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Characterization of dengue virus prevalence in Taiwan

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Summary:

In Taiwan, where dengue is not endemic and the constant importation of dengue viruses from the neighboring Southeast Asian countries through close commercial links and air travel is believed to cause local outbreaks. Due to unique geographic location, dengue is a major public health problem during summer and fall seasons in southern Taiwan. To reduce the introduction of dengue viruses into Taiwan and its further spread, fever screening of body temperature by infrared thermal camera was implemented at airport to screen dengue and other infectious agents after SARS epidemic. A panel of diagnostic tests including dengue, Malaria, enteric bacteria and other diseases suspected (such as yellow fever, plague, et al.) was carried out for selected fever patients. We have set up a laboratory based dengue surveillance system for virological surveillance and rapid diagnosis. Major elements of laboratory based dengue surveillance system includes virus isolation, rapid diagnosis based on real-time reverse transcription-polymerase chain reaction (RT-PCR) and capture IgM and IgG ELISA, and molecular epidemiologic study based on nucleotide sequencing and phylogenetic analysis. The laboratory based dengue surveillance system has been proved to be valuable in the identification of imported dengue cases and the reduction of local outbreaks. The collection of dengue viruses circulated in the Southeast Asian would be a precious resources for the understanding of the evolution of dengue viruses between interepidemic and epidemic periods. Future collaboration on the establishment of dengue network and genomic database would provide the catalyst for a more effective communication among the Southeast Asian countries on dengue surveillance, prevention and control of DF and DHF.

Purpose:

To set up a laboratory based dengue surveillance systems for virologic surveillance and molecular epidemiology in Taiwan.

Methods:

1 **Virus isolation and identification**

The isolation of dengue virus was performed using mosquito cell line (clone C6/36 of Aedes albopictus cells). For each acute phase serum, 4 μ l of serum sample was diluted in 200 μ l cultured medium (RPMI , Gibco/BRL, Life

Technologies, containing 1% FCS) and added to a 96-well microtiter plate, 50 μ l/well in quadruplicate. Then, 10^5 cells/100 μl/well of C6/36 were added into the microtiter plate and incubated at 37℃ for 7 days. Cells were harvested and virus isolates were identified by the indirect fluorescent antibody test with dengue virus group-specific and serotype-specific monoclonal antibodies. (3)

2 **ELISA**

Capture IgM and IgG ELISA was performed to measure dengue-specific IgM and IgG antibodies in the acute-phase and convalescent-phase serum samples of patients infected with dengue virus as previously described (2).

3 **Molecular diagnosis by one-step SYBR Green I quantitative RT-PCR**

One-step SYBR Green I RT-PCR amplification was performed in the Mx4000^{TM} quantitative PCR system (Stratagene) to detect and differentiate the dengue virus serotype in the acute-phase serum samples as previously described (1).

4 **Extraction of viral RNA**

Viral RNAs (70 μl) were extracted from 140 μl of cell culture supernatants or serum samples using the QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -70℃.

5 **Primers used for nucleotide sequencing of total structural protein genes** (See Table1)

6 **Nucleotide sequencing**：using ABI Prism 3700 DNA sequencer (Applied Biosystems).

7 **Phylogenetic analysis**

7.1 BLAST:http://www.ncbi.nlm.nih.gov/BLAST/

7.2 DNAStar

7.3 PHYLIP: http;//evolution.genetics.Washington.edu/phylip/software.html，

7.4 MEGA 3 http://www.megasoftware.net

Results: (if necessary, figures and tables will be included)

1. **Strategies for laboratory-based dengue surveillance**

In Taiwan, where dengue is not endemic and the constant importation of dengue viruses from the neighboring Southeast Asian countries through close commercial links and air travel is believed to cause local outbreaks (5). The main strategy to prevent dengue epidemic is to effectively reducing the introduction of

new dengue viruses into Taiwan and its further spread by effective control measures. We have previously reported the laboratory based dengue surveillance systems in Taiwan and the application of airport fever screening to reduce the introduction of dengue and other infectious diseases. Major elements of laboratory based dengue surveillance system include virus isolation, rapid diagnosis based on real-time reverse transcription-polymerase chain reaction (RT-PCR) and capture IgM and IgG ELISA, and molecular epidemiologic study based on nucleotide sequencing and phylogenetic analysis (3, 4). Acute phase serum samples from all suspected dengue cases were analyzed by real-time RT-PCR to detect and differentiate dengue virus serotype. The nucleotide sequences of partial nonstructural 5 (NS5), Core (C)-premature Membrane (prM), and envelope (E) gene fragments were determined directly from the serum samples of all imported and selectively indigenous cases. Total structural gene sequences were determined after viral isolation. A dengue virus genetic database was generated and constantly updated from gene sequences available from GenBank and Taiwan Center for Disease Control (CDC) dengue genomic database.

2. **Dengue virus genomic database**

For phylogenetic analysis, a dengue virus genetic database was generated and constantly updated from gene sequences available from GenBank and Taiwan CDC dengue genomic database. A total of more than 210 and 30 distinct dengue virus strains were isolated from imported and indigenous cases, respectively in Taiwan during 1982-2005.

3. **Nucleotide sequencing and phylogenetic analysis**

Figure 1 illustrating the evolutionary relationship of DEN-3 virus strains using E genes sequences available from Taiwan CDC dengue genomic database and GenBank Phylogenetic tree was constructed by neighbor-joining method and four genotypes were identified. Bootstrapping with 1000 replicates was used to place confidence value on groupings within the trees. The dengue virus strains responsible for the three major DEN-3 epidemics in Taiwan in 1994, 1998, and 2005 were highlighted. The results demonstrated that the application of molecular epidemiological investigation on virological surveillance could help monitoring the transmission dynamics of existing dengue virus strains and the introduction of new dengue virus strains.

Discussion:

The establishment of laboratory based dengue surveillance plays an important role in the effective control of dengue. Recently, rapid advances in molecular diagnosis, serological diagnosis and molecular epidemiology have revolutionized the laboratory diagnosis of dengue infection. This application of the laboratory based dengue surveillance system has been proved to be valuable in the identification of imported dengue cases and the

reduction of local outbreaks in Taiwan.

For better control and understanding of transmission dynamics of dengue epidemics in Southeast Asia, it is desired to establish a dengue network with laboratory based surveillance systems among all countries, especially those with heavy endemic. The initiation of this collaboration project lead by NIID, Japan with 5 or 6 countries' participation at this time is certainly a big step toward the success of this goal.

We at Taiwan CDC will continue to support this collaboration project through active participation and technical support. It is hoped that standard protocols can be established and made available for all participating laboratories in the future. Great efforts should be paid on the collection and characterization of distinct dengue virus strains circulated in various nations. Nucleotide sequences of these dengue virus strains should be determined and made available together with other epidemiologic data. At this stage, it is desirable to develop a genetic database network using the same electronic platform with the functions of searching and analysis among participating countries. The final goal of this program is to develop an electronic dengue network in that genetic and epidemiologic data can be freely shared in real time among participating countries. We are in the process developing an electronic dengue data base in Taiwan CDC that could become one of the candidates for this purpose.

