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Monoclonal antibody-escape variant of dengue virus serotype 1: Genetic composition and envelope protein expression

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Abstract. Monoclonal antibody-escape variant of dengue virus type 1 (MabEV DEN-1) was discovered and isolated in an outbreak of dengue in Klang Valley, Malaysia from December 2004 to March 2005. This study was done to investigate whether DEN152 (an isolate of MabEV DEN-1) is a product of recombination event or not. In addition, the non-synonymous mutations that correlate with the monoclonal antibody-escape variant were determined in this study. The genomes of DEN152 and two new DEN-1 isolates, DENB04 and DENK154 were completely sequenced, aligned, and compared. Phylogenetic tree was plotted and the recombination event on DEN152 was investigated. DEN152 is sub-grouped under genotype I and is closely related genetically to a DEN-1 isolated in Japan in 2004. DEN152 is not a recombinant product of any parental strains. Four amino acid substitutions were unique only to DEN 152. These amino acid substitutions were $^{Ser}[326]^{Leu}$, $^{Ser}[340]^{Leu}$ at the deduced E protein, $^{Ile}[250]^{Thr}$ at NS1 protein, and $^{Thr}[41]^{Ser}$ at NS5 protein. Thus, DEN152 is an isolate of the emerging monoclonal antibody-escape variant DEN-1 that escaped diagnostic laboratory detection.

INTRODUCTION

Dengue was first documented in Malaysia in 1902 and was made notifiable in 1973 (George & Lam, 1997). One of the largest outbreaks was seen in 1996 with 14,255 dengue cases reported and 32 deaths in Malaysia (George & Lam, 1997). Most of the dengue cases were reported among the urban population, with the highest incidence in the working and school-going age groups. Patients infected by dengue virus could develop dengue with or without warning signs and may progress to severe dengue. If no appropriate and timely management were given, patients with severe dengue usually die. Dengue is caused by dengue virus which belongs to the family of *Flaviviridae*, under the genus *Flavivirus*. There are four serotypes of dengue virus namely, DEN-1, DEN-2, DEN-3 and DEN-4 all of which are found co-circulating in Malaysia. Dengue viruses share similar antigens, however, the minute differences in these viruses were enough to elicit transient partial cross-protection after infection by each one of them (Henchal & Putnak, 1990).

The genome of dengue virus is a positive-sense single stranded RNA of more than 10 kb that codes for structural and nonstructural proteins. The 5' and 3' terminal ends of the genome are flanked by short non-coding regions. One of the structural proteins, the envelope (E) protein is the major target for neutralizing antibodies. In addition, the short term cross protection against each serotype is induced by the E protein. There are three

structural domains of the E protein which have been demonstrated to induce cross-reactive neutralization against all four serotypes (Wahala *et al.*, 2012). Besides E protein, monoclonal antibodies against NS1, NS2, NS3 and core proteins were used to investigate further on the pathogenesis of the dengue virus (Steidel *et al.*, 2012).

Isolation and detection of monoclonal antibody- escape variants (MabEVs) of DEN-1 in Malaysia during an outbreak of dengue posed a new challenge in dengue surveillance (Chua *et al.*, 2006). As the name suggested, these dengue viral variants were not typeable by commercially- available monoclonal antibodies nor by the conventional molecular technique, reverse transcription- polymerase chain reaction (RT-PCR) using dengue virus typing primers published by Lanciotii *et al.* (1992). The discovery of these genetic variants indicates that the dengue virus is changing rapidly due to its nature as a single stranded RNA virus (Tolou *et al.*, 2001; Worobey *et al.*, 1999). Dengue viruses are able to mutate and/or recombine to generate a vast genetic diversity pool for efficient dispersal and closely related dengue viral strains often present in distant parts of the world. As vaccine is not available currently, prevention and management of dengue outbreak rely heavily on dengue virus surveillance and dengue vector control. The existence of MabEVs poses a challenge in the detection and serotyping of dengue viruses during dengue virus surveillance. Thus, this study was done in an attempt to answer the question of why the routinely used commercial monoclonal antibody failed to detect the MabEVs of DEN-1.

