Isolation of monoclonal antibodies-escape variant of dengue virus serotype 1

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ABSTRACT

Introduction: During an outbreak from December 2004 to March 2005, 138 isolates of dengue virus were prospectively obtained from acute-phase serum samples of 1,067 patients with the provisional clinical diagnosis of acute dengue illness admitted to the adult wards of Hospital Tengku Ampuan Rahimah, Klang, Malaysia. Of the 138 dengue virus isolates, 87, 11, 24 and 3 were typed as dengue serotypes 1, 2, 3 and 4, respectively, by a commercial dengue virus typing kit using monoclonal antibodies (Mab). 13 dengue virus isolates could not be assigned to any specific serotype by serotyping Mab and molecular typing using dengue-type specific molecular typing primer pairs. We report the associated clinical features and limited molecular genetics of this Mab-escape dengue virus variant.

Methods: Limited molecular characterisation of the Mab-escape dengue virus variants with respect to a few concurrently isolated dengue serotype 1 virus was performed by reverse transcriptase polymerase chain reaction (RT-PCR), followed by nucleic acid sequencing of the 500-bp dengue virus partial genomic capsid-PreM fragment.

Results: The aligned nucleic acid sequence of RT-PCR products showed that these Mab-escape variants were of identical nucleic acid sequence, and shared the highest sequence homology (99 percent) with dengue virus serotype 1 (GeneBank accession No. AB178040) isolated from a Japanese patient in 2004. Though these Mab-escape dengue virus variants were noted to replicate to a 2-log higher titre than the current circulating dengue virus serotype 1, there was no significant difference between these variants and the currently circulating dengue virus serotype 1 with respect to disease severity (dengue fever versus dengue haemorrhagic fever) and clinical features.

Conclusion: There was no significant difference in the proportion of patients developing dengue haemorrhagic fever following acute infection by Mab-escape dengue virus 1 variant in comparison with infection by the conventional dengue virus 1. Similarly, there was no significant difference in the pattern of clinical presentations following acute infection by the two different strains of virus.

Keywords: dengue haemorrhagic fever, dengue virus, molecular typing, monoclonocal antibody-escape variant

INTRODUCTION

There are at least 30 flaviviruses known to cause diseases in humans, of which the dengue virus is presently the most common and most important cause of human flaviviral infection in terms of both morbidity and mortality[1-3]. Annually, dengue viruses cause more than 50 million cases of human infections worldwide, resulting in around 24,000 deaths[1]. Dengue fever (DF) is an acute febrile viral disease frequently presenting with symptoms of headache, bone, joint and muscular pains, rash and leukopaenia. Dengue haemorrhagic fever (DHF) is characterised by four major clinical manifestations: high fever, haemorrhagic phenomena, often with hepatomegaly and in severe cases, signs of circulatory failure. Patients with DHF may develop hypovolaemic shock resulting from plasma leakage. This condition, called dengue shock syndrome, is associated with high mortality.

In Malaysia, DF was first reported in 1902 in Penang[4] and has since become a major public health problem, especially with the appearance of the first DHF outbreak, also in Penang, in 1962[5]. Rapid industrialisation and economic development over

the last two decades have brought about massive infrastructure development that led to the creation of many man-made environment and habitats for the breeding of *Aedes* mosquitoes, the vector of dengue virus. The reported incidence rate of clinically-diagnosed DF and DHF in Malaysia shows an upward trend from 8.5 cases per 100,000 population in 1988, to 123.4 cases per 100,000 population in 1998. For the year 2001, the incidence rate was 68.8 per 100,000 population. Most of the dengue cases were reported among the urban population (70-80%), with the highest incidence in the working and school-going age groups, correlating with the relatively high *Aedes* Index in construction sites, factories and schools[6].