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1. **Publication list for this work:**

None

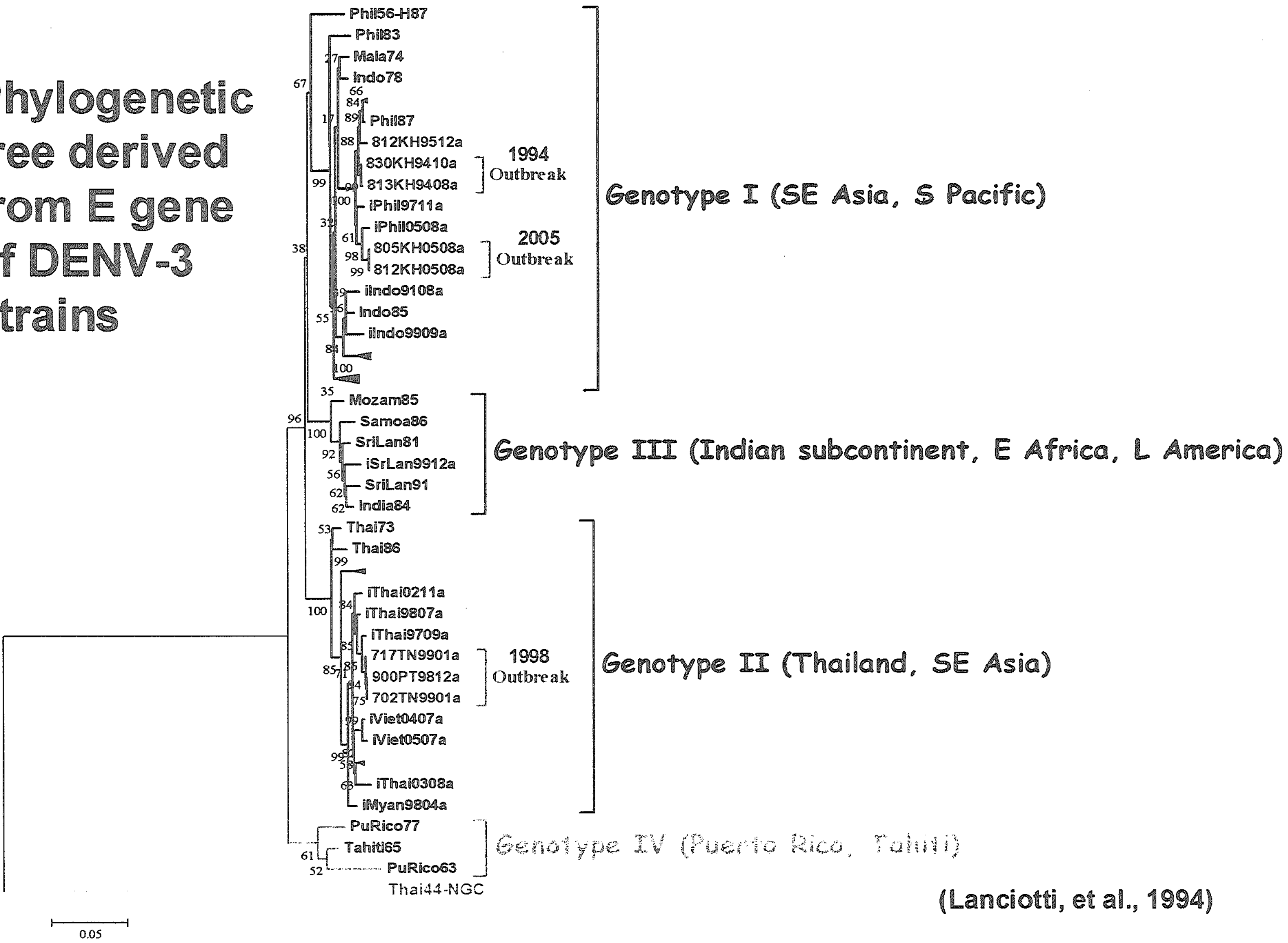
Table 1: Primers used for nucleotide sequencing of total structural protein genes

Primer	Sequence
DEN-1 primers	
DN5UTRF	AGT TGT TAG TCT ACG TGG ACC G
D1-545F	ATT GCG ATG GAT TTG GGA GAG
D1-631R	GTC AAC GTC ATC TGG TTC CG
D1-1217R	TTC GTC GRC ACA CAA AGT TCG
D1-1125F	AAA TAT CAA ACA CCA CCA CCG
D1-1548F	ATC ATG GCT TGT CCA CAA AC
D1-1631R	TTC CAA GTC TCT TGG GAT GT
D1-2046F	CCA CCY TTT GGT GAG AGC TAC
D1-2123R	TGC TTC CYT TCT TGA ACC AGC
D1-2583R	CAC ACA CCC TCC TCC CAT GCC
D1-2630R	TTT GYT TCC ACA TGA TGT TCT C
DEN-2 primers	
DN5UTRF	AGT TGT TAG TCT ACG TGG ACC G
D2-556F	GAC CTT GGT GAR TTG TGT GAA G
D2-642R	GCA CCA ACA ATC TAT GTC TTC
D2-1232R	TGT CTA CCA TGG AGT GTT TGC AG
D2-1157F	GCC CAA CAC AAG GRG AAC CCA
D2-1572F	CAT TGC CTG TGC ACC AGC CAA GC
D2-1644R	TGT CTC TTT CTG TAT CCA ATT TGA
D2-2028F	AGT CAA CAT AGA AGC AGA ACC
D2-2162R	GCT CCY CTC ATT GTT GTC TC
D2-2610R	CAR TCT TGT TAC TGA GCG GA
DEN-3 primers	
DN5UTRF	AGT TGT TAG TCT ACG TGG ACC G
D3-541F	CAA CAT GTG CAC ACT CAT AGC
D3-636R	CAG CAG TCA ATG TCT TCA GG
D3-1268R	TCC CTT GCC AAA CAA ACC AC
D3-1186F	GGA GCA GGA CCA GAA CTA C
D3-1568F	TTT GAC CTA CCY CTA CCA TGG
D3-1695R	TGC GAT CCA AGG ACT ACT ACT TCT TG
D3-2036F	GAA CCT CCT TTT GGG GAA AG
D3-2149R	TCT GGC AGT GGC CTC GAA C
D3-2638R	AGT TCA TTG GCT ATT TGC TTC C
D3-2621R	GCT TCC ACA AGA GGT TCT CCA TTC

DEN-4 primers

D4-5UTRF	AGT TGT TAG TCT GTG TGG ACC G
D4-540F	TGC ACT CTC ATT GCC ATG GAC
D4-658R	GTA GAC GTG AGG TTG CAC CAG C
D4-1461R	ATT TGA CTT CCA CCG ATG GTG ACC TAG GAG TTA T
D4-1108F	GAA CCT ATT GCA TTG AAG CCT C
D4-1543F	AAA AGA AAA CRT GGC TTG TGC
D4-1680R	GTC TCT TGG CAT GAG GAA CC
D4-2018F	CCA ACA GTG TAA CCA ACA TAG
D4-2114R	GAA CCA ATG GAG TGT TAA TGC
D4-2609R	CCT CGT GGT TGA TCT AAT TCC

Figure 1.



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Nineteen nucleotides in the variable region of 3′ non-translated region are dispensable for the replication of dengue type 1 virus in vitro

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Summary

In many flaviruses, first 50–400 nucleotides of 3′ non-translated region (3′ NTR) exhibit lower conservation level than other regions and are called "variable region". Two dengue type 1 virus (DENV-1) strains, which have 17- and 29-nt deletion in the variable region, were recently isolated from Japanese dengue fever patients. The effect of a small deletion in the 3′ NTR was analyzed using two DENV-1 viruses which were prepared from a newly developed infectious cDNA clone. These included a recombinant virus rDENV-1(02–20), without any deletion in 3′ NTR, and rDENV-1m10, with 19-nt deletion in the variable region of rDENV-1(02–20). These two viruses were compared for growth kinetics and plaque morphology in Vero, Huh-7 and C6/36 cells. No apparent difference was detected between rDENV-1(02–20) and rDENV-1m10 in replication efficiency and plaque size in these cell lines. The results suggest that the complete variable region of DENV-1 is dispensable for virus replication and propagation in vitro.
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Keywords: Dengue type 1 virus; cDNA clone; 3′ NTR

1. Introduction

Dengue viruses (DENV) are etiologic agents of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The viruses are transmitted to humans by *Aedes* mosquitoes (Halstead, 1997; Gubler, 1998). Dengue virus infections are a serious cause of morbidity and mortality in most tropical and subtropical areas of the world. Dengue cases are estimated to occur in up to 100 million individuals annually and the case fatality ratio is 1–5% in DHF and DSS patients (Halstead, 1997). DHF and DSS occurs more frequently in patients experiencing a secondary dengue virus infection than in those experiencing a primary infection, suggesting that the presence of heterotypic dengue virus antibodies is a risk factor for developing DHF and DSS in secondary infections (Halstead, 1988; Kurane and Ennis, 1997). On the other hand, the genotypic differences also appear to be associated with the difference in virulence (Rosen, 1997).

