from the NCBI GenBank. Each strain is indicated by country and strain designation followed by the year of isolation. (◄) Position of the Brunei DEN-2 strains. Phylogenetic analyses were conducted in MEGA4. The evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence.

2.4. Sequencing the PCR product

DNA sequencing of the PCR product were carried out by a commercial laboratory (MACROGEN, Korea) using the Sanger dideoxy sequencing methodology.

2.5. Nucleotide sequences used in the study

The other sequences used in this study were retrieved from the National Center for Biomedical Investigation (NCBI) database with the following GenBank accession no.: Indonesia BA05i (AY858035), Indonesia TB16i (AY858036), Indonesia 98900665DF (AB189123), Indonesia 98900663DHF (AB189122), Indonesia 98900666DSS (AB189124), Thailand 16681 (U87411), Thailand K0010 (AF100460), New Guinea C (AF038403), Taiwan 1008DHF (AY776328), Australia TSV01 (AY037116), Mexico 0131 (AF100469), Jamaica N.1409 (M20558), Venezuela Ven2 (AF100465), Thailand ThNH-7 (AF022434), Thailand K0008 (AF100459), Thailand CO167 (AF100464), Colombia I348600 (AY702040), Cuba 205/97 (AY702039), Domincan Rep. DR59/01 (AB122022), China FJ11/99 (AF359579), China FJ-10 (AF276619), China 04 (AF119661), Martinique 703/98 (AF208496), DEN-1 (AF513110), DEN-3 (AB189125) and DEN-4 (AF326573).

2.6. Sequence and phylogenetic analysis

Multiple sequence alignments were performed using the CLUSTAL W programme (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses were conducted using the MEGA4 programme (Tamura et al., 2007). The tree was constructed from aligned nucleotide sequences using the Maximum Parsimony method (Fig. 2). Branch topology was verified by generating 1000 bootstraps.

3. Results

Twenty-five sets of primers were designed to amplify 25 overlapping fragments across the whole sequence of DS31/291005, DS04/221205 and DS09/280106 virus genome. All were obtained from the 3rd passage of the virus culture in C6/36 mosquito cell line. The complete sequence length of the Brunei DEN-2 virus strains

were 10,709 nucleotides and the NCBI GenBank accession no. were EU179857 and EU179859, respectively.

Between the Brunei strains, the isolate DS09/280106 showed 70 amino acids substitutions as compared to the other two strains. These changes were found throughout the whole genome protein from the capsid to the NS5 region of the viral strain. The highest number was in the NS2A region with 35 amino acid substitutions occurring at positions 12 (V → I), 27 (V → I), 29 (T → A), 32 (A → T), 36 (V → T), 38 (V → I), 39 (S → T), 48 (M → L), 50 (F → Y), 51 (R → K), 57 (M → I), 58 (V → I), 63 (T → A), 67 (D → E), 68 (I → M), 89 (A → V), 92 (L → V), 104 (T → A), 115 (T → A), 116 (I → L), 118 (E → N), 119 (T → D), 120 (I → V), 122 (E → A), 131 (M → I), 133 (V → I), 136 (I → L), 139 (N → K), 147 (V → A), 149 (I → V), 152 (I → L), 158 (A → I), 159 (V → M), 161 (L → I) and 186 (Q → R). One amino acid substitution was found in each of the capsid, pre membrane, NS2B and NS4B protein at positions 108 (V → M), 160 (A → T), 73 (F → I) and 89 (I → M), respectively. The envelope region showed 3 amino acid changes at position 172 (D → E), 235 (E → K) and 419 (A → V). This strain also had 5 amino acid substitutions in the NS1 region at positions 69 (K → R), 191 (N → S), 205 (A → T), 227 (K → R) and 247 (F → I). There were 2 amino acid substitutions in the NS3 region at positions 122 (I → T) and 461 (I → V) while the NS4A show 3 changes in positions 19 (Q → H), 22 (R → I) and 93 (T → I). In the NS5 region, there were 5 amino acid substitutions at positions 273 (N → D), 570 (R → K), 634 (H → Q), 748 (L → M) and 899 (L → F). Strain DS31/291005 shows amino acid transition from serine to proline at position 125 in the NS1 region. A single amino acid change was also observed in the NS3 protein region at position 335 (E → D) and there were 3 similar transitions of glutamic acid at positions 157 (E → D), 169 (E → D), and 217 (E → Q) in the NS5 region. The strain DS04/221205 had 2 amino acid changes in the NS2A region at positions 15 (M → W) and 16 (A → H). In the NS5 protein region, there were 6 substitutions found at positions 131 (D → H), 142 (T → P), 175 (N → H), 176 (T → P), 198 (Q → P) and 215 (T → P).