MATERIALS AND METHODS

Dengue isolate

Three dengue virus strains (DEN 152, DEN B04 and DEN K154) were isolated at National Public Health Laboratory, Sungai Buloh, Selangor, Malaysia from serum samples derived from patients during the dengue outbreak in Klang Valley, Malaysia from late 2004 to early 2005. The MabEV DEN 152 was identified by indirect immuno-fluorescence assay using commercially available monoclonal antibody (Mab) against dengue complex (Chemicon Int., CA, USA; Cat. No. MAB8705) but could not be further typed to any dengue serotype by commercially-available monoclonal antibodies against each serotype dengue virus (Chemicon Int., CA, USA; Cat. No. MAB8701, MAB8702, MAB8703, MAB8704 for DEN-1, DEN-2, DEN-3 and DEN-4, respectively). The serotype of DEN 152 could not even be determined by RT-PCR using the primer pairs published by Lanciotti *et al.* (1992). On the other hand, DEN B04 and DEN K154 were serotyped as DEN-1 by indirect immuno-fluorescence assay using commercially-available monoclonal antibodies and RT-PCR using the method devised by Lanciotti *et al.* (1992). The complete genomes of three isolates DEN 152, DEN B04 and DEN K154 were targeted by 27 pairs of primers designed in-house to amplify overlapping fragments of approximately 300-630 bp spanning the complete DENV 1 genome. RT-PCR was performed in a single reaction tube using SuperScript® III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, USA) according to manufacturer's protocol. All RT- amplified PCR products were purified using the QIAquick Gel Purification Kit (Qiagen, Germany) and sequenced directly. The 5' and 3' ends of the viral genome were mapped by using improved rapid amplification cDNA ends (RACE) as previously described (Li *et al.*, 2005). In brief, the viral RNA genomic ends were ligated prior to cDNA synthesis and then followed by nested PCR amplification. PCR products of expected sizes were purified using the QIAquick Gel Purification Kit (Qiagen, Germany), sequenced directly or, cloned into pCR-Blunt-II TOPO vector (Invitrogen, USA) for sequencing using vector-specific primers. Multiple sequence alignments were done and homology search was conducted using the BLAST. Phylogenetic analysis was conducted with MEGA 4 (Martin *et al.*, 2005; Tamura *et al.*, 2007) and bootstrap with 1000 replicates. Recombination analysis was performed by using Recombination Detection Program 2 which is available freely (Martin *et al.*, 2005).

Expression of envelope (E) protein in baculoviral system

With the complete viral genome available, the envelope gene was amplified using SuperScript® III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen, USA) according to manufacturer's protocol. Amplified E genes of respective DEN 152 and DEN K154 were purified using the QIAquick Gel Purification Kit (Qiagen, Germany), sub-cloned into pFastBacHTc (Invitrogen, USA) donor plasmid after digestion with appropriate enzymes. The insert within the recombinant vector was confirmed by PCR, digestion, and finally sequenced. The recombinant donor plasmid was transformed into the *E. coli* dH10Bac (Invitrogen, USA) competent cells containing baculo-derived bacmid DNA. The recombinant bacmid was constructed by transposing between the mini-Tn7 element on pFastBac and the *att* Tn7 attachment site on the bacmid. The transformants were plated onto LB containing 50 mg/ml kanamycin, 7 mg/ml gentamicin, 10 mg/ml tetracycline, 100 mg/ml X-gal and 40 mg/ml isopropylthio-b-galactoside and incubated at 37°C for 24 hrs. The recombinant bacmids were isolated from the overnight cultures by alkaline lysis purification and confirmed by PCR. Insect Sf9 cells were cultured in Grace's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) and incubated at 27°C. Recombinant baculovirus was constructed by transfecting the recombinant bacmid to Sf9 monolayer cells using Cellfectin (Invitrogen, USA) according to manufacturer's protocol and monitored daily until the cytopathic effect appeared. The recombinant baculovirus was harvested from the cell culture medium at 48 hrs post transfection and stored at 4°C or used for inoculating more Sf9 cells to amplify the virus stock. To produce the E protein, the Sf9 cells were inoculated with the recombinant baculovirus and incubated at 27°C for 48 hrs. Harvested infected SF9 cells were resuspended, spotted on individual wells of Teflon-coated slide (ICN Biomedicals, USA) and allowed to air-dry prior to fixation with acetone. A 1:1000 dilution of specific DEN-1 monoclonal antibody MAB8701 (Chemicon Int, CA, USA) was added onto each well and incubated at 37°C for 30 minutes in a moist chamber prior to staining with anti-human IgG-conjugated with fluoroscein isothiocyanate (FITC). The above procedure was also repeated with a 1:10 dilution of human serum which is a known positive for anti-dengue virus IgG antibody. The cells were then examined under a UV-fluorescent microscope.