DENV belong to the family Flaviviridae and there are four dengue viruses (DENV-1 to -4). Flaviviruses are single-stranded, positive-sense RNA viruses. The approximately 11-kb genome encodes three structural proteins (C, prM and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame, and non-translated regions (NTR) in its 5′ and 3′ terminal (Lindenbach and Rice, 2001). Full length infectious clone of flaviviruses has been appreciated as powerful tool for studying replication, pathogenesis, and vaccine development.

3′ NTR of flaviviruses has crucial roles in virus replication (Lindenbach and Rice, 2001; Markoff, 2003). The 3′ NTR is divided into three regions based on the difference in the conservation level: (1) the variable region is located immediately after the open reading frame; (2) the 3′-terminal region is well conserved and contains a cyclization sequence motif (CS1) and stable stem-loop structure; (3) the region between the variable and 3′-terminal regions exhibits moderate conservation level and contains several hairpin motifs (Markoff, 2003; Bryant et al., 2005). CS1 is thought to interact with a complementary sequence, which is located in C region of the 5′ side of the virus genome, and the "base-pairing" is essential for the RNA synthe-

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sis of mosquito-borne flaviviruses (Hahn et al., 1987; Khromykh et al., 2001; Lo et al., 2003). The stem-loop structure of the 3′ NTR stabilizes viral RNA genome and also enhances translation initiation (Holden and Harris, 2004). Other group also showed that the stem-loop structure is required for RNA replication, but not for viral translation (Tilgner and Shi, 2004; Tilgner et al., 2005). Furthermore, well-conserved 3′-terminal dinucleotides ($CU_{OH}3'$) of the flavivirus genome is indispensable for virus replication (Khromykh et al., 2003). On the other hand, the variable region shows high sequence variability and nucleotide deletions in this region were detected in some strains of Japanese encephalitis virus (JEV) (13–26 nt), tick born encephalitis virus (TBEV) (59–207 nt) (Gritsun et al., 1997; Nam et al., 2001; Yang et al., 2004), and yellow fever virus (40–80 nt) (Wang et al., 1996; Bryant et al., 2005). We recently identified two new DENV-1 strains, which has 17- and 29-nt deletion in the variable region, from Japanese DF patients who had stayed in Seychelles and Yap island in Micronesia, respectively (Nukui et al., manuscript in submision). Such small nucleotide deletion is also observed in some strains of DENV-2 and DENV-3 (unpublished data). The deletion in the variable region of the TBEV genome was spontaneously induced during growth of the virus in cell culture and in mice, although length of the deletion was much larger (68–362 nt) than that observed in DENV and JEV (Mandl et al., 1998). Mutational analysis of the 3′ NTR in TBEV using recombinant cDNA clones indicated that the dele-

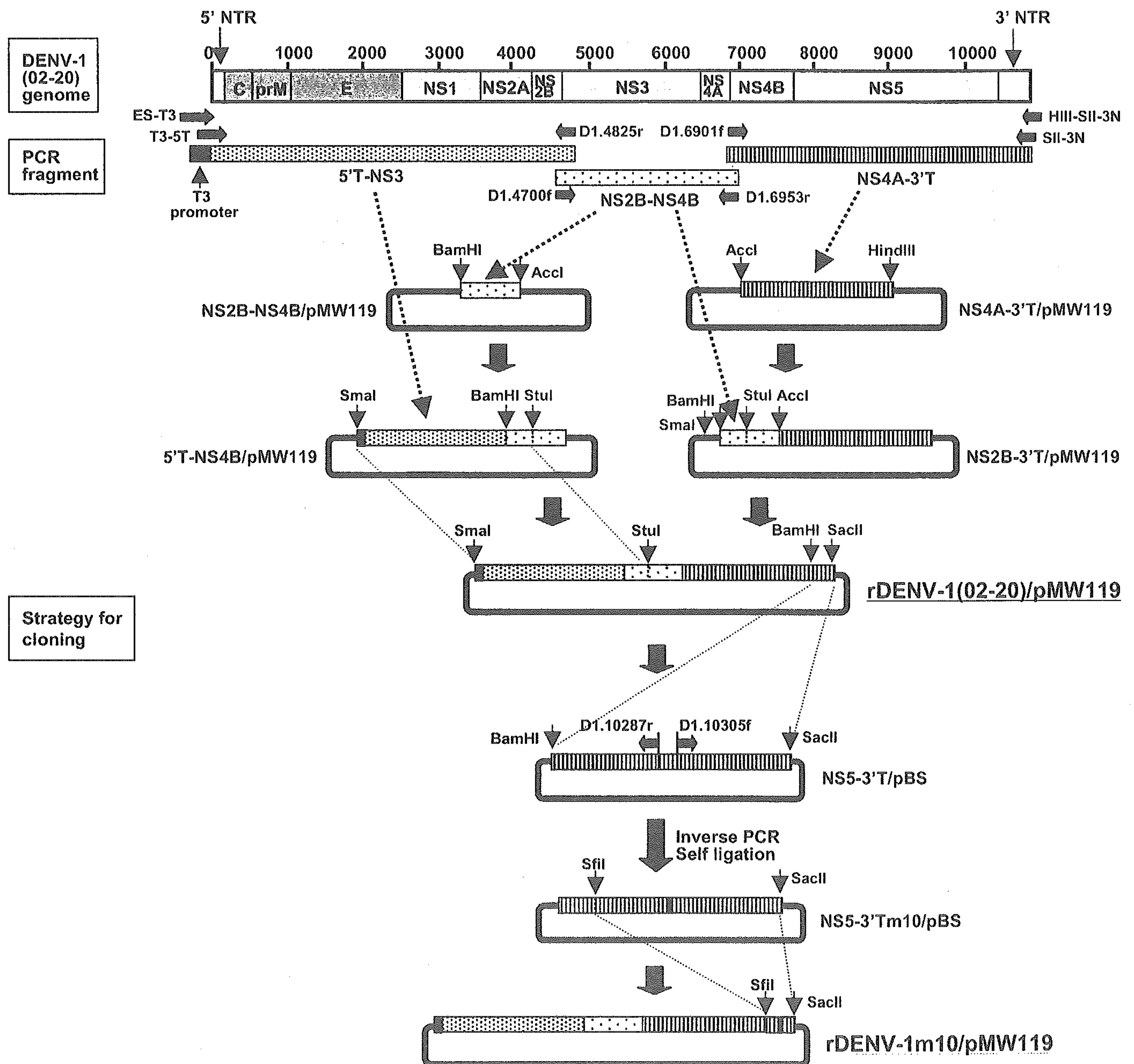

Fig. 1. Schemetic representation of the strategy for construction of recombinant DENV-1 clone rDENV-1(02–20)/pMW119 and mutant clone rDENV-1m10/pMW119. The upper illustration of the figure shows genomic structure of DENV-1 and its RT-PCR fragments used for the following cloning. A detailed explanation of the strategy is described in Section 2.

tion in the variable region may not affect growth properties in vitro and virulence in mice (Mandl et al., 1998). In contrast, similar analysis in DENV-4 suggested that recombinant viruses with deletion in the variable region exhibited small plaque morphology on mosquito C6/36 cells and slightly low replication efficiency on simian LLC-MK_2 cells (Men et al., 1996). It was also reported that the variable region may play a role in the rate of JE viral RNA replication (Nam et al., 2001). Thus, the function of the variable region of 3′ NTR in flavivirus remains to be determined.

In the present paper, we examined the effect of a small deletion in the 3′ NTR on the nature of DENV-1 virus. We first developed recombinant full-length cDNA clones of conventional type of DENV-1 rDENV-1(02–20) using low copy number plasmid pMW119. We then constructed mutant DENV-1 clone rDENV-1m10, which has 19-nt deletion in the 3′ NTR almost corresponding to the deletion site observed in the DENV-1 isolate (Nukui et al., manuscript in submission), and compared the growth kinetics and plaque morphology of the recombinant virus between rDENV-1(02–20) and rDENV-1 m10.

