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Sequence analysis of E/NS1 gene junction of Dengue Type-1 viruses isolated in Klang Valley 2010 to 2012

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Abstract. Dengue is a mosquito-borne viral disease caused by four serotypes of dengue virus, affecting the human population for decades in many tropical and subtropical regions of the world. In Malaysia, all four dengue serotypes co-circulates in a dengue season even though any one of the serotypes can predominate. In this study, serum samples were collected from dengue fever and severe dengue fever patients within Klang Valley from 2010-2012 to determine the prevailing dengue serotypes. In addition, sequencing of the envelope/non-structural 1 (E/NS1) gene junction of the virus isolated was performed to identify the presence of any mutations that are suggestive of increased virulence in the virus. The results showed that Dengue-1 (DEN-1) was the predominant circulating serotype. The E/NS1 gene sequences of the isolates were analysed to trace the evolutionary knowledge of the strains. All sequences of the isolates were compared with DEN-1 prototype Hawaii strain as the reference sequence. The E/NS1 sequences of other dengue strains from neighbouring regions as well as other parts of the world obtained from the GenBank database were also included in the phylogenetic tree analysis. Analyses showed that there was 97% to 100% similarity among the ten isolates at the nucleotide level. Similarly, the amino acid analogue also showed 98% to 100% homology. However, all five non-severe dengue isolates showed variation at position 780, resulting in an amino acid change from valine to alanine as compared to severe dengue isolates. A rooted phylogenetic tree was performed using neighbour-joining method with DEN-2 and DEN-3 as the outgroups. Results showed that all ten isolates were classified as genotype I. In addition, the five isolates from severe dengue patients were found to be clustered together with JN697057 and JN697058, Malaysian DEN-1 strains from the 2005 outbreak.

INTRODUCTION

Dengue is a mosquito-borne viral disease caused by four serotypes of dengue virus namely DEN-1, DEN-2, DEN-3 and DEN-4. The virus is a single stranded, positive sense strand RNA virus with a genome size of approximately 11 kb comprising of three structural and seven non-structural proteins flanked by 5' and 3' untranslated region (UTR) (Osman *et al.*, 2008). The order of the proteins encoded in the open reading frame (ORF) are 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (Speight & Westaway, 1989). Dengue is known to affect the human population for decades. The incidence of this disease has not declined over the years and is in fact soaring to an estimate of 400 million cases annually, especially in the tropical and subtropical countries (Centers for Disease Control and Prevention, 2014).

The first dengue outbreak recorded in Malaysia was in 1902 that occurred in Penang and more cases were reported in other regions following that (Abu Bakar & Shafee, 2002). The Malaysian Ministry of Health reported that there was a 10% increase in the number of dengue cases in 2012 as compared

to 2011 in which Selangor recorded the highest number of dengue cases with a total record of 9 113 cases followed by Kuala Lumpur and Putrajaya (1 814 cases). In terms of serotype prevalence in Malaysia, the Ministry of Health reported in their revised second edition of Clinical Practice Guidelines on Management of Dengue Infection [MOH/P/PAK/209.10(GU)] showed that in the early 1990s, DEN-3 was the predominant circulating serotype but declined rapidly in 1996 after being displaced by DEN-1 and DEN-2. In the year 1997, however, DEN-2 started to grow steadily and peaked in 1999. DEN-1 began to increase in the early 2000s particularly in 2003 until 2006. Mohd-Zaki *et al* (2014) reported that DEN-1 became the major circulating serotype again from 2010 until 2011. They also reported that in 2012, DEN-3 was the predominant circulating serotype in Malaysia. DEN-1 and DEN-2 had an equal distribution while DEN-4 was the least circulating serotype. Interestingly, from 1st January until 12th July 2012, Sentinel Surveillance System, Ministry of Health Malaysia recorded that there was a heterogenous distribution of dengue virus serotypes in separate states of the country (Mohd-Zaki *et al*., 2014). The increasing prevalence of DENV-1 that is currently occurring is not only in Malaysia but is also present in more than 100 countries as reported by Villabona-Arenas & Zanotto, 2013. The Virology Unit, Institute for Medical Research (IMR) and The National Public Health Laboratory, Sungai Buloh regularly conducts dengue serotype surveillance as part of research to predict dengue outbreak. Based on the number of dengue cases received by the Virology Unit, DEN-1 continued to be the major circulating serotype since late 2010. Hence, this serotype has become the subject of interest in this study. Primary control measures such as fogging, larvicidal and source reduction have been introduced by the government to curb the increasing mosquito population. Even so, controlling dengue will always be a great challenge.

