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Short communication

A method for full genome sequencing of all four serotypes of the dengue virus

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A B S T R A C T

The availability of whole genome sequencing has contributed to many aspects of dengue research, and its use in dengue virus (DENV) surveillance for early epidemic warning has been proposed. Methods to sequence the genomes of individual dengue serotypes have been described previously, but no single method is known to be applicable for all four serotypes. This report describes a method for sequencing the entire genome of all four DENV serotypes. Using tagged oligonucleotide primers designed for the 3′ end, viral RNA was reverse transcribed into a cDNA spanning the entire genome of each of the four serotypes (DENV-1 to -4). This was followed by amplification of the entire cDNA in five overlapping amplicons. A sequence tag was added to the sense primer annealing to the 5′ UTR sequence and the antisense primer annealing to the 3′ UTR sequence to ensure no terminal nucleotides were omitted during PCR. Sixty-one virus isolates were sequenced: 58 DENV-2, one DENV-1, one DENV-4 and one DENV-3 published previously. The method described could be applied readily for viral biology studies and incorporated into proactive dengue virologic surveillance.

The dengue virus (DENV) is a *Flavivirus* that comprises four antigenically distinct serotypes (DENV-1 to -4) that co-circulate in tropical cities of the world (Gubler, 1998). The World Health Organization estimates that 50–100 million individuals contract dengue fever (DF) each year; of these, 500,000 people suffer from dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) with approximately 20,000 deaths resulting annually (Mackenzie et al., 2004). The geographic spread of the mosquito vector *Aedes aegypti*, uncontrolled urbanization, and increases in global travel between countries have changed the epidemiology of dengue fever and are largely responsible for the re-emergence of epidemic disease since 1970 (Gubler, 1998, 2004). Presently, all four serotypes co-circulate in Africa, Central and South America, and Asia.

While outbreaks of DF and DHF are influenced by multiple ecological and host factors (Halstead, 2007, 2008), viral factors also contribute to epidemic transmission (Gubler and Trent, 1993). The high genetic variability of the virus, differential selection pressures, and population bottlenecks subject the DENV to *in situ* and adaptive evolution, which can lead quickly to virus population heterogeneity or cause circulating strains to disappear (Bennett et al., 2003, 2006; Holmes, 2003; Rico-Hesse, 2003; Rico-Hesse et al., 1997; Twiddy et al., 2002). Such forces are most likely responsible for the formation of distinct clades, subtypes, or genotypes, within all four serotypes (Bennett et al., 2003; Rico-Hesse, 1990, 2003) and some of these changes may have in turn resulted in epidemic DHF (Bennett et al., 2006; Messer et al., 2003). Since viral evolution is a significant force that drives epidemiologic change, full viral genome sequencing is thus an important tool both for studies that probe the mechanism of viral virulence as well as virologic surveillance for advanced warning of dengue epidemics (Ooi et al., 2007).

The highly conserved envelope gene has been sequenced most frequently as it is a major antigenic determinant of humoral immunity. However, *in situ* and adaptive molecular evolution occurring in the nonstructural genes and in the 5′ and 3′ untranslated regions (UTR) have been shown to impact epidemiologic activity and virulence (Bennett et al., 2003; Leitmeyer et al., 1999; Rico-Hesse, 2003). Phylogenetic analysis of the full genome provides greater resolution on the dynamics of dengue epidemics than partial genome sequencing (Ong et al., 2008; Schreiber et al., 2009). However, a single method optimized to sequence all four viral serotypes has not been described in the literature. This report describes an approach to sequence all four DENV serotypes. The hallmark of this method is the generation of full genome cDNA using tagged oligonucleotide primers designed to anneal to strains across all genotypes for each serotype as well as the use of tagged primers

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Table 1
Alignment of the RT primers with the consensus sequences of the 3′ untranslated region for each genotype within the four DENV serotypes (DENV-1 to DENV-4).

