COMPARISON OF SEQUENCES OF E/NS1 GENE JUNCTION OF DENGUE TYPE 3 VIRUS FOLLOWING CULTURE SUBPASSAGE IN C6/36 CELLS TO STUDY THE POSSIBLE OCCURRENCE OF MUTATIONS

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Abstract. The aim of this study was to determine whether mutations could occur in the dengue virus genome following three subpassages of the virus in a mosquito cell line. This was done because sources of virus isolates used for sequencing studies are usually maintained in cell lines rather than in patients' sera. Therefore it must be assured that no mutation occurred during the passaging. For this purpose, sequencing was carried out using the polymerase chain reaction (PCR) products of the envelope/non-structural protein 1 junction region (280 nucleotides) of dengue type 3 virus. Sequence data were compared between the virus from a patient's serum against the virus subpassaged three times in the C6/36 cell line. We found that the sequence data of the virus from serum was identical to the virus that was subpassaged three times in C6/36 cell line.

INTRODUCTION

Dengue is an arthropod borne viral disease characterized by fever, headache, rash and leukopenia (Halstead, 1980). Dengue hemorrhagic fever (DHF) is a severe febrile disease with abnormalities of hemostasis and increased vascular permeability (World Health Organization, 1976). In some countries the occurrences of dengue can be seasonal but in Malaysia dengue occurs throughout the year.

Dengue viruses are positive-sense, single-stranded RNA viruses and like other known flaviviruses, have a gene order of 5'-C-prM(M)-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3'(Rice, 1990). There are four antigenically distinct serotypes of the dengue virus, dengue 1, dengue 2, dengue 3 and dengue 4 (Porterfield, 1980). The nucleotide and protein sequence data of the dengue viruses is necessary so as to understand various aspects such as genomic structure, protein functions and expression, identification of new strains for vaccine design and construction of phylogenetic trees. In addition, the sequencing of selected regions of dengue viruses isolated from dengue fever and DHF patients may help to understand the pathogenicity of the disease as the presence of a virulence gene has not been ruled out (Rosen, 1977).

Dengue virus isolated from patients' sera and maintained in tissue culture cell lines is frequently used for sequencing. The objective of this study is to find out whether any mutations can occur in the dengue virus genome during the first three passages of the dengue virus using the C6/36 cell line. We selected three as the number of subpassages as this is the standard passage number in our laboratory for the isolation of dengue virus. For this purpose we had compared sequencing data of the virus from two sources, namely from serum and from C6/36 cell line after three subpassages. The target viral genome is the envelope/non-structural protein 1 (E/NS1) junction comprising 280 nucleotides. This region was chosen as it showed a uniform rate of random mutation with no hypervariable regions (Rico-Hesse, 1990). The selected dengue serotype was dengue 3, the dominant serotype in Malaysia in 1994 (Thayan *et al*, 1995 b).

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MATERIALS AND METHODS

Virus isolates

Dengue viruses were isolated from patients by inoculating 20µl of serum samples from patients during the acute stage of the disease into a monolayered C6/36 cell line (Igarashi, 1978). After incubating at 28°C for 2 hours, the culture medium was removed by centrifuging. Then 2 ml of maintenance medium (2% fetal calf serum in Eagle's Minimum Essential Medium with non-essential amino acids) were added. The tube was incubated at 28°C for 7 days. The culture fluids were harvested and subpassaged three times in C6/36 cells. The presence of virus was confirmed by immunostaining (Igarashi *et al*, 1982) and the final culture fluids were stored at -80°C before use.

Primers

Primers used in this study were synthesized using an Applied Biosystems DNA synthesizer (Model 380B). The nucleotide sequences of the primers and the references are listed in Table 1 (Osatomi and Sumiyoshi, 1980).