Dengue virus belongs to the family *Flaviviridae*, under the genus *Flavivirus*. Traditionally, it was classified under the Group B arboviruses. There are four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4). They are antigenically very similar to each other but different enough to elicit only transient partial cross-protection after infection by each one of them[7,8]. Information on the molecular epidemiology, molecular genetics and evolution of dengue viruses circulating in Malaysia, currently and previously, is scanty. During a recent outbreak of dengue from December 2004 to March 2005 in Klang Valley, peninsular Malaysia, all four serotypes of dengue virus were isolated, although dengue virus serotype 1 predominated. Interestingly, a couple of dengue virus variants concurrently isolated were not typeable by commercially-available typing monoclonal antibodies (Mab) nor by a molecular technique, reverse transcriptase polymerase chain reaction (RT-PCR), using dengue virus typing primers published by Lanciotti et al[9]. We report the associated clinical features and limited molecular genetics of this Mab-escape dengue virus variant.

METHODS

A prospective study was carried out to determine the clinical features of laboratory confirmed dengue virus infection occurring from late December 2004 to early March 2005 and to compare these features to those of other acute viral febrile illnesses. The study population included all patients admitted to the adult wards of Hospital Tengku Ampuan Rahimah (HTAR), Klang, Malaysia, with the provisional clinical diagnosis of acute dengue illness. Acute-phase venous blood samples were collected from patients at the time of admission, and convalescent-phase venous blood samples were collected three or more days after the first blood

samples. Serological, virological and molecular techniques were used to confirm acute dengue virus infection in these patients.

C6/36 mosquito cell-line (ATCC CRL-1660) was used for the isolation of dengue virus from the patient's serum. 20 μL of each patient's acute-phase serum sample was carefully transferred into a Jui Meng (JM) cell culture tube[10] containing a newly confluent monolayer of C6/36 cells. The inoculated JM tubes were placed in a specifically-designed culture rack and incubated at 30°C. Daily examination of the culture was made under an inverted light microscope for the presence of microbial contamination and cytopathic effects. At the end of the tenth day of culture, 200 μL of cell suspension containing C6/36 cells was carefully harvested and washed twice with 1× phosphate buffered saline (PBS) by the process of centrifugation and re-suspension of pelleted cells.

After the last wash, the cells were suspended in PBS at a cell concentration of approximately 100 cells per μL. 10 μL of the cell suspension were carefully transferred onto each well of a 12-well Teflon-coated slide and allowed to air-dry over a warm-plate. The Teflon slide containing dried cells was fixed in cold acetone for 10 min and probed for the presence of dengue virus infected cells by an indirect immunofluorescence assay using a commercially-available monoclonal antibody (Chemicon Int. Inc, Temecula, CA, USA; Cat. No. MAB8705). The remaining cells harvested from the JM tube that gave positive fluorescence were re-seeded onto four wells of a new Teflon slide. After air-drying and acetone fixation, serotyping of dengue virus in the infected cells was carried out using dengue virus typing Mab (Chemicon Int. Inc, Temecula, CA, USA; Cat. No. MAB8701, MAB8702, MAB8703, MAB8704).

Dengue virus RNA was extracted from 200 μL of culture supernatant using a viral RNA extraction kit (Roche Diagnostics, Indianapolis, IN, USA). Dengue virus generic and serotype-specific oligonucleotide primers were used for the amplification of dengue virus genomic sequence fragments according to Lanciotti et al[9]. RT-PCR was performed in a single reaction tube using the Access RT-PCR Kit (Promega Corporation, Madison, WI, USA). Each genomic fragment was amplified in a 50 μL reaction mix containing the respective dengue virus generic pair of forward and reverse primers of 20 pmol each and 2 μL of the extracted viral RNA as template. Each reaction mix was subjected to 60 min of reverse transcription at 42°C, reverse transcriptase inactivation of 98°C for

5 min, followed by 35 cycles of amplification at a denaturing temperature of 95°C for 30 s, annealing temperature of 55°C for 60 s and an extension temperature of 72°C for 1 min per cycle.