RESULTS AND DISCUSSION

The complete genomes of DEN B04, DEN K154 and DEN 152 were uploaded into GenBank with accession code JN697056, JN697057 and JN697058, respectively. The complete genome length of each of the three isolates (DEN K154, DEN B04 and DEN 152) was 10,735 nucleotides and the deduced translated polyprotein consists of 3392 amino acids. BLAST searches identified 18 complete genome sequences of DEN-1 with high similarity to the three isolates. Phylogenetic tree (Figure 1) showed that the DEN K154 and DEN 152 were of the same genotype I and more closely related to the dengue virus isolated in Singapore, 2003 and in Japan, 2004 with 99% amino acid similarity, respectively. DEN B04 appears to be of genotype IV and is clustered with dengue virus isolated in France, 2004, in Seychelles, 2003 and in Indonesia, 1998 with 98%, 97% and 98% amino acid similarity, respectively. By comparing the complete genome and translated polyprotein sequences, mutation analysis showed that four amino acid substitutions are unique only to DEN 152, an isolate of the MabEV DEN-1. These amino acid substitutions are $^{Ser}[326]^{Leu}$, $^{Ser}[340]^{Leu}$ at the deduced E protein, $^{Ile}[250]^{Thr}$ at NS1 protein, and $^{Thr}[41]^{Ser}$ at NS5 protein as shown in Figure 2. These four non-synonymous mutations were not detected by comparing the partial nucleotide sequence of capsid-preM in the previous study (Chua *et al.*, 2006). Whether $^{Ser}[326]^{Leu}$ and $^{Ser}[340]^{Leu}$ at the deduced E protein and $^{Ile}[250]^{Thr}$ at NS1 protein influence the viral replication kinetics remain to be investigated. Nevertheless, the