Amplification of dengue virus RNA

Reverse transcriptase-PCR was performed as described by Morita *et al* (1991). Briefly, 5 µl of serum were incubated with 5 µl of a detergent mix (1% Nonidet P-40), 50Units of RNase Inhibitor (Takara Co, Kyoto, Japan) in phosphate-buffered saline (-) for 1 minute at room temperature. Following this, 90 µl of reverse transcriptase (RT-PCR) mix which contains 100 pmole of each primer, 0.2mM deoxynucleoside triphosphate, 10mM Tris (pH 8.9), 1.5mM MgCl, 80mM KCl, 0.5mg bovine serum albumin per ml, 0.1% sodium cholate, 0.1% Triton X-100, 10 Units of reverse transcriptase (Life Science Inc, USA) and 4 Units of Tth DNA polymerase (Toyobo Co, Osaka, Japan) was added into the reaction tube. Dengue infected C6/36 cell line was used as a positive control while sterile water was used as negative control. The reaction mix was covered by a drop of mineral oil after which the tube was incubated at 51°C for 15 minutes for reverse transcription reaction. Following this, amplification (Denaturation at 92°C for 60 seconds, annealing at 51°C for 60 seconds and extension at 72°C for 60 seconds) and a final extension step for 5 minutes was carried out using a thermal cycler (Iwaki Co, Tokyo, Japan) for 35 cycles. The resulting PCR product (12µl) was subjected to agarose gel electrophoresis (3% Nusieve 3:1, FMC, USA) and amplified DNA fragments were identified by ethidium bromide staining.

PCR product purification

PCR product used for direct sequencing was purified from excess primers and short oligonucleotides by the method as described by Thayan *et al* (1995a). Briefly, 80µl of PCR product was dispensed into the upper part of microfilter spin unit columns (Millipore Ultrafree MC filter, 30,000 NMWL, USA) and centrifuged at 7,000 rpm for 5 minutes. The filtrate containing excess primers and short oligonucleotides was discarded and DNA (which remains on the filter) was washed three

Table 1

Nucleotide sequences of dengue virus primers.

Primer*	Sequence (5' to 3')	Position	Ref
D3S	ATTGGAGACAAAGCCCTGAA	2072-2091	1
D3C	TCCATTCTCCCAAGCGCCTG	2572-2553	2

*-S = sense primer; C = complementary primer; D3 = Dengue virus type 3.
1 and 2 = Osatomi and Sumiyoshi (1980).

times with 200µl of TE buffer (pH 8.0). After the final washing, DNA was resuspended in TE buffer. DNA concentration was quantitated using optical density at 260 nm and stored at -20°C before use.

Sequencing

Purified PCR products were used as template for terminator cycle sequencing using Taq Dye Deoxy terminator Cycle Sequencing Kit (Applied Biosystems, USA). The sequence reaction was purified of excess dyedeoxy terminators by using phenol-chloroform extraction. Polyacrylamide gel electrophoresis was carried out using the Applied Biosystems DNA Sequencer Model 373A according to the manufacturer's protocol. The resulting sequencing data was analysed using a DNASIS-MAC Version 2.2 Software System (Hitachi). The prototype dengue 3 isolate, H87 from the Philippines was used as the standard (Osatomi and Sumiyoshi, 1980).

RESULTS

Sequencing data :

	5' (2273)												
MaKH00693 (serum)	AGT	GGA	GTC	TCA	TGG	ATA	ATG	AAA	ATT	GGA	ATA	GGT	GTC
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	---	--C	---	---	---	---	---	---	---	---	---

MaKH00693 (serum)	CTC	TTA	ACC	TGG	ATA	GGG	TTG	AAT	TCA	AAA	AAC	ACT	TCC
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	---	---	---	---	---	---	---	---	---	---	--T

MaKH00693 (serum)	ATG	TCA	TTT	TCA	TGC	ATT	GCG	ATA	GGA	ATT	ATT	ACA	CTC
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---

MaKH18094 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	---	---	---	---	---	---	---	--C	---	---	---

MaKH00693 (serum)	TAT	CTG	GGA	GCC	GTG	GTA	CAA	GCT	GAC	ATG	GGG	TGT	GTC
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	--G	-T-	---	--G	---	---	---	---	---	---	---

MaKH00693 (serum)	ATA	AAC	TGG	AAA	GGA	AAA	GAA	CTC	AAA	TGT	GGA	AGC	GGA
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	---	---	--C	---	---	---	---	---	---	--T	---

MaKH00693 (serum)	ATT	TTC	GTC	ACC	AAT	GAG	GTC	CAC	ACC	TGG	ACA	GAG	CAG
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH18094 (serum)	---	---	---	--T	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	--T	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	----	--T	---	---	---	---	---	---	---	---	--A

MaKH00693 (serum)	TAC	AAA	TTT	CAA	GCA	GAC	TCC	CCT	AAA	AGA	CTG	GCG	ACA
MaKH00693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH07093 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH08693 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH09193 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---

MaKH18094 (serum)	---	---	---	---	---	---	---	---	---	---	---	---	---
MaKH18094 (virus iso)	---	---	---	---	---	---	---	---	---	---	---	---	---
H87 (Phili)	---	---	---	---	---	---	---	--C	---	---	G--	--A	---