2. Materials and methods

2.1. Cells and virus

Vero (NIBSC strain) and C6/36 cells were cultured at 37 and 28 °C, respectively, in 5% CO_2 in Eagle's minimum essential medium (MEM) supplemented with heat-inactivated fetal bovine serum (FBS) and 100 U of penicillin–streptomycin/ml. Human hepatoma Huh-7 cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 U of penicillin–streptomycin/ml. DENV-1 strain NIID02–20 (DENV-1(02–20), accession no. AB178040) was isolated in 2002 from Japanese DF patient who came back from Thailand and the virus was propagated in Vero cells.

2.2. Construction of recombinant DENV-1 clones

Schematic flow of the construction of recombinant DENV-1 clones is shown in Fig. 1. Primers used for constructing the clones are listed in Table 1. DENV-1(02–20) RNA was extracted from culture supernatant fluid using High Pure Viral RNA Kit (Roche Diagnostics) and used for synthesis of viral cDNA using the SuperScript III reverse transcriptase (Invitrogen). 5′ terminal- NS3 region of DENV-1 genome was amplified with primers T3-5N and D1.4825r for 1st PCR and primers ES-T3 and D1.4825r. The primer ES-T3 contains complete T3 polymerase promoter sequence (Fig. 1 and Table 1). NS2B–NS4B region of DENV-1 genome was amplified with primers D1.4700f and D1.6953r. NS4A-3′ terminal region of DENV-1 genome was amplified with primers D1.6901f and SII-3N for 1st PCR and D1.6901f and HIII-SII-3N for 2nd PCR. These PCR reactions were done using a thermostable high-fidelity DNA polymerase KOD-plus (Toyobo). PCR products of the NS2B–NS4B region and the NS4A-3′ terminal region were subcloned into the low copy number plasmid pMW119 (Nippon Gene) at BamHI–AccI site (NS2B–NS4B/pMW119) and AccI–HindII (NS4A-3′T/pMW119) site, respectively, using competent cell Stbl2 (Invitrogen). PCR fragment of the 5′ terminal–NS3 region was subcloned into SmaI–BamHI site of NS2B–NS4B/pMW119 (5′T-NS4B/pMW119) and the NS2B–NS4B fragment was also subcloned into BamHI–AccI site of NS4A-3′T/pMW119 (NS2B-3′T/pMW119). Complete recombinant DENV-1 clone (rDENV-1(02–20)/pMW119) was constructed by replacing a SmaI–StuI small fragment of NS2B-3′T/pMW119 with a SmaI–StuI fragment, which contains the 5′ terminal–NS3 region, of 5′T-NS4B/pMW119.

To construct a recombinant DEN-1 clone with small deletion in variable region of 3′ NTR, BamHI–SacII fragment on the NS5–3′ terminal region was subcloned into BamHI–SacII site of pBluescript II SK(+) (Stratagene)(NS5-3′T/pBS). The plasmid was amplified by inverse PCR method with primers D1.10287 r and D1.10305f and then the products were treated with DpnI to digest plasmid templates, purified, self-ligated and then transformed into Stbl2. A plasmid clone NS5-3′Tm10/pBS, which has 19-nt deletion in the variable region of 3′ NTR, was obtained (Fig. 3). SfiI–SacII fragment of rDENV-1(02–20)/pMW119 was replaced with SfiI–SacII fragment of NS5-3′Tm10/pBS which contained the part of NS5-3′ terminal region to complete mutant recombinant DENV-1 clone rDENV-1m10/pMW119. Nucleotide sequence

Table 1
Primers used for construction of cDNA clones

Direction	Primer[a]	Sequence (5′–3′)[b]
Forward (sense)	ES-T3	ATCCCGGG*AATTAACCCTCACTAAAGGG*AGTTGT
	T3-5N	*CCCTCACTAAAGGG*AGTTGTTAGTCTACGTGGACC
	D1.4700f	CAAGGGAAGAGAC TGGAACCGAG
	D1.6901f	TGCCGCAATGCTGGACGTAGAC
	D1.10305f	GAAATCAAACAAGGCAAGAAG
Reverse (antisense)	D1.4825r	TGGTTCAACAGCAATCACCTGCAC
	D1.6953r	CATAGAGGGTCCAGGCTGAAGC
	DI.10287	GTAAATGTGTTGACTCACCAG
	SII-3N	CCGCCGAGAACCTGTTGATTCAACAGC
	HIII-SII-3N	CGCAAGCTTCCGCGGAGAACCTGTTGATTC

[a] Regions that amplified by the primers are indicated in the Fig. 1.
[b] T3 polymerase promoter sequence in ES-T3 and T305N primers was shown in italic.

of the viral genome region of the recombinant clones was checked after amplification of the plasmids in *E. coli*.

2.3. *In vitro transcription, transfection and recovery of virus solution*

rDENV-1 clones were digested at the 3′ end of viral genome with SacII, purified using GenElute PCR Clean-Up kit (Sigma, St. Louis, Mis), and 1 μg linearized DNA was transcribed at 37 °C for 3 h using mMESSAGE mMACHINE RNA transcription kit (Ambion, Austin, Tx). After DNase I treatment, synthesized RNA was purified using RNeasy MinElute Cleanup kit (Qiagen, Hiden, Germany). Three microgram of RNA was transfected into Vero cells (2×10^6 cells) using DMRIE-C reagent (Invitrogen) and cells were incubated at 37 °C for 6 days in 5% CO_2. Five-hundred microlitres of the culture supernatant of transfected Vero cells was then inoculated to Vero cells (1×10^6 cells) and the cells were incubated at 37 °C in 5% CO_2. Six days after inoculation, culture supernatant fluid was recovered, titrated and used for further inoculations as virus solution. The nucleotide sequence of the recombinant virus was determined and no nucleotide mutation was detected.

2.4. *Analysis of virus growth and plaque size*

Cells (1×10^6) were plated in 25-cm^2 culture flask and infected with rDENV-1(02–20) and rDENV-1m10 at a multiplicity of infection (m.o.i.) of 0.02 plaque forming units (pfu)/cell. Small aliquots of the media were recovered on days 0, 1, 2, 4, 6 and 8 and the aliquots were titered by plaque assay on Vero or Huh-7 cells grown in 12-well culture plates. To evaluate the plaque size, Vero or Huh-7 cells were fixed with 3.7% (v/v) formaldehyde solution in phosphate-buffer saline for 1 h, the methyl cellulose overlay was removed and the cells were stained with methylene blue solution for 2 h.

2.5. *Immunoblotting*

Culture supernatant was mixed with SDS-PAGE sample buffer containing 2-mercaptoethanol, heated at 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. The proteins on the gel were transferred to a polyvinylidene difluoride membrane filter (PVDF; Millipore) and the filter was incubated in buffer that contained ascitic fluid from mice immunized with DENV-2, which reacts with the E protein. After washing, the filter was incubated with sheep horseradish peroxidase-conjugated anti-mouse IgG and products were detected with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

3. Results

3.1. *Establishment of the system for production of recombinant DENV-1 virus*

Full-length cDNA clone of DENV-1 was constructed from DENV-1 virus strain NIID02-20, an isolate from a Japanese DF patient who was infected in Thailand in 2002. Three parts of the virus genome were amplified by RT-PCR and serially ligated into low copy number plasmid pMW119 (Fig. 1). Plasmid rDENV-1(02–20)/pMW119 contained full length of DENV-1 NIID02–20 cDNA and T3 promoter sequence and additional *Sac*II site, which were located immediately upstream and downstream of the virus cDNA, respectively. Recombinant virus RNA