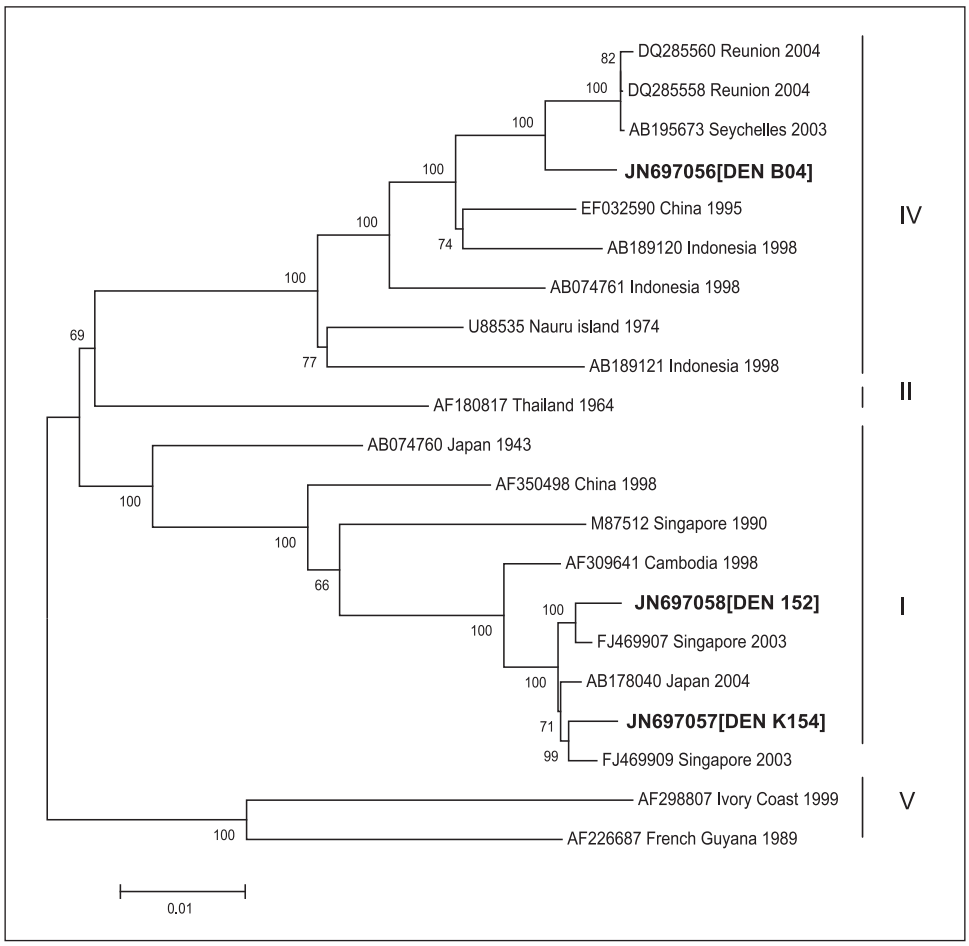
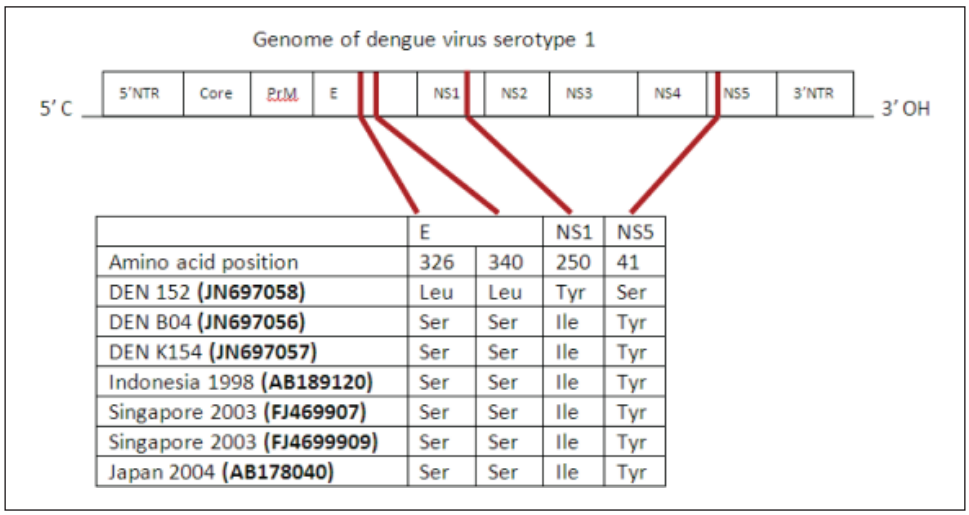


Figure 1. Phylogenetic tree of monoclonal antibodies-escape variant DEN 152 with complete genome nucleotide. Each strain is indicated by accession number followed by country and the year of isolation. Bootstrap values are indicated at the branch point and the four genotypes of DEN-1 are indicated on the extreme right.

isolate DEN 152 showed 2-log higher titre in mosquito cells in comparison to other DEN-1 isolates (Chua *et al.*, 2006).

No recombination was detected in DEN 152 genome using RDP 2, thus indicating that this monoclonal antibody-escape variant is not a product of any recombination event. As the polymerase enzyme of RNA virus is known for its lack of proof reading, high mutation rate accounts for diversity of the genetic pool among dengue viruses. Even though recombination does not play a role in the existence of this MabEV, genomic mutation contributes largely to the diversity of genetic pool among dengue viruses and possibly to the emergence of MabEVs. Other DEN 1 and MabEVs were reported to be isolated from 11.5% (10/87) and 46.2% (6/13) of foreign workers (Chua *et al.*, 2006) and this suggests the possibility of MabEV being brought into Malaysia as an alternative route of the emergence of monoclonal antibody escape variant.