	3' (2552) GCC	ATC	G
MaKH00693 (serum)			
MaKH00693 (virus iso)	---	---	-
MaKH07093 (serum)	---	---	-
MaKH07093 (virus iso)	---	---	-
MaKH08693 (serum)	---	---	-
MaKH08693 (virus iso)	---	---	-
MaKH09193 (serum)	---	---	-
MaKH09193 (virus iso)	---	---	-
MaKH18094 (serum)	---	---	-
MaKH18094 (virus iso)	---	---	-
H87 (Phili)	---	--T	-

Fig 1–Comparison of nucleotide sequences of the E/NS1 gene junction of five dengue type 3 virus isolates, from serum and also after three subpassages in C6/36. Coding for the isolates are as follows : Ma-Malaysia, K-Kuala Lumpur, H-Human, 3 digit middle numbers-isolate numbers, 2 digit last numbers-year of isolation. Nucleotide differences from MaKH00693 are shown; dashes indicate identities. Nucleotide positions are numbered according to Osatomi and Sumiyoshi (1980). H87 refers to the prototype dengue type 3 isolated in the Philippines.

	727
MaKH00693 (serum)	SGVSWIMKIGIGVLLTWIGLNSKNTSMSFSCIAIGIITLYLGAVVQADMGC
MaKH00693 (virus iso)	----------
MaKH07093 (serum)	----------
MaKH07093 (virus iso)	----------
MaKH08693 (serum)	----------
MaKH08693 (virus iso)	----------
MaKH09193 (serum)	----------
MaKH09193 (virus iso)	----------
MaKH18094 (serum)	----------
MaKH18094 (virus iso)	----------
H87 (Phili)	----------A---------

	819
MaKH00693 (serum)	VINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAI
MaKH00693 (virus iso)	----------
MaKH07093 (serum)	----------
MaKH07093 (virus iso)	----------
MaKH08693 (serum)	----------
MaKH08693 (virus iso)	----------
MaKH09193 (serum)	----------
MaKH09193 (virus iso)	----------
MaKH18094 (serum)	----------
MaKH18094 (virus iso)	----------
H87 (Phili)	----------V----

Fig 2–Deduced 93-amino acid sequences of the E/NS1 gene junction of five dengue type 3 virus isolates, from serum and also after 3 subpassages in C6/36. Amino acid differences are shown; dashes indicate identities. Amino acid positions are numbered according to Osatomi and Sumiyoshi (1980).

DISCUSSION

DNA sequencing is a very useful technique by which the precise order of nucleotides can be determined. Since the mid-1970's, when molecular cloning techniques in general were rapidly improving, simple methods were also developed to determine the nucleotide sequence of DNA (Sanger *et al*, 1977; Maxam and Gilbert, 1977). In addition, the nucleotide sequences of cDNA clones of a protein of unknown function often provides valuable clues regarding the function of the protein (Davis *et al*, 1994).

Virus isolates used for sequencing studies are normally maintained in cell-lines rather than in patients' sera which are very limited in quantity and normally have a lower yield of the virus. It is noted that extensive passaging of a virus in cell culture is a standard method for selecting attenuated virus for vaccines (Lustig *et al*, 1988). Bhamarapravati *et al* (1987) used a live attenuated dengue-2 virus passaged 53 times in primary dog kidney cells as a candidate vaccine for evaluation among adult volunteers, while Lustig *et al* (1988) found that extensive passaging leads to the substitution of certain amino acids in the envelope region of the Sindbis virus. This change is responsible for the attenuation as that particular amino acid was found to be the virulence determinant. Therefore it must be assured that the virus does not undergo any mutations during passaging in the cell-line, at least for the first three passages as normally three passages are needed to confirm the presence of virus.

In this study we carried out sequencing of the PCR products of selected regions of the dengue virus from serum and also after three passages in C6/36 cell line to determine whether any mutations occurred. We found that the sequence data for the region of interest of the virus from serum was identical to the virus that was passaged three times in the C6/36 cell line. This was the same for all the five isolates (Fig 1, 2). The C6/36 (Igarashi, 1978) cell-line derived from *Aedes albopictus* is the most established cell-line for dengue virus. The absence of any mutations during passaging proves that C6/36 is a very stable and suitable cell-line for the dengue virus. We conclude that tissue culture isolates (subpassaged three times) is a suitable source for sequencing experiments of dengue virus.

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