When comparing with the 1998 Indonesian isolates, all 3 strains showed a V → I substitution at position 104 in the capsid region while isolates DS04/221205 and DS31/291005 had an M → V substitution at position 108. In the prM gene, all 3 isolates had a substitution of A → V at position 96. Isolate DS09/280106 had another substitution in the prM region at position 160 causing the changes from A → T. In the envelope region, all 3 had a V → I substitution at position 129. The amino acid change of E → D had occurred at position 172 in isolates DS04/221205 and DS31/291005. Two additional substitutions at positions 235 (E → K) and 419 (A → V) occurred in isolate DS09/280106. Five amino acid changes were located in the NS1 region of the isolate DS09/280106 at positions 117 (K → R), 191 (N → S), 205 (A → T), 227 (K → R), and 247 (F → I) while isolate DS31/291005 had a single change in this region at position 125 (S → P). At position 272, all the three isolates had a K → R substitution. In the NS2A gene region, DS09/280106 had a total of 35 amino acid changes occurring at positions 12 (V → I), 27 (V → I), 29 (T → A), 32 (A → T), 36 (V → T), 38 (V → I), 39 (S → T), 48 (M → L), 50 (F → Y), 51 (R → K), 57 (M → I), 58 (V → I), 63 (T → A), 67 (D → E), 68 (I → M), 89 (A → V), 92 (L → V), 104 (T → A),115 (T → A), 116 (I → L), 118 (E → N), 119 (T → D),120 (I → V), 122 (E → A), 131 (M → I), 133 (V → I), 136 (I → L), 139 (N → K), 147 (V → A), 149 (I → V), 152 (I → L), 158 (A → I), 159 (V → M), 161 (L → I) and 186 (Q → R) while DS04/221205 had two substitutions at positions 15 (M → W) and 16 (A → H). Isolates DS04/221205 and DS31/291005 had a single amino acid change in the NS2B region at position 73 (I → F). Both these isolates showed two changes at locations 122 (T → I) and 461 (V → I) in the NS3 gene. DS31/291005 had an additional substitution at position 335 (E → D). In this gene also all the three isolates had a change of N → S at position 177. In the NS4A gene, isolate DS09/280106 had three changes at positions 19 (Q → H), 22 (R → I) and 93 (V → I) while the other two isolates had only a single change at position 93 (V → T). In the NS4B region, all the three had an amino acid change at position 19 (A → T), an additional substitution at location 89 (I → M) was found in isolate DS09/280106. All three isolates had an E → K change at position 46 and I → T at position 271 in the NS5 gene. Isolate DS04/221205 had a total of eight changes throughout the NS5 at positions 131 (D → H), 142 (T → P), 175 (N → H), 176 (T → P), 198 (Q → P), 215 (T → P) and 570 (K → R). Four changes were found in DS31/291005 at 157 (E → D), 169 (E → D), 217 (E → Q) and 570 (K → R) and in isolate DS09/280106 it was found at 273 (N → D), 634 (H → Q), 748 (L → M) and 899 (L → F).

In addition the Indonesian strains TB16i and DF98900665 had a change of S → N when compared to the Brunei DEN-2 isolates at position 390 in the envelope region. In the NS1 gene, the strain TB16i had an I → V substitution at position 21. In this region also it was found that both strains also have an amino acid change at position 117 (A → T). In the NS2A gene, Strain Tb16i had a single change at position 212 (S → A). Another Indonesia strain BA05i isolated in 2004 showed a single changed at position 106 (I → V) in the NS2B gene region. This strain also had a single change at position 549 (R → V) of the NS3 gene while the strain DF98900665 had a change in position 453 (S → I). Strain TB16i had a single change in the NS4A region at position 36 (A → T). Strain BA05i had four changes in the NS5 region at positions 19 (A → T), 391 (P → R), 634 (H → Q) and 648 (A → E) while in the same region strain TB16i had three changes at positions 430 (G → E), 570 (K → R) and 648 (A → V). Strain DF98900665 had three substitutions at positions 64 (W → C), 173 (N → K) and 590 (M → L) while strain DHF98900663 had a single change at position27 (I → V) in the same gene region.

4. Discussion

To date, there is no data on the molecular epidemiology of dengue viruses in Brunei and this study is the first to provide such documentation. In the 2005–2006 dengue infection, only two serotypes were found circulating in the country and there were no deaths due to DF and DHF/DSS reported. This event is of concern as neighbouring countries were reporting deaths due to dengue haemorrhagic manifestation annually and yet the extent of the country's dengue infection was minimal. The main objective of the study is to establish the virus genotype, speculate the source of transmission and detect the development of any variation in the nucleotide and the amino acid sequences of the local dengue virus.

The full-length analysis of the 3 Brunei DEN-2 viruses at the nucleotide and amino acids sequences showed that they were most closely related to the Indonesian strains of 1998 and 2004 and the Australian strains of 1993 in the Cosmopolitan genotype of the DEN-2 genotype classification by Twiddy et al. These data corroborate our previous results obtained from the E/NS1 limited sequence analysis (Osman et al., 2008) and also with others (Deubel et al., 1993; Rico-Hesse et al., 1997; Miagostovich et al., 1998 and Twiddy et al., 2002). This Cosmopolitan genotype has been said to spread as a result of geographic proximity as well as social and economic activities with its neighbours (Sarawak and Sabah) Malaysia and (Kalimantan) Indonesia. The presence of this genotype could also be due to absence of this genotype in the country and hence a more susceptible population.

In the Envelope protein sequence of the strain DS09/280106, the transition of amino acid E (Glutamic acid) to K (Lysine) will cause charge reversal from negative to positive. This could have an effect on cell tropism or entry and also on immunogenecity. The changes in the NS1 protein might be caused by the single amino acid transition at position 205 (alanine to threonine) making it hydrophilic. Transitions of the amino acid in the NS2A region that causes chang-

ing of the amino acid groupings were only found in the positions 29 (threonine to alanine), 32 (alanine to threonine), 50 (phenylalanine to tyrosine), 63 (threonine to alanine), 104 (threonine to alanine), 118 (glutamic acid to asparagine), 119 (threonine to aspartic acid), 122 (glutamic acid to alanine), 139 (asparagine to lysine) and 186 (glutamine to arginine). It is not known what these changes have on function of this region of the virus. Currently we are working to investigate the biological characteristics of our strain and determine the correlation with the changes that had occurred in the Brunei strain.

It was also noticed that the three DEN-2 strains isolated in Brunei did not originate from a single branch of the phylogeny suggesting that multiple introductions might have occurred in the country from the neighbouring countries. As there were limited full genome sequences of DEN-2 from nearby states in the GenBank, origin of the virus infecting the population of Brunei during the 2005–2006 dengue infection cannot be determined.

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