Many other researchers used the E/NS1 gene junction to determine molecular epidemiology of dengue viruses (Ricco-Hesse, 1990; Osman *et al*., 2008; Thayan, 1997). The purpose of the research was to determine the prevailing serotype and also to identify presence of any 'virulence factor' in the virus.

MATERIAL AND METHODS

Dengue patients' samples

In the current study, serum samples from patients of severe and non-severe DEN-1 were collected from two hospitals in Klang Valley, namely the Selayang Hospital and Kuala Lumpur Hospital. A total number of 269 serum samples were obtained from admitted dengue patients who met the inclusion criteria in the study. Institutional ethical approval for blood collection was obtained prior to study (Registration number: NMRR-09-883-4768). A total number of 155 samples were tested positive by SYBR Green Real time RT-PCR. Among these, 74 samples were of DEN-1 and it was found that five of these were from severe dengue cases. For comparison purposes, another five samples were randomly picked among the non-severe dengue cases (Table 1). All samples were collected from late 2010 until 2012.

Virus isolation

Five serum samples from each of severe dengue and non-severe dengue patients were collected for virus isolation. This was done by inoculating into C6/36 *Aedes albopictus* cell lines. 25 cm^3 screw capped flat bottom flask was seeded with 5.0 ml of C6/36 cells at a concentration of 5 x 10^5/ml, which were then further seeded into a flat-sided culture tube with a volume of 1.0 ml of confluent monolayer cells. Cells were incubated at room temperature until confluent and later inoculated with 20 µl of DEN-1 positive patients' sera and left at room temperature for 1 hour to allow for viral adsorption. A tube of non-inoculated cells was used as a negative control. Incubation at room temperature was maintained for 7 to 10 days and cells were observed regularly to detect any possible cytopatic effect (CPE), which was determined by the formation of syncitia. Once CPE was observed, the culture fluids were then harvested through a freeze-thaw method

Table 1. List of dengue virus type-1 used in the study

ISOLATES	CLINICAL STATUS	GENBANK ACCESSION NO
MaSH01011	Non-severe dengue	KR053537
MaSH01511	Non-severe dengue	KR053540
MaSH07011	Non-severe dengue	KR053552
MaSH10812	Non-severe dengue	KR053557
MaSH11712	Non-severe dengue	KR053559
MaSH00110	Severe dengue	KR053536
MaSH02111	Severe dengue	KR053541
MaSH03211	Severe dengue	KR053543
MaSH06011	Severe dengue	KR053550
MaSH06511	Severe dengue	KR053551

The first two letters stand for country of isolation (Malaysia) followed by states (Selangor), source of sample (Human) and the lab number. The last two digits indicate the year of sample collection.

where the tubes were left in a -70°C freezer overnight before thawing at room temperature that causes cell disruption and the release of viruses. Verification for the presence of dengue virus was done by performing Real time RT-PCR (Chutinimitkul *et al.*, 2005).

RNA extraction and Real time RT-PCR to determine dengue serotypes

Extraction of viral RNA was carried out using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's protocol. Real time RT-PCR was performed to identify dengue serotypes by using QuantiTect® SYBR® Green RT-PCR kit (Qiagen, Germany) according to the method performed by Chutinimitkul *et al.* (2005). Briefly, a volume of 20 µl of the RT-PCR master mix was added into a sterile 0.2 ml PCR tube before adding 5 µl of the extracted viral RNA and subsequently briefly vortexed and centrifuged before conducting the RT-PCR reaction. The thermal cycling conditions for the reaction are as follows: reverse transcription for 30 minutes at 50°C, followed by initial denaturation at 95°C for 15 minutes. Subsequently, 40 cycles of PCR with the following steps were conducted: denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 40 seconds. Final extension was done at 72°C for 7 minutes. Later, melt curve analysis was performed at 75°C with an interval of 5 seconds every 0.5°C increase, until 95°C. The final incubation was at 4°C for infinity.