The amplified products were confirmed by electrophoresing 3 μL of each of the amplified products in a 1% agarose gel. The desired RT-PCR products were gel purified and extracted using QIAquick Gel Purification Kit (Qiagen, Mainz, Germany), sequenced by ABI Prism Big-Dye (Pharmacia, Piscataway, NJ, USA) dideoxyl termination cycle sequencing using respective forward and reverse primers, and then analysed on an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Molecular serotyping of dengue virus was carried out by a semi-nested PCR using respective pairs of molecular typing primers and 2 μL of the first RT-PCR product after 100-fold dilution.

The sequence data derived from the PCR amplified fragments were routinely managed using the Clone Manager 5 and Align Plus 4 programme package (S&E Software, Ohio, USA). Multiple sequence alignments were done using standard linear scoring matrix with the following parameter settings: mismatch penalty of 1, open gap penalty of 4, extended gap penalty of 1 and similarity significance value cut-off of 60%. Deduced proteins were translated and aligned using the protein analysis module included in the Clone Manager 5 package. Multiple sequence alignments and neighbour-joining phylogram were generated using the Clustal X programme[11]. Phylogenetic analysis was conducted using the PHYLIP software package (University of Washington, Seattle, WA, USA). Most likely genetic relationships were determined by evaluation of trees from 1,000 randomised re-sampling cycles. Final tree files were visualised using the TreeView programme[12].

The concentration of virus in the supernatant was determined according to the method described by Dougherty[13]. Briefly, 200 μL of clarified culture supernatant from a JM tube containing dengue virus infected culture was inoculated into a 25-cm^2 culture flask containing a confluent monolayer growth of C6/36 (ATCC, CRL-1660) cells. After ten days of culture at 30°C, the supernatant fluid was clarified by centrifugation at 1,000× g, and 0.5 ml aliquots were made for storage at -80⁰C. Meanwhile, a serial ten-fold dilution of the virus in clarified culture supernatant was made up from 10^{-1} to 10^{12} dilution using Roswell Park Memorial Institute (RPMI) culture medium with 1% foetal bovine serum (FCS) as the diluent by initially pipetting 100 μL of the neat supernatant into 900 μL of RPMI.

After a good mixing by pipetting up-and-down, 100 μL of the diluted fluid was successively transferred into another 900 μL of RPMI. Upon completing the titration, 100 μL of the diluted supernatant from each dilution was carefully transferred into each well of a 24-well tissue culture plate which was already pre-seeded with 1 μL of RPMI growth medium supplemented with 10% foetal calf serum and containing 1×10^5 C6/36 cells. For each dilution, four cultures were set up. The culture plates were then carefully sealed with adhesive tape and incubated at 30°C. After ten days of culture, an aliquot of cells from each well was harvested, washed with phosphate buffer saline and probed for the presence of dengue infected cells by indirect immunofluorescence as described earlier. The titre of dengue virus particles in the supernatant was then enumerated as 50% tissue culture infective dose ($TCID_{50}$) according to the formula described by Dougherty[13].

The patients' epidemiological and clinical data as well as viral test results were tabulated in a Microsoft Excel spreadsheet. Data analysis was performed using Epi Info 6 computer free software programme from the Centers for Disease Control and Prevention, Atlanta, USA. A p-value of 0.05 or less was taken as the level of significant association for each ordinal variable with the relevant adjusting variables.

RESULTS

During the outbreak period, from late December 2004 to early March 2005, 1,067 adult patients with the provisional clinical diagnosis of acute dengue illness were admitted to HTAR, Klang. 138 isolates of dengue virus were obtained from their acute-phase serum samples. Of the 138 dengue virus isolates, 87, 11, 24 and 3 were typed as dengue serotype 1, 2, 3 and 4, respectively, by commercially-available dengue typing Mab. However, 13 isolates that gave a positive staining reaction with the dengue complex typing monoclonal antibody could not be assigned to any specific serotype by the serotyping Mab. Using the published generic primer pair[9], an expected 500+ base-pair amplicon (nt 134 to 644) was obtained by a one-step RT-PCR for all dengue virus isolates including the variants that could not be serotyped. In contrast, all the dengue isolates could be correspondingly molecular-typed using dengue type-specific molecular typing primer pairs except for the non-serotypeable Mab-escape variants.