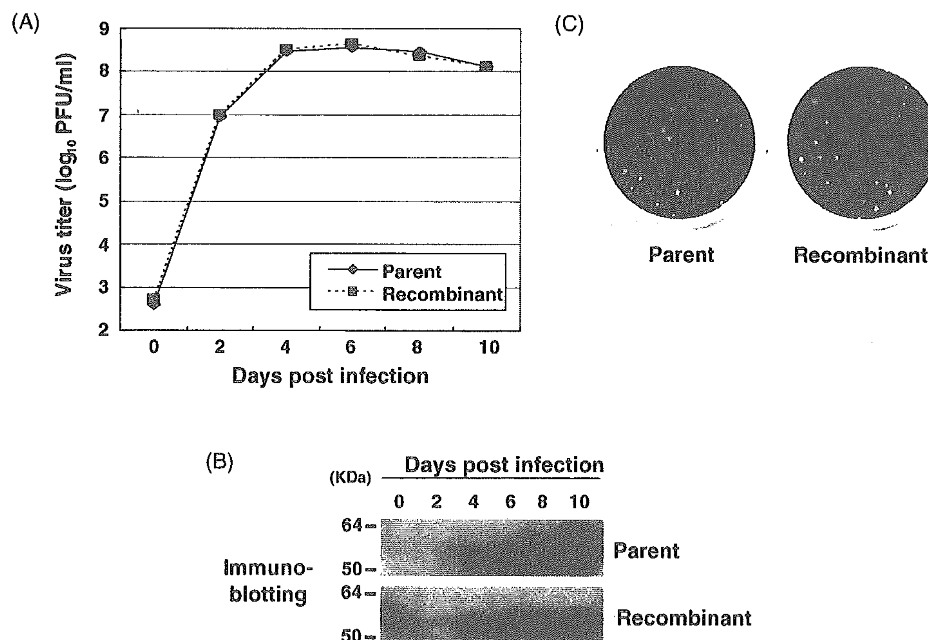

Fig. 2. Growth properties of parent and recombinant DENV-1s in Vero cells. (A) Growth curves of the viruses. The cell culture medium was sampled at the days indicated and the virus titers were determined on Vero cells. (B) Detection of the virus antigens in culture medium of Vero cells infected with the viruses. The cell culture medium sampled was also analyzed by immunoblotting with ascitic fluid of mice immunized with DENV-2. (C) Plaque phenotypes of the viruses at seven days postinfection in Vero cells.

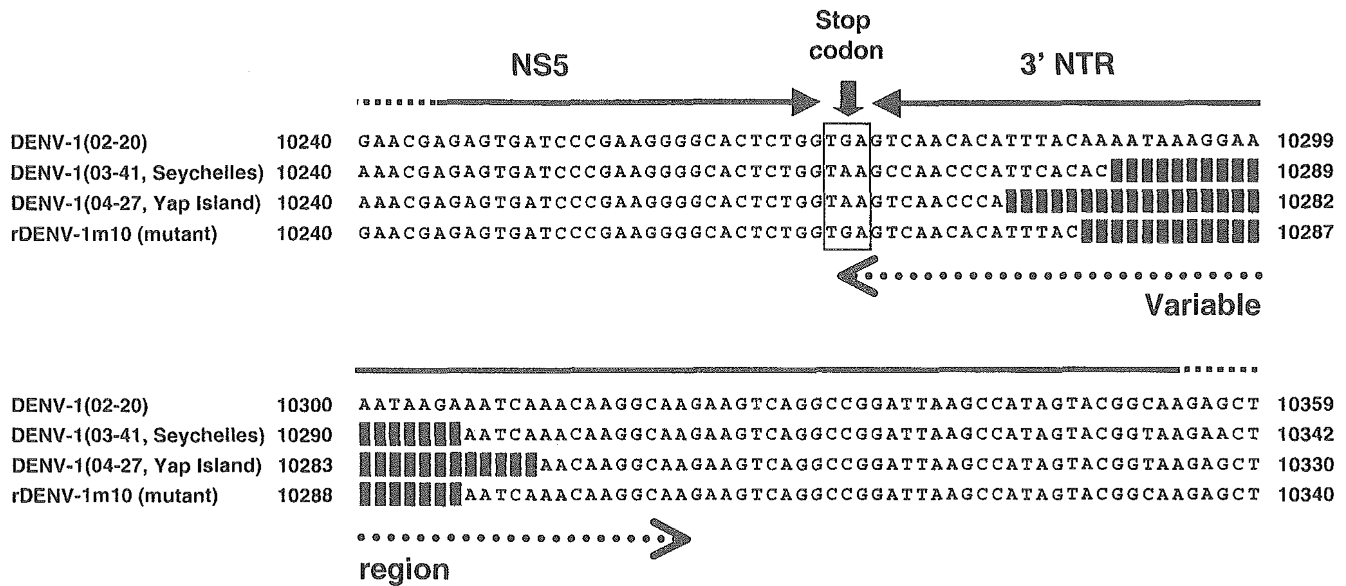

Fig. 3. Nucleotide sequences of the variable region in 3′ NTR of DENV-1(02–20), DENV-1(03–41, Seychelles), DENV-1(04–27, Yap island) and recombinant mutant clone (rDENV-1m10). Gray boxes indicate deleted nucleotides in the region.

was synthesized from the cDNA clone by T3 polymerase system and then transfected into Vero cells. After one blind inoculation, culture supernatant fluid was recovered and used as recombinant virus solution. In order to determine the growth property of the recombinant virus, the growth kinetics, secretion of virus antigens and plaque morphology of the recombinant virus were examined in comparison with the parent virus (Fig. 2). The growth curve and plaque size of the recombinant virus were almost identical to the parent virus (Fig. 2A and C). The levels of secreted viral antigens (E protein) were similar for both viruses (Fig. 2B). The recombinant virus had no mutation in nucleotide sequence (data not shown). The results indicated that the system for producing infectious recombinant DENV-1 viruses was established.

3.2. Construction of mutant DENV-1 cDNA clone

Two novel DENV-1 strains, NIID03–41 and NIID04–27, isolated in our laboratory had a small deletion in the variable region of the 3′ NTR (Fig. 3). To examined the effect of a small deletion on the nature of DENV-1 virus, we attempted to introduce the 17-nucleotide deletion found in NIID03–41 strain into the 3′ NTR of recombinant DENV-1(02–20) cDNA clone by using site-directed mutagenesis method after subcloning of NS5-3′ terminal region of the virus cDNA (Fig. 1). Several mutant clones with different deletion length were obtained. However, unfortunately, a clone which has 17-nucleotide deletion was not obtained (data not shown). Therefore, we picked up a clone NS5-3′Tm10, which has 19-nucleotides deletion similar in size and region to the 17-nucleotide deletion (Figs. 1 and 3). NS5-3′ terminal fragment with the deletion was returned into the recombinant cDNA clone to complete rDENV-1m10/pMW119 (Fig. 1). Recombinant DENV-1m10 virus was prepared and the nucleotide sequence of the virus genome was confirmed.

3.3. Comparison of the growth properties between rDENV-1(02–20) and rDENV-1m10 in vitro

To examine the effect of the small deletion in the 3′ NTR on the nature of DENV-1, growth kinetics and plaque morphology were compared between rDENV-1(02–20) and rDENV-1m10. The recombinant viruses were inoculated to Vero cells and the amounts of the infectious virus in the culture supernatant fluids were assessed at various timepoints (Fig. 4A). The growth

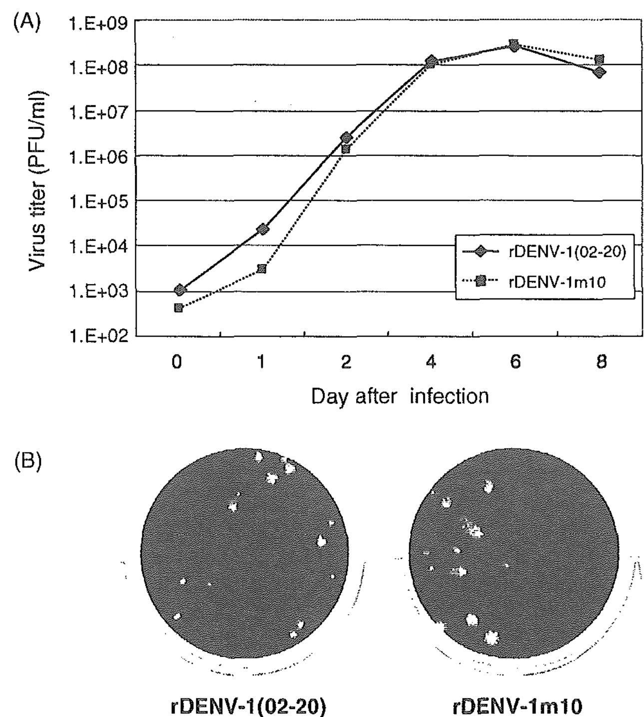

Fig. 4. Comparison of (A) growth kinetics and (B) plaque phenotypes of virus between rDENV-1(02–20) and rDENV-1m10 in Vero cells. Similar results were obtained in two independent experiments.