Amplification of the target gene

For the sequencing study, the E/NS1 gene junction was chosen where conventional RT-PCR on DEN-1 target was done using QIAGEN One-step RT-PCR kit (Qiagen, Germany). Primer sequences used in this study were designed using OligoAnalyzer and Primer3 online software based on Nauru Island strain (U88535). The forward primer 5'-TCAGCGGTGTTTCTTGGAC-3' and reverse primer 5'-ATTCCACACACACCCT CCTC-3' amplified 313 bp of the E/NS1 junction virus genome encompassing the 240 nucleotides of the E/NS1 junction gene described by Ricco-Hesse (1990). The RT-PCR parameters were as follows: reverse transcription at 50°C for 1 hour, initial denaturation at 95°C for 15 minutes, 40 PCR cycles consisting of denaturation at 94°C for 30 seconds, annealing at 57.3°C for 45 seconds and extension at 72°C for 1 minute. There was a final elongation at 72°C for 10 minutes. The PCR products were subsequently subjected to electrophoresis on 2% agarose gel and purified using QIAquick PCR purification kit (Qiagen, Germany).

Sequencing and sequence analysis

Sequencing of purified PCR product was carried out by out-sourcing to a commercial lab. Clean-up and analysis of sequencing data was done using Lasergene software Version 8.1 where both forward and reverse primer sequences were aligned and edited to comprise only the 240 bp E/NS1 gene junction. Multiple sequence alignment was achieved using Clustal W. Phylogenetic analyses were conducted using MEGA 5 software and phylogenetic tree was constructed using neighbour-joining method with bootstraps value of 1000 replicates.

RESULTS

Dengue serotype determination

All 269 serum samples from suspected dengue patients were tested by SYBR Green Real time RT-PCR to determine the serotype of dengue virus. The results indicate that 159 of the samples were positive of dengue using SYBR Green Real time RT-PCR method. Among those positive samples, the breakdowns of dengue serotypes were as follows:- DEN-1: 78, DEN-2: 32, DEN-3: 34 and DEN-4: 15. DEN-1 was the pre-dominant serotype circulating and therefore has become the focus of the study.

Dengue virus isolation

Ten serum samples were selected for viral propagation and gene sequencing. These included the 5 samples from confirmed severe dengue patients and another 5 samples randomly selected from non-severe cases. The virus was propagated in C6/36 cells and successfully isolated as confirmed by SYBR Green Real time RT-PCR.

Dengue E/NS1 sequencing

The E/NS1 gene sequence data of all ten DEN-1 isolates are showed in Figure 1. Nucleotide positions were numbered according to Mason *et al.* (1987). In this particular study, DEN-1 prototype Hawaii strain was used as the reference sequence. Sequence analyses of the ten isolates showed that there were 7 differences in the nucleotides within the sequenced region. However, there was 97% to 100% identity among them. In contrast, comparison with prototype Hawaii strain showed that the nucleotide percentage identity was only around 91% to 92% with 25 differences in the nucleotide.