Serotype	Genotype	Sequence	Genome Position
DENV-1	I	–	
	II	–	
	III	–	10715–10735
	d1a5B	CGRCAACTTAGTTGTCCAAGA	
DENV-2	Sylvatic	–	
	American	–	
	Cosmopolitan	–	
	American/Asian	–	
	Asian I	–	
	Asian II	–	10704–10723
	d2a5B	GACAACTTAGTTGTCCAAGA	
DENV-3	I	–	
	II	–	
	III	–	10687–10707
	d3a5B	CGACAACTTAGTTGTCCAAGA	
DENV-4	I	–	
	II	–	
	III	–	10633–10653
	d4a5B	CAACAACTAGGTTGTCCAAGA	

Oligonucleotide primers matched each consensus sequence with 100% similarity and degenerate bases were inserted at appropriate positions to ensure this accuracy. The genome position for DENV-1 refers to isolate AF311958.1 in GenBank; for DENV-2, isolate AF204178.1; for DENV-3, isolate DQ675533.1; for DENV-4, isolate AY618989.1.

during PCR amplification to ensure no omissions from terminals of the two untranslated regions.

Low passaged clinical viral isolates were obtained from Singapore, Puerto Rico and Indonesia. The Singapore samples were collected through an on-going early dengue infection and outcome study, which was approved by the Domain Specific Review Board of the National Healthcare Group (DSRB/B/05/013) and described previously (Low et al., 2006). The samples from Puerto Rico and Indonesia were obtained as part of dengue virologic surveillance (conducted by DJG). These viruses were passed in C6/36 cells and serotyped by indirect immunofluorescence using DENV serotype-specific monoclonal antibodies (ATCC: HB46-49). The cells from the virus culture were scraped off with a rubber policeman, washed once with 1× PBS, resuspended in 100 μL of 1× PBS and spotted onto a Teflon coated glass slide, air-dried and then immersed in 80% acetone for 10 min. The slide was rinsed with 1× PBS and air-dried. Two microliters of serotype-specific monoclonal antibody was added onto each well, incubated at 37 °C for 45 min in a humidified chamber, and washed two times with 1× PBS before drying. FITC-tagged secondary antibodies were diluted 1:30 with 0.1% Evan's Blue and 2 μL was added onto each well. Slides were then incubated at 37 °C for 45 min in the humidified chamber and then washed twice with 1× PBS. Slides were dried and mounted with buffered glycerol before imaging under a fluorescent microscope.

The primers used in this study were developed by aligning consensus sequences for 5 complete genomes for each genotype within each of the four serotypes in ClustalW. Primers for reverse transcription of the four serotypes were designed with 100% similarity to these consensus sequences (Table 1). A degenerate nucleotide R, which is a mixture of A and G bases, was used in primer d1a5B to match position 10717 of the DENV-1 genome where both T and C are present within genotype I viruses. Degenerate bases were not needed for primers d2a5B, d3a5B, and d4a5B (Table 1). A 21 base pair tag was added to the 5′ end of each of the four RT primers (Table 2). The tagged regions of each primer were analyzed in NCBI BLAST to ensure that they did not anneal to the DENV genome. The primers used to amplify the cDNA of all four serotypes are listed in Supporting Information Table S2. The primer binding positions and the expected amplicon sizes are shown in Fig. 1.

DENV RNA was extracted from the aliquoted culture supernatant using a QIAamp Viral RNA Mini Kit (Qiagen™, Valencia, CA, USA) according to the manufacturer's protocol and stored at −80 °C until use. cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen™, Carlsbad, CA, USA). In a 0.2 mL RNAse-free microtube, 5 μL of viral RNA was mixed with 1 μL of 10 μM gene-specific synthetic oligonucleotide primer, 1 μL of 10 mM dNTP mix, and 3 μL of DEPC-treated water, for a final volume of 10 μL. This was incubated at 65 °C for 5 min to denature RNA secondary structures on an Applied Biosystems™ (Foster City, CA, USA) Veriti 96 Well Thermal Cycler and then placed on ice for 5 min. Next, 2 μL 10× RT buffer (Invitrogen™), 4 μL of 25 mM $MgCl_2$, 2 μL 0.1 M DTT, 1 μL RNaseOUT™ (40 U/μL), and 1 μL SuperScript™ III RT enzyme (200 U/μL) was mixed in a separate RNAse-free 0.2 mL microtube and added to the RNA mixture on ice. This was then incubated at 50 °C for 50 min for cDNA synthesis and then 85 °C for 5 min to terminate the reactions on an Applied Biosystems™ Veriti 96 Well Thermal Cycler. The cDNA product was then chilled on ice for 5 min, and 1 μL of RNase H was added to each tube and incubated at 37 °C for 20 min to degrade viral and carrier RNA. First strand cDNA products were stored at −80 °C.