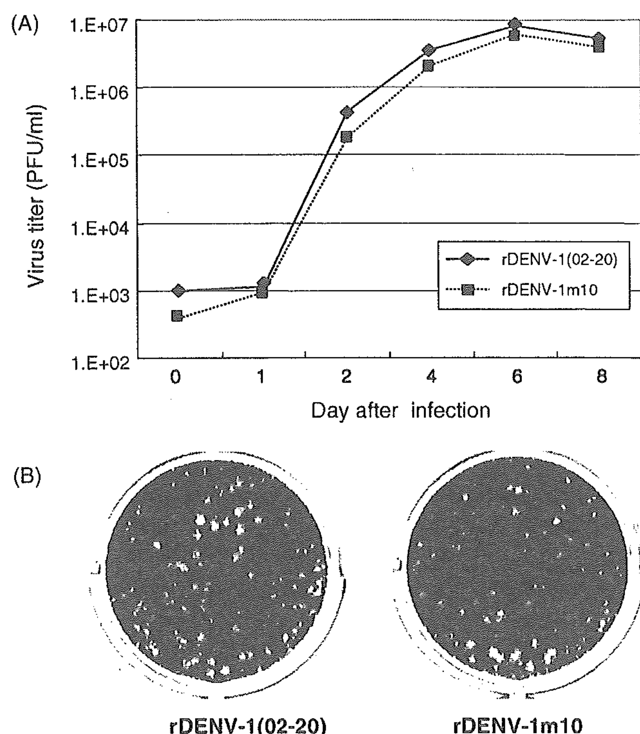

Fig. 5. Comparison of (A) growth kinetics and (B) plaque phenotypes of virus between rDENV-1(02–20) and rDENV-1m10 in Huh-7 cells. Similar results were obtained in two independent experiments.

curve of the mutant virus was similar to that of the virus without deletion. Moreover, no apparent difference was observed in the plaque size between the viruses (Fig. 4B). The growth property of the viruses were next compared in human hepatoma Huh-7 cells (Fig. 5A and B). Growth kinetics and plaque size of the mutant virus were similar to those of the virus without deletion. The growth kinetics were also compared in mosquito C6/36 cells and no significant difference was detected (Fig. 6). These results indicate that the 19-nt deletion (nucleotide 10288–10306) in the 3′ NTR of DENV-1 does not affect the growth nature in human, African green monkey, and *Aedes* mosquito cells in vitro.

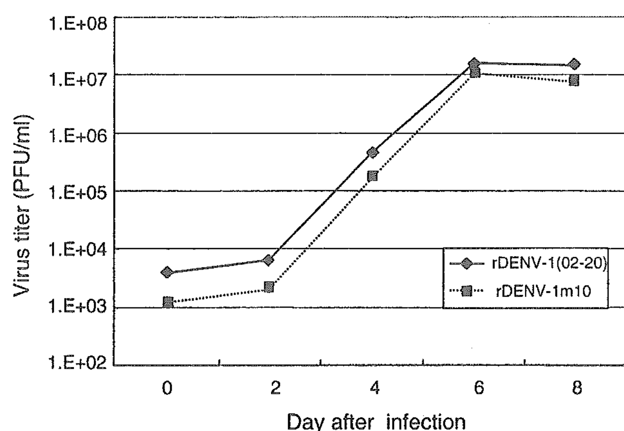

Fig. 6. Comparison of the growth kinetics of virus between rDENV-1(02–20) and rDENV-1m10 in C6/36 cells. Similar results were obtained in two independent experiments.

4. Discussion

To examine the effect of a 19-nt nucleotide deletion in the 3′ NTR of DENV-1 genome, two full-length cDNA clones of DENV-1 with and without the deletion were constructed. The 19-nt deletion was prepared according to the deletions identified in two recent DENV-1 isolates in our laboratory (Nukui et al., manuscript in submission). The virus with 19-nt deletion was compared with that without deletion in replication efficiency in three types of cells derived from human, African green monkey, and mosquito. There was no significant difference in the virus growth between the two viruses.

Full length infectious clone of flaviviruses has been appreciated as a powerful tool for studying replication, pathogenesis, and vaccine development. Several groups constructed full length infectious clones for dengue viruses (Lai et al., 1991; Kinney et al., 1997; Polo et al., 1997; Gualano et al., 1998; Puri et al., 2000; Sriburi et al., 2001). In the present study, we established the system for production of infectious recombinant DENV-1. We overcame the problem associated with the genetic instability of full-length DENV-1 cDNA in *E. coil,* using a very low copy number (1–5 copies/cell) plasmid pMW119 and a competent *E. coli* strain Stbl2. No spontaneous mutation or deletion was detected during several re-transformation of the cDNA clone into Stbl2. It is easy to construct mutant DENV-1 viruses, which have some artificial mutations into its genome, using the developed infectious cDNA clones. Thus, our clone is a useful tool not only for analyzing biology of DENV but also for developing a live-attenuated vaccine.

Recent studies on the function of the well-conserved core region of 3′ NTR of flaviviruses has revealed the importance of this region in virus replication; however, the role of the variable region of 3′ NTR in flaviviruses remains controversial. A TBEV mutant lacking the entire variable region was indistinguishable from the wild-type virus, which has 400-nt variable region in growth properties in vitro and virulence in mice (Mandl et al., 1998). These findings are consistent with our present data on DENV-1, suggesting that the variable region in the 3′ NTR is an accessory sequence in flavivirus. In contrast, it was also reported that a series of DENV-4 which have deletion in 5′ side of 3′ NTR appeared to replicate less efficiently in cultured cells and to reduce the level and duration of viremia in rhesus monkey, compared to the wild-type virus (Men et al., 1996). In the study by Men et al., however, the deletions were larger than that we prepared in DENV-1 and included both the variable region and a conserved sequence motif. Therefore, the attenuation may be caused by the deletion of the conserved sequence rather than variable region. Recent studies using reporter replicon of West Nile virus showed that deletion of most of 3′ NTR containing the variable region did not affect translation efficiency, although the region was indispensable for virus replication (Tilgner et al., 2005). On the other hand, it was reported that terminal 3′ stem-loop domain enhanced translation initiation in DENV-3 (Holden and Harris, 2004). Detailed analysis is needed to further elucidate the function of 3′ NTR and role of the variable region.

The 19-nt deletion in the variable region did not demonstrate any apparent effect in in vitro growth analyses in the present

study. It is not known why the variable region exists in 3′ NTR in flaviviruses. JEV, a flavivirus, is classified into five genotypes. Major JEV strains isolated in Japan and Vietnam has shifted from genotype III to genotype I in 1990s (Ma et al., 2003; Nga et al., 2004). Interestingly, almost all the genotype III JEV strains analyzed so far had small deletions in the variable region in the 3′ NTR. These deletions were similar to that observed in the DENV-1 strains in the size and locations. Deletion of a similar size was detected in the similar location when a genotype III JEV strain was passaged many times in suckling mouse brain (Nerome, unpublished data). Moreover, spontaneous deletions in the variable region were also detected during passage of TBEV in cultured cells (Vero cells, etc.) and mouse brains (Mandl et al., 1998). These findings suggest that the deleted region of flaviviruses is not required for virus replication in the cultured cells and mouse brain; however, it is still possible that the deletion may effect on the growth of viruses in natural vectors, amplifiers, and humans. Further studies are needed to clarify the functions of the deleted region in natural hosts and ex vivo cultured human cells.

Acknowledgments

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Novel Dengue Virus Type 1 from Travelers to Yap State, Micronesia

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Dengue virus type 1(DENV-1), which was responsible for the dengue fever outbreak in Yap State, Micronesia, in 2004, was isolated from serum samples of 4 dengue patients in Japan. Genome sequencing demonstrated that this virus belonged to genotype IV and had a 29-nucleotide deletion in the 3′ noncoding region.