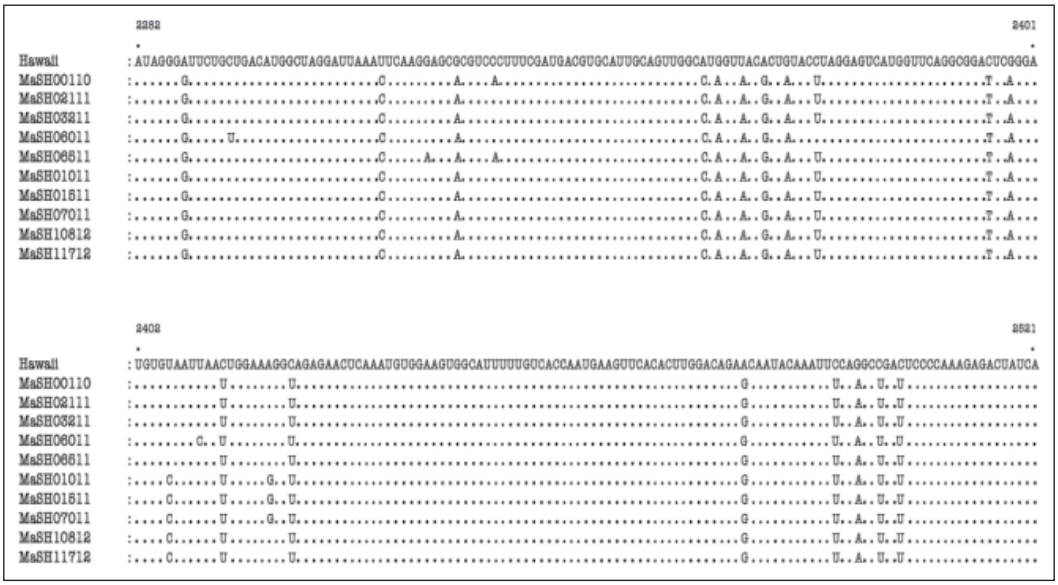

Figure 1. Nucleotide sequence (240 bp) of all isolates in the study. The top sequence is DENV-1 prototype Hawaii strain.

In terms of amino acid percentage identity, there was a 98% to 100% identity among the ten dengue isolates. Nevertheless, there was a variation showed by all five non-severe dengue isolates whereby at amino acid position 780, there is a change in the amino acid from valine to alanine when compared to severe dengue isolates. This change was caused by a single base substitution at nucleotide position 2406 that change the codon from GUA → GCA. The amino acid percentage homology between all isolates in this study and the prototype strain was from 95% to 96%. The amino acid position 780 of the prototype strain was the same as the severe dengue isolates, which is Valine (Figure 2).

However, all the sequenced isolates showed another three amino acid variations when compared with prototype DEN-1 Hawaii strain (amino acid position 741, I → V; amino acid position 753, A → T; and amino acid position 764, M → L) at nucleotide positions 2288, 2324, 2357 and 2359 respectively. The first two changes were caused by a single base substitution at the first codon (AUU → GUU and GCG → ACG). The third amino acid change on the other hand was caused by two bases substitution at the first and third codon (AUG → CUA).

Phylogenetic analysis

Phylogenetic tree analysis was generated by comparison between the isolates and other DEN-1 strains from neighbouring countries and around the world obtained from GenBank (Table 4).The tree placed all the isolates, both from severe and non-severe dengue in genotype I (Figure 3). DEN-2 and DEN-3 were used as the outgroup. Based on the phylogenetic tree, the results indicate that even though both DEN-1 virus from severe and non-severe patients were typed as genotype I, there is further division within the genotype between severe and non-severe dengue. The severe dengue isolates were clustered together with Malaysian strains (Accession no: JN697057 and JN697058) isolated in 2005, Singapore strain (GU370049) isolated in 2008, and Thailand strain (FJ687432) isolated in 2001. The non-severe dengue on the other hand, were grouped together with strain from China (JQ048541) isolated in the year 2011.

DISCUSSION

The growing number of dengue incidence in Malaysia has enhanced efforts to find the probable cause of dengue outbreaks which can be achieved by conducting dengue surveillance and serotype determination. This is important as any introduction of new genotypes or strains can result in dengue outbreak. As such, molecular epidemiology of dengue viruses can either be a precautionary step or complementary to active vector surveillance and activation of vector control, if necessary. DNA sequencing is a very useful tool to analyse nucleotide variations and amino acid differences in both