cDNA was amplified by PCR in five overlapping amplicons. 2 μL of cDNA was mixed with 5 μL of 10× *PfuUltra*® II Fusion HS DNA Polymerase Buffer (Stratagene©, La Jolla, CA, USA), 1.25 μL of 10 μM

Table 2
Serotype, name and sequence of oligonucleotide antisense primers used to reverse transcribe the dengue virus RNA genome.

Serotype	Primer name	Primer sequence
DENV-1	d1a5B	5′-*TTTGTCGGTCTGGGGGGGTAT*AGAACCTGTTGATTCAACRGC-3′
DENV-2	d2a5B	5′-*TTTGTCGGTCTGGGGGGGTAT*AGAACCTGTTGATTCAACAG-3′
DENV-3	d3a5B	5′-*TTTGTCGGTCTGGGGGGGTAT*AGAACCTGTTGATTCAACAGC-3′
DENV-4	d4a5B	5′-*TTTGTCGGTCTGGGGGGGTAT*AGAACCTGTTGGATCAACAAC-3′

Nucleotides in italics were added as a sequencing tag and do not anneal to the viral genome.

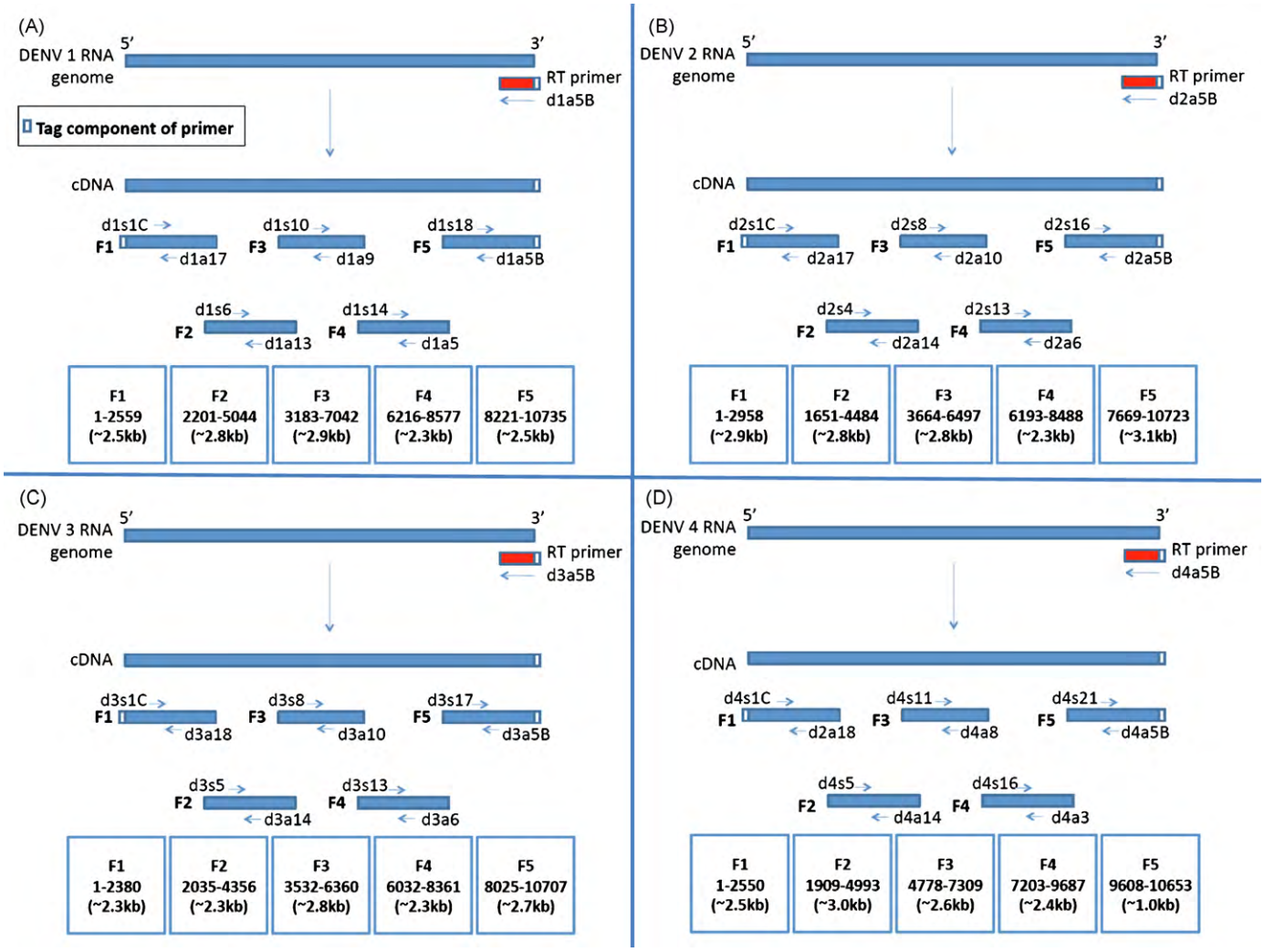

Fig. 1. Schematic representation of the strategy employed in sequencing the complete genomes of DENV. Panels A–D represent DENV-1 to DENV-4, respectively. From top to bottom for each panel: genome organization, full genome cDNA, overlapping PCR amplicons with their respective sequencing primer pairs. The sizes of each respective fragment are indicated as well. Sense primers (5′ → 3′) are designated by arrows pointing to the right and antisense primers (3′ → 5′) are designated by arrows pointing to the left.

dNTP, 1 μL each of 10 μM sense and antisense oligonucleotide serotype-specific primers, 1 μL *PfuUltra*® II Fusion HS DNA Polymerase, and was filled to 50 μL with autoclaved water. The cycling conditions for the five amplicons for each serotype are listed in Supporting Information Table S1. PCR products were separated in a 1% agarose TAE gel and visualized under UV light with ethidium bromide staining (Fig. 2). After confirming the bands of interest were present without non-specific products, the PCR product was purified using a Qiagen QIAquick® PCR Purification Kit. Purified products were quantified using a Thermo Scientific Nanodrop™ 8000 Spectrophotometer.