Dengue virus (DENV) is a mosquitoborne flaviviruses; there are 4 serotypes, DENV-1, -2, -3 and -4. DENV has been found in ≥100 countries and 2.5 billion people live in areas where dengue is endemic. Fifty to one hundred million cases of dengue infection are estimated to occur annually (*1*). In Japan, outbreaks of dengue fever occurred in Nagasaki, Hiroshima, Kobe, and Osaka from 1942 to 1945, but none thereafter (*2*). However, ≈50 imported dengue cases occur annually in Japan.

The DENV genome is a single-stranded positive-sense RNA of ≈11,000 nucleotides (nt) that encodes 3 structural proteins (capsid, membrane, and envelope) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (*3*). Surrounding the open reading frame (ORF) are 5′ and 3′ noncoding regions (NCRs) that form RNA secondary structures (*4*). These regions are ≈100 and ≈400 nt, respectively (*5*).

Dengue fever developed in 9 Japanese patients in 2004 after they returned from Yap state. We report the genetic characterization of RNA from DENV-1 isolates from these patients.

The Study

Yap is the westernmost state of the Federated States of Micronesia and composed of 4 major islands. Yap has a total area of 102 km^2 and a population of 11,241 (2000 census). The climate is moderate and fairly constant. The mean annual temperature is 27°C. Relative humidity ranges from 65% to 100% (annual mean 83%). Rainfall averages 120 inches a year and is seasonal.

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In 1995, a dengue epidemic caused by DENV-4 occurred in Yap state (*6*), but no dengue outbreaks have since been reported. However, the Yap Epinet Team reported a dengue outbreak caused by DENV-1 in Yap state that began in the last week of May 2004. A total of 658 reported dengue fever cases (defined by the World Health Organization) occurred as of December 29, 2004. No deaths or dengue hemorrhagic fever/dengue shock syndrome cases were reported (*7*).

Fever, headache, and diarrhea developed in 7 Japanese adults who visited Yap after their return to Japan in August 2004. DENV infection was serologically confirmed in 5 patients (patients 1–5) by an immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) (Focus Diagnostics Inc., Herndon, VA, USA) and an IgG ELISA (PANBIO Ltd., Brisbane, Queensland, Australia) at the National Institute of Infectious Diseases in Tokyo, Japan. Of these 5 patients, 4 had a primary DENV infection and 1 had a primary dengue infection and a secondary flavivirus infection. DENV infection was serologically confirmed in the sixth patient at another institute. The seventh patient did not visit a medical facility but had symptoms of dengue fever. In addition, 2 other Japanese patients who traveled to Yap in September 2004 were diagnosed with dengue (data not shown).

Four virus isolates (NIID04-27, -31, -41, and -47) were obtained from serum samples from patients 1–4, respectively. Two hundred microliters of serum samples diluted 1:40 was injected onto C6/36 cells in minimal essential medium supplemented with 2% fetal calf serum. The cells were incubated at 28°C for 7 days and culture supernatant fluids were collected. DENV isolates were used for analysis without any further passage.

Complete nucleotide sequencing of RNA of NIID04-27, -31, and -47 and partial sequencing of NIID04-41 were performed. Viral RNA was extracted by using a High Pure RNA extraction kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, transcribed to cDNA, and amplified by polymerase chain reaction, as described previously (*8*). The cDNA was purified and sequenced by using the ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Seventeen pairs of primers were designed based on the DENV-1 NIID02-20 sequence (GenBank accession no. AB178040) and used in the analyses (*9*).

The nucleotide sequences of the viral isolates were compared with published complete sequences of DENV-1 (Table 1). Sequence alignment and analysis were performed by using ATGC analysis programs (version 4.02; Genetyx Corp., Tokyo, Japan). Phylogenetic analyses of nucleotide sequences were conducted with ClustalX software version 1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClusterW/). A phylogenetic tree was reconstructed for

Table 1. Dengue virus (DENV) strains used in the study

Virus	Strain	Origin	Year isolated	GenBank accession no.
DENV-1	NIID04-27	Yap Island	2004	AB204803
DENV-1	NIID03-41	Republic of Seychelles	2003	AB195673
DENV-1	FGA/89	French Guiana	1989	AF226687
DENV-1	BR/90	Brazil	1990	AF226685
DENV-1	BR/97-111	Brazil	1997	AF311956
DENV-1	BR/01-MR	Brazil	2001	AF513110
DENV-1	Abidjan	Côte d'Ivoire	1998	AF298807
DENV-1	Mochizuki	Japan	1943	AB074760
DENV-1	S275/90	Singapore	1990	M87512
DENV-1	16007	Thailand	1964	AF180817
DENV-1	GZ/80	China	1980	AF350498
DENV-1	A88	Indonesia	1988	AB074761
DENV-1	Cambodia	Cambodia	1998	AF309641
DENV-1	Djibouti	Ethiopia	1998	AF298808
DENV-1	West Pac 74	Nauru	1974	U88535
DENV-1	98901530	Indonesia	1998	AB189121
DENV-1	98901518	Indonesia	1998	AB189120
DENV-1	259par00	Paraguay	2000	AF514883
DENV-1	295arg00	Argentina	2000	AF514885
DENV-1	ARG9920	Argentina	1999	AY277664
DENV-1	NIID02-20	Thailand	2002	AB178040
DENV-1	99-36-1HuNIID	Paraguay	1999	AB111065
DENV-1	01-27-1HuNIID	The Philippines	2001	–
DENV-1	01-32-1HuNIID	The Philippines	2001	–
DENV-1	01-36-1HuNIID	Singapore, Malaysia	2001	AB111067
DENV-1	01-42-1HuNIID	Thailand, Cambodia	2001	AB111069
DENV-1	01-44-1HuNIID	Tahiti	2001	AB111070
DENV-1	01-54-1HuNIID	India	2001	–
DENV-1	01-54b-1HuNIID	India	2001	–
DENV-1	01-61-1HuNIID	Cambodia	2001	AB111071
DENV-1	01-65-1HuNIID	Thailand	2001	AB111072
DENV-1	01-66-1HuNIID	Thailand	2001	–
DENV-2	DENtype2-TB16i	Indonesia	2004	AY858036
DENV-3	DENtype3-TB55i	Indonesia	2004	AY858048
DENV-4	DENtype4-8976/95	Indonesia	2004	AY762085

aligned nucleotide sequences by using the neighbor-joining method. Bootstrap reassembling analysis of 1,000 replicates was used to assess confidence values for virus groupings. The phylogenetic tree was constructed by using Treeview software version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.htm).

The full-length RNA genomes of NIID04-27, -31, and -47 were 10,706 nt. A previous study reported that the full-length RNA genome of DENV-1 was 10,735 nt (8). The differences in the genome sequence between NIID04-27 and the other 2 isolates (NIID04-31 and -47) were subtle; identities with NIID04-31 and -47 were 99.94% and 99.92%, respectively. The results suggest that these 3 isolates belong to the same strain. Therefore, we used NIID04-27 as a representative isolate for further analysis.

To characterize the molecular structure of the genome, the complete NIID04-27 nucleotide sequence was compared with those of other DENV-1 strains available in GenBank (Table 1). NIID04-27 shared sequence identity ranging from 90.9% to 96.9% (Table 2) with 12 other DENV-1 strains. With respect to the alignment of full-length genomes, some alterations were found in the 3′ NCR. These alterations included a deletion of 29 nt starting at the 13th position from the ORF termination codon (Figure 1). The same deletion in the 3′ NCR was found in the viral genome amplified directly from the serum sample from patient 1 and was also observed in NIID04-31, -41, and -47.

To further analyze the genetic variation in the 3′ NCR of DENV-1, we analyzed the sequence of 24 other DENV-1 strains. Only the NIID03-41 strain, which was isolated in our laboratory from a patient returning from the Republic of Seychelles, had a 17-nt deletion in the 3′ NCR (Figure 1). The complete genomes of the 25 DENV-1 strains analyzed showed high levels of nucleotide sequence identity in the 3′ NCR, except for a small region of 50 nt immediately after the ORF, which is the hypervariable region. The nucleotide sequence identities in the 3′ NCR between NIID04-27 and 12 other DENV-1 strains ranged from 89.3% to 92.5% (Table 2).