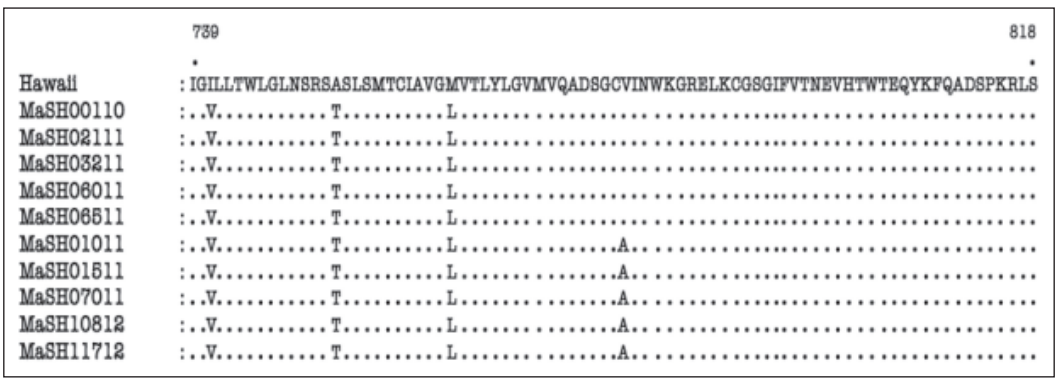

Figure 2. The amino acid sequence (80bp) of both severe and non-severe dengue isolates in the study. The top sequence is DENV-1 prototype Hawaii strain.

Table 2. Country of origin and year of isolation of DEN-1 viruses used in the study

Genotype	GenBank Accession no.	Country	Year
I	JQ048541	China	2011
	JN697057	Malaysia	2005
	GU370049	Singapore	2008
	FJ687432	Thailand	2001
	JN697068	Malaysia	2005
	AF309641	Cambodia	1998
	AF350498	China	1980
	M32915	Taiwan	1987
	AF298808	Djibouti	1998
	AB609588	Hawaii	1944
	AB074760	Japan	1943
II	AF180817	Thailand	1964
	D10513	Thailand	1954
III	EF457905	Malaysia	1972
IV	AB189121	Indonesia	1998
	EU179861	Brunei	2006
	U88535	Nauru Island	1974
	M32919	Philippines	1974
	AB074761	Indonesia	1988
	AB204803	Japan	2004
	DQ285561	Seychelles	2004
	JN697056	Malaysia	2005
	AB189120	Indonesia	1998
	GQ328924	Indonesia	1998
	DQ016636	Indonesia	2003
V	AY762084	Singapore	1993
	EU179860	Brunei	2005
	M32898	Malaysia	1982
	M32913	Sri Lanka	1969
	AF514889	Argentina	2000
	M32901	Mexico	1983
	M87512	Singapore	1990
	AY306098	Sao Paolo	2001
	AF514876	Argentina	2000
	S64849	Brazil	1990