The amplicons from the one-step RT-PCR of three known dengue serotype 1 isolates (DEN125, DEN155, DEN695) and three Mab-escape variants
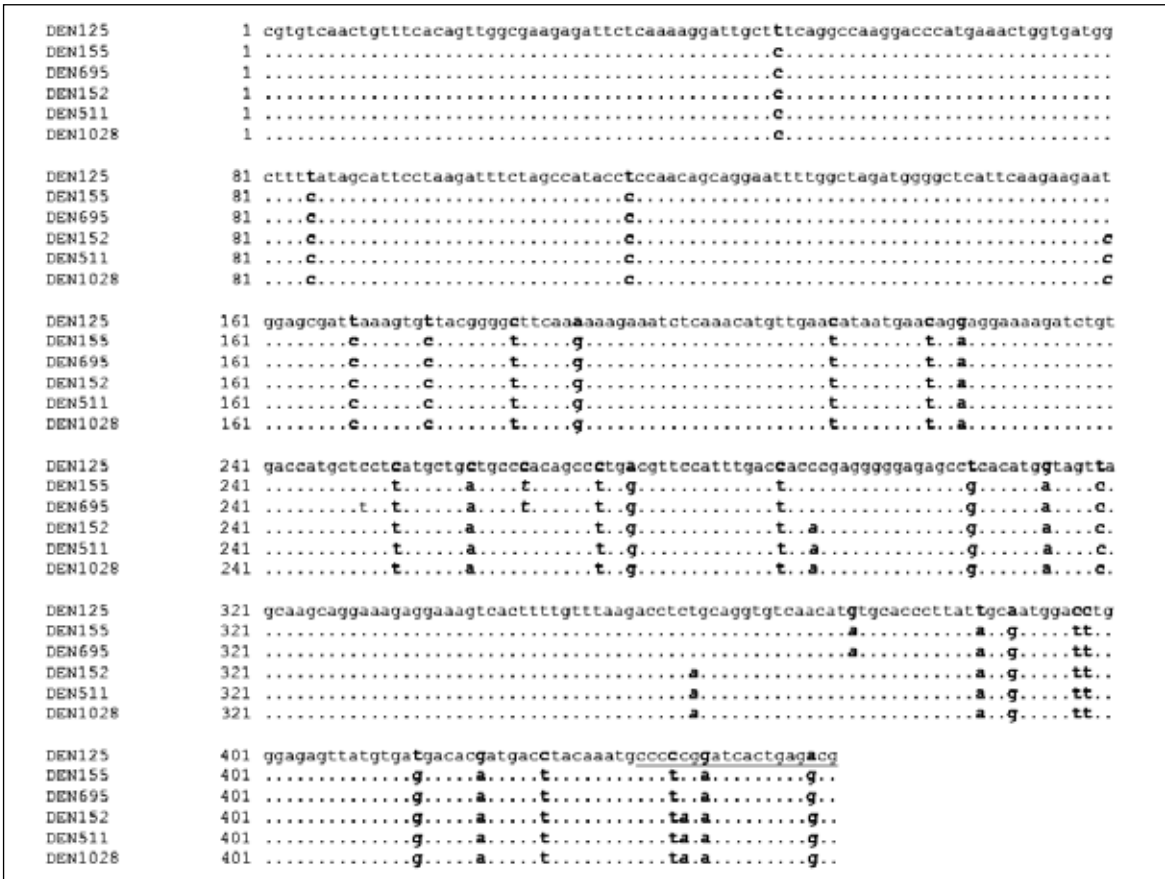
Fig. 1 Nucleic acid sequence alignment of RT-PCR amplified products derived respectively from three dengue virus serotype 1 (DEN 125, DEN 155, DEN695) and three dengue virus 1 Mab-escape variants (DEN152, DEN511, DEN1028) isolated in this study. RT-PCR products were obtained using dengue virus generic primer-pair described by Lanciotti et al[9]. The nucleic acid sequences of both primers used in the amplification were not included in the alignment. DEN155 and DEN695 appeared to be belonging to the same lineage and differed from DEN125 of a different lineage by 30 nucleotides (in bold). Mab-escape variants appeared to have evolved from lineage DEN155 and DEN695 with six nucleotide diversions in this amplified fragment (in bold and italic). The downstream primer binding site for molecular typing of dengue virus serotype 1 is underlined.
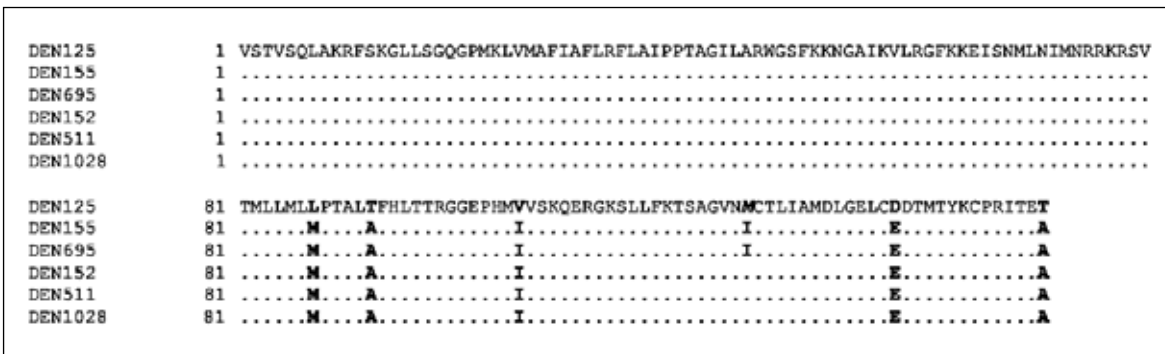