Table 2. Pairwise comparisons of full-length genome and 3′ noncoding region sequences of dengue virus type 1 (DENV-1) strains*

Virus strain	NIID 04-27	FGA/89	BR/90	BR/97 -111	Abidjan	Mochizuki	S275/90	16007	GZ/80	A88	Cambodia	Djibouti	West Pac74
	% identify of nucleotide												
	Full-length genome												
NIID04-27		**91.5**	**91.5**	**91.3**	**90.9**	**93.4**	**91.9**	**93.1**	**92.3**	**96.9**	**91.7**	**91.7**	**95.6**
FGA/89	**89.5**		98.3	97.9	94.5	93.7	93.4	93.5	92.5	92.3	92.0	92.1	93.3
BR/90	**89.5**	99.4		98.8	94.5	93.9	93.7	93.7	92.7	92.3	92.2	92.0	92.9
BR/97-111	**89.3**	98.7	99.4		94.4	93.7	93.5	93.5	92.5	92.2	92.1	91.8	92.8
Abidjan	**89.7**	94.0	93.8	94.2		92.9	92.8	92.9	92.1	92.0	91.7	91.6	92.3
Mochizuki	**91.2**	94.8	94.7	94.9	93.4		95.0	95.4	96.1	94.6	95.3	95.2	95.1
S275/90	**91.2**	91.7	91.5	91.9	96.6	94.4		93.8	96.1	93.0	96.4	95.2	93.7
16007	**90.8**	96.2	95.9	95.7	93.2	97.4	93.4		94.2	94.2	93.7	93.5	94.7
GZ/80	**90.2**	94.1	93.8	94.4	93.6	97.4	94.4	95.7		93.3	96.7	98.0	93.9
A88	**92.5**	93.8	93.6	93.8	92.3	96.8	92.9	96.8	95.5		92.7	92.7	97.2
Cambodia	**89.9**	92.1	92.1	92.1	96.4	94.4	96.9	93.6	95.1	92.8		95.9	93.4
Djibouti	**89.5**	92.1	91.9	91.9	95.3	95.3	96.9	94.0	95.5	93.2	97.6		93.3
WestPac74	**91.4**	95.1	94.9	95.1	93.8	93.8	93.2	97.2	95.1	97.2	93.0	93.4	
	3′ noncoding region												

*The percentage nucleotide sequence identities of the complete genomes are shown in the upper right half of the table. The percentage nucleotide sequence identities of the 3′ noncoding region of the genomes are shown in the lower left half of the table. The percentage sequence homologies between NIID04-27 and each of 12 other DENV-1 strains are shown in boldface.

To understand the genetic relationships and evolution of DENV-1 strains, we also performed phylogenetic analysis of the fully sequenced DENV-1 strains that included NIID04-27 (Figure 2). NIID 04-27 belonged to genotype IV along with A88, 98901518, 98901530, NIID03-41 and West Pac74 .This cluster was called the Pacific group in a previous report (*10*). NIID04-27 and NIID03-41 are the first DENV-1 strains to have deletions in 3′ NCR.

Conclusions

We have genetically characterized DENV-1 isolate NIID04-27 by determining its complete nucleotide sequence and comparing the sequence with most of the available DENV-1 full-length sequences. Sequence heterogeneity in the 3′ NCR of the genus *Flavivirus* has been reported for tickborne encephalitis virus, Japanese encephalitis virus, DENV-2, and DENV-4 (*11–13*). For example, DENV-2 isolated in Texas, Peru, Venezuela, Mexico, and Puerto Rico had a 10-nt deletion starting at the 19th nucleotide position from the ORF termination codon (*13*).

The terminus of the 3′ NCR has a conserved sequence and secondary structure. The functions of the 3′ NCR of flaviviruses have not been fully determined. The 3′ NCR in flaviviruses affects RNA replication but does not affect viral translation (*14,15*). Introduction of a 30-nt deletion starting at the 212th position from the ORF termination codon in the 3′ NCR of DENV-4 reduced the ability of the virus to propagate in vivo and in vitro (*16*).

We have identified a 29-nt deletion in the 3′ NCR of DENV-1 isolated from a dengue patient returning to Japan from Yap. Isolates from 3 other patients infected in the same outbreak also had the same deletion. The DENV-1 strain with a 29-nt deletion in the 3′ NCR was responsible for the dengue epidemic in Yap in 2004. The biologic characteristics induced by this deletion should be further analyzed.

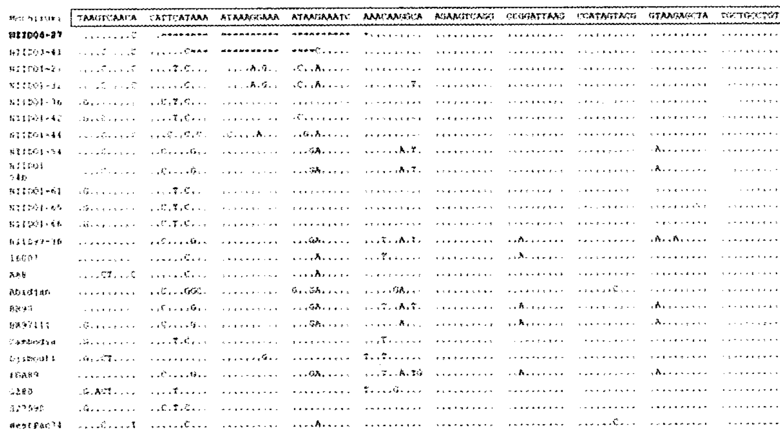

Figure 1. Nucleotide sequence alignment of the variable region in the 3′ noncoding region of dengue virus type 1 strains, including NIID04-27 sequenced in the present study. The Mochizuki strain was used as the consensus sequence, and the sequence of 100 nucleotides immediately downstream of the open reading frame termination codon is shown at the top. Solid dots indicate nucleotides identical to the consensus sequence and hyphens indicate deletions.

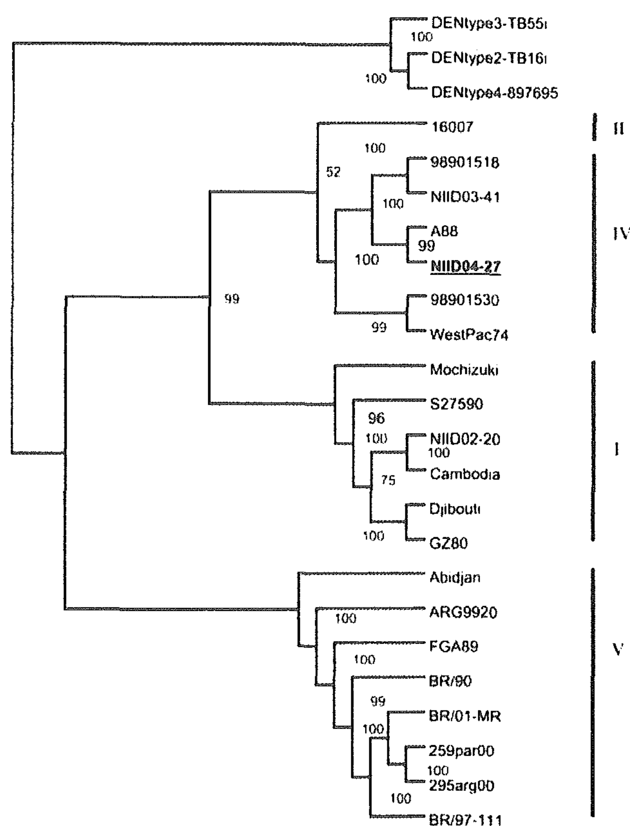

Figure 2. Phylogenetic tree based on the full-length genome sequence of 21 available dengue virus (DENV) type 1 strains and DENV-2, -3, and -4. The multiple sequence alignments were obtained with ClustalX, and the tree was constructed by the neighbor-joining method. The percentage of successful bootstrap replicates is indicated at the nodes. The NIID04-27 strain is indicated in boldface. Genotypes I, II, IV, and V correspond to DENV-1 genotypes as defined by Goncalvez et al. (*10*).

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