Serotyping of dengue isolates by molecular method according to Lanciotti *et al.* (1992) is widely used for diagnosis and surveillance of dengue, particularly in Southeast Asian countries (Vaughn *et al.*, 2000; Reynes *et al.*, 2003; Chua *et al.*, 2006). It is a semi-nested PCR targeting the partial capsid–precursor M gene of dengue virus. Our laboratory results showed that DEN B04 and DEN K154 were successfully amplified by semi-nested PCR using D1 and TS1



	E		NS1	NS5
Amino acid position	326	340	250	41
DEN 152 **(JN697058)**	Leu	Leu	Tyr	Ser
DEN B04 **(JN697056)**	Ser	Ser	Ile	Tyr
DEN K154 **(JN697057)**	Ser	Ser	Ile	Tyr
Indonesia 1998 **(AB189120)**	Ser	Ser	Ile	Tyr
Singapore 2003 **(FJ469907)**	Ser	Ser	Ile	Tyr
Singapore 2003 **(FJ4699909)**	Ser	Ser	Ile	Tyr
Japan 2004 **(AB178040)**	Ser	Ser	Ile	Tyr

Figure 2. Unique substitution amino acid found in DEN 152, the monoclonal antibodies escape variant. Dengue isolates of genetically closely related that are indicated by country, by the year of isolation and followed by accession number were included in the mutation analysis.

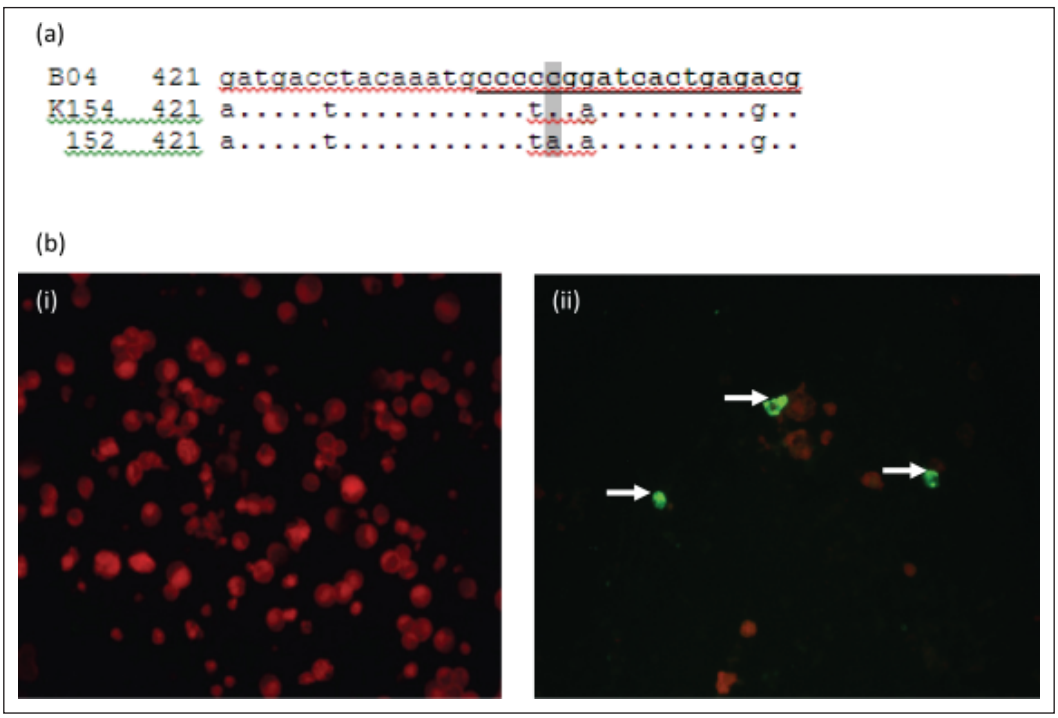


Figure 3. Features of MabEV DEN-152. (a) Partial capsid-precursor M gene segment of DEN B04 and DEN K154 aligned to DEN 152. Dots indicate nucleotides identical to DEN B04. The downstream primer binding site for molecular typing of DEN-1 is underlined. DEN 152 has a transversion of cytidine (C) at position 440 to adenine (A) as highlighted in grey which affect primer annealing and thus leading to escape detection by molecular technique. (b) Envelope protein of DEN 152 in transfected Sf9 cells were stained using (i) commercial Mab (Cat. No. 8701, Chemicon Inc., USA) and (ii) positive human IgG antibody against dengue virus. Positive bright green fluorescence indicated by arrows showed positive detection of E proteins by using positive human serum (polyclonal IgG) against DEN-1. [Magnification 200X]