Fig. 2 Amino acid alignment of respective translated proteins derived from RT-PCR amplified nuclei acid sequences of three dengue virus serotype 1 (DEN 125, DEN 155, DEN695) and three dengue virus 1 Mab-escape variants (DEN152, DEN511, DEN1028) isolated in this study. Though Mab-escape variants differed from DEN155 and DEN695 by six nucleotide (Fig. 1), the nucleic acid changes only led to one amino acid change from isoleucine to methionine (in bold and italic).

(DEN152, DEN511, DEN1028) were gel-purified and sequenced. The aligned nucleic acid sequences of amplified genomic fragments from these isolates, excluding the nucleotide sequences of both primers (nt 162 to 615, amino-terminal of capsid protein and carboxyl-terminal of pre-membrane protein) are shown in Fig.1. The aligned amino acid sequence

of their respective translated protein fragments is shown in Fig. 2. The aligned sequence information showed DEN155 and DEN695 to be of the same lineage (differing by one nucleotide) which is different from that of DEN125 (differing by 30 nucleotides) (Fig. 1, in bold) although all three isolates were typed as dengue serotype 1 (Fig. 3).
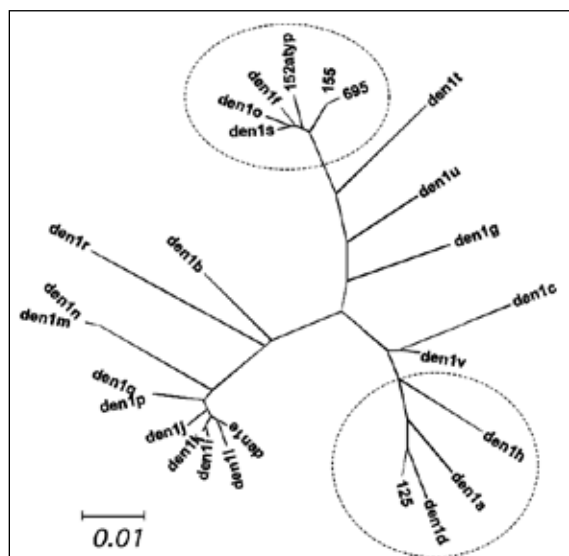
Fig. 3 Unrooted phylogenetic tree based on the partial nucleotide sequence of capsid-preM genetic segment (454 nucleotides) of a number of strains of dengue virus serotype 1. The neighbour-joining tree was generated using Clustal-X and PHYLIP programmes. The partial nucleotide sequence of capsid-preM genetic segment of dengue 1 virus isolates DEN125, DEN155, DEN695 and monoclonal antibody-escape variant were represented by 125, 155, 695 and 152atyp, respectively. The respective GenBank Accessions numbers of other strains of dengue virus serotype 1 used in this analysis are in parentheses: den1a (AB195673), den1b (AY762084), den1c (AB189121), den1d (AB189120), den1e (AY277665), den1f (AB178040), den1g (AB074760), den1h (AB074761), den1i (AF311956), den1j (AF311957), den1k(AF311958), den1l (AF513110), den1m (AY277664), den1n (AY277666), den1o (AY708047), den1p (AF226686), den1q (AF226687), den1r (AF298807), den1s (AF309641), den1t (AF350498), den1u (M87512), den1v (U88535).