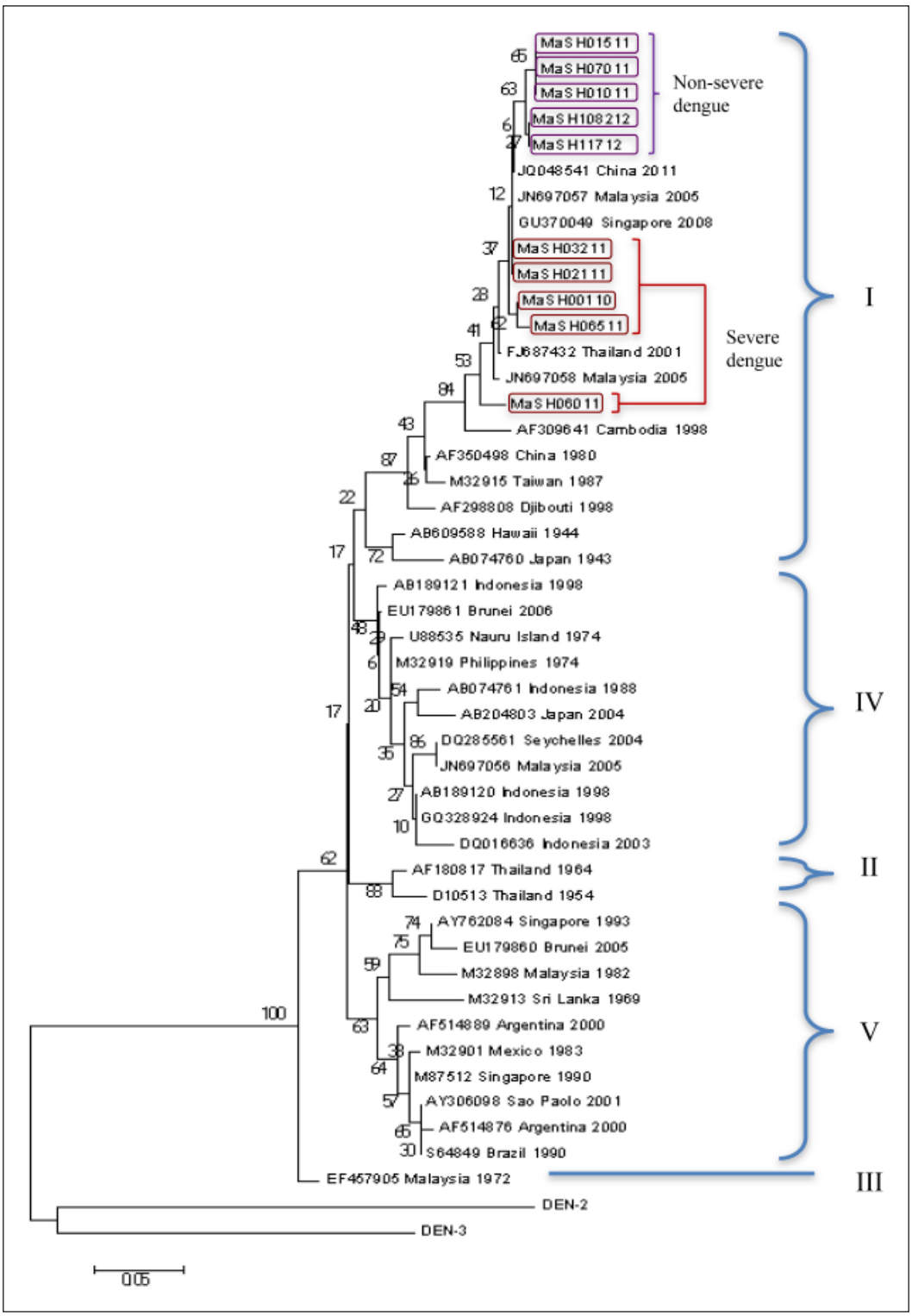

Figure 3. The neighbour-joining tree showing the relationship of the E/NS1 gene sequences of the ten DEN-1 isolates in the study and 35 other DEN-1 strains in GenBank. Bootstrap value is shown above the connecting branch and the tree is rooted using DEN-2 and DEN-3 strains as the outgroup.

molecular and evolutionary biology and therefore can help further understand the molecular epidemiology of dengue. Molecular epidemiology is important for investigation of the patterns, etiology, transmission and risk factors of dengue. It is also useful for disease surveillance, which can be achieved by performing the phylogenetic analysis.

Presently, published DNA sequences of Malaysian E/NS1 gene junction dengue strain, especially DEN-1 are limited, as indicated in the GenBank. Also, most researchers have opted for E protein gene as the target of interest in studying dengue genetic variation and evolutionary analysis as the envelope protein contains sites required for virus binding and penetration into susceptible cells and mutations on these sites may affect virus-mediated membrane fusion and neurovirulence (Laille & Roche, 2004). Nevertheless, the E/NS1 junction gene has been selected as the target gene of interest since it showed a uniform rate of random mutation with no hypervariable regions and is therefore suitable for comparison of variations among the same serotype and other phylogenetic studies (Rico-Hesse, 1990). By examining the E/NS1 junction region, the genotype of the virus can be established, apart from localizing the source of transmission and the possible association with dengue outbreaks.