primers (Lanciotti *et al.*, 1992) while DEN 152 could not be amplified using a similar set of primer-pair. To understand how DEN 152 escapes molecular detection, the sequence of the intended semi-nested PCR was identified, aligned and analysed. The DEN 152 has a transversion of cytidine (C) at position 440 to adenine (A) as shown in Figure 3a. This observation indicated that the amplification of the partial capsid-precursor M genetic segment (482 nt) failed because of a primer-template mismatch; however this mismatch does not lead to any change in amino acid sequence at the binding site. It was noted that the same observation has been described in dengue viruses isolated in Cambodia using the same method (Reynes *et al.*, 2003).

To further demonstrate how the DEN 152 escapes detection by commercially-available monoclonal antibodies, the envelope (E) gene was expressed in baculovirus. A number of studies have utilized baculovirus expression systems to produce high yields of recombinant flaviviral proteins, including those derived from St. Louis encephalitis virus (Venugopal *et al.*, 1995), tick-borne encephalitis virus (Marx *et al.*, 2001), yellow fever virus (Despres *et al.*, 1991), Japanese encephalitis virus (McCown *et al.*, 1990) and dengue virus (Chan *et al.*, 2002). The use of baculovirus to express recombinant proteins has a number of advantages, such as the ability to process the recombinant proteins in a manner that is similar to the processing of native proteins (Gubler, 1998). The transfected Sf9 cells were collected and analysed for recombinant protein expression at 48 hours post-transfection by immunofluorescence assay using specific monoclonal antibody against DEN-1 (Chemicon Inc., CA, USA; Cat No. MAB8701). Negative results were observed for DEN 152 and DEN K154, indicating that the DEN-1 monoclonal antibody binding site was not specifically mapped to E protein. Following that, positive results as shown in Figure 3b were obtained when the transfected cells expressing the E protein were stained using positive human serum which was known to be positive for anti-dengue virus IgG antibody. This result not only indicated that the E protein was successfully expressed in the Sf9 cell but also proved that the commercial available MAB8701 antibody did not bind to E proteins.

The inability of DEN 152 to be stereotyped by RT-PCR methods according to Lanciotti *et al.* (1992) and commercially-available monoclonal antibodies support that genetic mutation in dengue virus leading to genetic drifts occur in nature. Different genotypes were commonly reported co-circulating in a single serotype (Jiang *et al.*, 2012; Khan *et al.*, 2008) and the genotypes were shown to be continuous shifting genetically while co-circulating with each other (Kotaki *et al.*, 2014). There is a 2-4% difference in amino acids among genotypes in dengue virus due to continuous genetic shifts in the single serotype (Kotaki *et al.*, 2014). The observation of four unique non-synonymous mutations in DEN152 further supports the occurrence of continuous genetic shifts in nature leading to amino acid change. It is unknown whether these minimal amino acid changes among genotypes will lead to better fitness in viral replication or survival. Nevertheless, more MabEVs were isolated subsequently from other parts of Peninsular Malaysia after the outbreak period implying that the variants have better surviving advantage (Chua *et al.*, 2006).

In conclusion, MabEV DEN 152 was clustered under genotype I. Several mutations leading to non-synonymous substitutions in amino acid were noted and substitutions unique only to DEN 152 were first reported in this study. The emerging of MabEV DEN-1 showed that dengue virus is continually mutating and perhaps adapting to the changing environment of rapid urbanization in Malaysia. Hence it is of importance to incorporate better dengue surveillance to have a better understanding of the molecular epidemiology of these circulating variants.

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