The three Mab-escape variants were of identical nucleic acid sequence and shared the highest sequence homology (99%) (differing by four nucleotides) to dengue virus serotype 1 (GenBank accession No. AB178040) isolated from a Japanese patient in 2004,

followed by lineage DEN155 and DEN695 (differing by 6 nucleotides) (98% homology) (Fig. 1, in bold and italics, and Fig. 3). However, the nucleotide sequence of the three Mab-escape variants differed from lineage DEN125 by 32 nucleotides (93% homology). Although the three Mab-escape variants differed from DEN155 and DEN695 by six nucleotides, the nucleotide changes only led to one amino acid change (isoleucine/methionine) at position 125 (Fig. 2, in bold and italics). The phylogenetic relationship of these six dengue virus isolates with respect to the other strains of dengue virus serotype 1 based on the same partial capsid-preM genetic segment (454 nts) nucleotide sequences deposited in GenBank is shown in Fig. 3. The nucleic acid sequences based on the same partial genomic fragment of the remaining 10 Mab-escape dengue virus variants obtained later were found to be identical to each other and also to the earlier three Mab-escape variants.

In relation to epidemiological and clinical features, the mean age of patients with positive isolation of dengue virus serotype 1 was 27.4 years old (range 12 to 58 years, SD=9.8) and the mean age of patients with positive isolation of Mab-escape variant was 30.3 years old (range 16 to 59 years, SD=11.3). The male-to-female ratio was 2:1 for dengue virus serotype 1 verses 5.5:1 for Mab-escape variants but there was no statistically significant gender difference between the two groups (Fisher's exact test, p=0.3345). Dengue virus serotype 1 was isolated from 11.5% (10/87) of foreign workers whereas Mab-escape variants were isolated from 46.2% (6/13) of foreign workers (χ^2=7.69, p=0.0055).

Table I. The frequency of different clinical features presented in 87 patients infected with dengue virus serotype 1 and 13 patients having acute dengue virus infection due to Mab-escape dengue virus 1 variant.

Clinical feature	No. with DEN1 (%)	No. with DENv (%)	χ^2-value	p-value
Rash	14 (16.1)	2 (15.4)	Fisher	1.0000
Myalgia	70 (80.1)	10 (76.9)	Fisher	0.7200
Retro-orbital pain	60 (69.0)	9 (69.2)	Fisher	1.0000
Abdominal pain	41 (47.1)	5 (38.5)	0.08	0.7746
URTI symptom	5 (5.7)	0 (0)	Fisher	1.0000
Sore throat	8 (9.2)	3 (23.1)	Fisher	0.1526
Diarrhoea	28 (32.2)	4 (30.8)	Fisher	1.0000
Jaundice	2 (2.3)	0 (0)	Fisher	1.0000