DEN-1 was chosen as it was the predominating dengue serotype in 2011. It was observed that the number of dengue cases and deaths have reduced in Malaysia between 2010 and 2011 where there were 46 171 dengue cases with 134 deaths in 2010 as compared to 19 884 dengue cases and 36 deaths in 2011. Thereafter, the number of dengue cases started to escalate where there were a total of 108,698 cases with 215 deaths in 2014 and a total of 120,836 cases with 336 deaths in 2015. Even though, over the years the number of cases and deaths were increasing, the only exception being in 2011 where there was an actual decrease in dengue cases in Malaysia. This may suggest an introduction of a less virulent strain of dengue virus in the population. In addition, unpublished data from Virology Unit, IMR showed that the pre-dominating dengue serotype in Klang Valley was DEN-2 in 2010 compared to 2011 where DEN-1 was the predominating serotype. Hence, the drop in both total number of dengue cases and death tolls in 2011 is a result of a change in predominating dengue serotype where DEN-1 has replaced DEN-2. It can be further postulated that the circulating DEN-1 strain/genotype could be of less virulent. In this study, it was found that DEN-1 was the predominant circulating serotype in year 2012. This was opposed to what was reported by Mohd-Zaki *et al.*, 2014 whereby DEN-3 was the predominant circulating serotype in Malaysia that year. The findings did not coincide because the data in this study represent the serotype distribution in Klang Valley alone compared to serotype distribution from all states in the country as published by Mohd-Zaki *et al.* (2014).

Results from this study showed that there were four amino acid changes within the sequenced region, which were at amino acid position 741, 753, 764 and 780. Also, the change detected at amino acid position 780 between the severe and non-severe dengue isolates is not exactly at the junction gene itself but rather at the NS1 gene, which is at the nucleotide position 2406. NS1 gene plays an important role in early stages of viral replication. Further analysis needs to be done to determine whether the change in this amino acid position affects the viral replication process. The three amino acid changes that occurred at positions 741, 753, and 764 of local dengue serotypes strains compared to prototype dengue 1 Hawaii strain were located within the envelope gene region (nucleotide position 2288, 2324, 2357 and 2359) which is at the C-terminal domain III containing stem region and two transmembrane anchor. Domain III was found to interact with cellular receptors for entry (Chu *et al.*, 2007, 2005; Hung *et al.*, 2004; Lee & Lobigs, 2000) and has been showed to comprise of residues that are important for determination of host range, tropism and virulence (Rey *et al.*, 1995). It also contains epitopes that bind to neutralizing antibodies

(Weaver & Vasilakis, 2009). Mutations within this domain, namely N390D is located within the putative glycosaminoglycan binding motif (386L-411M) responsible for the binding of DENV onto the host cell membrane via the non-Fc receptor (Chen *et al.*, 1996) and thus has been suggested as a potential virulence determinant in American DENV-2 genotype (Leitmeyer *et al.*, 1999). Sanchez & Ruiz (1996) on the other hand reported that mutation in this region altered virulence in mice. A more detail research and analysis are required to determine whether the three amino acid changes mentioned previously in this study fall within this particular mutation domain. Moreover, the amino acid change in the severe dengue isolates of this study did not occur in the C-terminal domain III and therefore no relation to the mutation mentioned above that caused virulence.

All DEN-1 isolates in this study were classified as genotype group I and were closely related to those strains that were isolated in recent years around the neighbouring countries. The non-severe dengue isolates were shown to originate from dengue strain isolated in China during the year 2011 suggesting that they may have originated from there. Based on the phylogenetic tree (Figure 3), all five isolates from severe dengue patients in the present study seem to have originated from the 2005 DEN-1 strain. Furthermore, even though there was only a single amino acid change at position 780 between isolates from severe and non-severe cases, but based on the phylogenetic tree, strains from both groups were separated into two different clusters, well within the same genotype. The significance of this finding remains unknown as whether the single amino acid change results in phenotypic changes can be proven through *in vivo* study.

The data from this study has provided some insights on the patterns and evolutionary origin of the strains. The results have indicated that very little variation has occurred in DEN-1 for the past 5 years between 2005 and 2011. Therefore we conclude that all the isolates of DENV-1 in this study falls within genotype group I. Further study is needed to determine whether the single amino acid change between isolates from severe and non-severe dengue is indicative of virulence.

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