DEN1: dengue virus serotype 1

DENv: monoclonal antibody-escape dengue virus 1 variant

URTI symptom: cough and runny nose

20 of the 87 (23%) patients having acute dengue serotype 1 infection developed DHF, whereas only one of 13 (7.7%) patients having acute infection due to Mab-escape variants developed DHF. However, statistically, there was no significant difference between the two groups of patients having acute dengue infection developing DHF (Fisher's exact test, p=0.2904). The proportion of patients having various patterns of clinical manifestations due to acute dengue virus serotype 1 infection in comparison to those due to the Mab-escape variants is shown in Table I. There is no statistically significant difference between the two groups of patients with respect to clinical presentations. With regard to the titre of virus in the supernatant at the end of ten days of culture in C6/36 cells, DEN125 attained a virus concentration of 10 $TCID_{50}$ per millilitre of clarified culture supernatant[6], DEN155 and DEN695 achieved a virus titre of 10 $TCID_{50}$/ml[7] whereas all the three dengue virus Mab-escape variants reached a virus titre of 10 $TCID_{50}$/ml[9].

DISCUSSION

Dengue virus is a small enveloped virus measuring 50 to 60 nm in size, with two types of viral-encoded glycoproteins embedded in its envelope membrane. Within the viral envelope, viral-encoded basic capsid proteins enclose the viral genome. The viral genome comprises a single-stranded positive sense RNA of about 11 thousand nucleotides. Like many other RNA viruses, the dengue virus shows considerable genetic diversity. In the evolutionary genetics of dengue viruses, most attention has been directed towards mutations, natural selection, and genetic drift[14-17]. The isolation of a number of Mab-escape dengue virus 1 variants in this outbreak supports the occurrence of genetic mutation leading to genetic drift in nature. The natural selection process in this study is not known but may be related to the ability of the Mab-escape variants to replicate in mosquito cells to a 2-log higher titre than the current circulating strains of dengue virus serotype 1. The survival advantage for these variant viruses in nature is implicated in the subsequent isolation of more Mab-escape variants from other parts of peninsular Malaysia after the outbreak period, from March to September 2005 (unpublished data). However, to substantiate the hypothesis, further investigations are required to see if similar higher viral replication kinetics can be demonstrated in adult female *Aedes* mosquitoes.

The nucleic acid sequence information of six isolates of dengue virus 1 in this study revealed the reason behind the failure of earlier attempts to type the variant virus by RT-PCR using the classical dengue typing primers described by Lanciotti et al[9]. A base substitution (Fig. 1, cytosine to adenine) at the primer binding site caused a failure in the molecular typing of dengue virus serotype 1 but did not lead to any change in amino acid sequence at the binding site, suggesting that this site may not be the dengue serotype 1 monoclonal antibody binding site. Further research will be undertaken to sequence and analyse the complete genomic sequence of this Mab-escape variant.

Dengue virus serotype 1 has been the dominant dengue virus among the four dengue serotypes co-circulating in peninsular Malaysia since 2003. Reports from previous studies indicated the possibility that genetic changes in the virus lead to change in viral virulence[18,19]. The isolation of this Mab-escape variant led to two major concerns. Firstly, it is possible that the Mab-escape dengue virus variant may cause more serious disease leading to more cases of DHF. Secondly, the Mab-escape variant may also escape cross-neutralisation from previous exposure to dengue serotype 1 infection. Data from this limited study show that there was no significant difference in the proportion of patients developing DHF following acute infection by Mab-escape dengue virus 1 variant in comparison with infection by the conventional dengue virus 1. Similarly, there was no significant difference in the pattern of clinical presentations following acute infection by the two different strains of virus. Research work is on-going to study the kinetics of cross-neutralisation between the two strains of dengue 1 virus using convalescent sera from respective patients infected with conventional dengue virus serotype 1 and Mab-escape dengue serotype 1 variant.

Interestingly, the Mab-escape dengue virus 1 variant was isolated in a significantly higher proportion of foreign workers than the local population in this outbreak. This finding suggests that the variant could possibly have evolved *de novo* in the locality that housed the foreign workers or have been brought into the community by migrant workers and subsequently spread to the local population as dengue serotype 1 virus (that are non-typable by Lanciotti method) have been described in Cambidian strains[20]. Further work is urgently needed to investigate whether a similar Mab-escape dengue virus 1 variant is co-circulating in neighbouring countries.

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