Purified PCR products were sequenced using an Applied Biosystems Big Dye ddNTP capillary sequencer as described previously (Schreiber et al., 2009). The chromatograms from capillary sequencing were assembled into a specimen consensus sequence using SeqScape version 2.5 (Applied Biosystems) with steps such as base calling, low-quality base trimming and alignment of specimen sequence to reference sequence handled internally by the software. The consensus sequences were then aligned to sequences of other known genomes of the same serotype. These consensus sequences then went through a further round of manual editing to ensure its accuracy and to weed out artifacts from the base-calling algorithm of the automated sequencer. Whenever possible, two individuals carried out manual editing independently. The two sets of results were then compared and merged to produce the final version of a specimen's genome sequence (Ong et al., 2008). The nucleotide sequences published in this study have been deposited in the GenBank database, and the accession numbers of these sequences are shown in Table S3.

In total, the whole genome of 61 DENV isolates was sequenced in this study. The use of tagged primers both in reverse transcription and amplification enabled us to obtain the full sequence of DENV-1 to -4 viruses, including the 5′ and 3′ UTRs. Capturing the sequences in the UTRs is important as nucleotide differences in these regions have been correlated with virus replication efficiency (Cahour et al., 1995; Leitmeyer et al., 1999; Men et al., 1996), epidemic activity and differential phenotypic disease expression (Bennett et al., 2003; Messer et al., 2003; Rico-Hesse et al., 1997). Internal primers sequencing towards the two ends of the genome were used to determine the terminal sequences. However, as the cDNA was constructed using a single RT primer, the actual terminal sequence of the 3′ end (21 bp) was thus not determined. While methods have been described to obtain the actual sequence of the 3′ ends (Brock et al., 1992; Mandl et al., 1991), they add a level of complexity to a process that is otherwise simple.

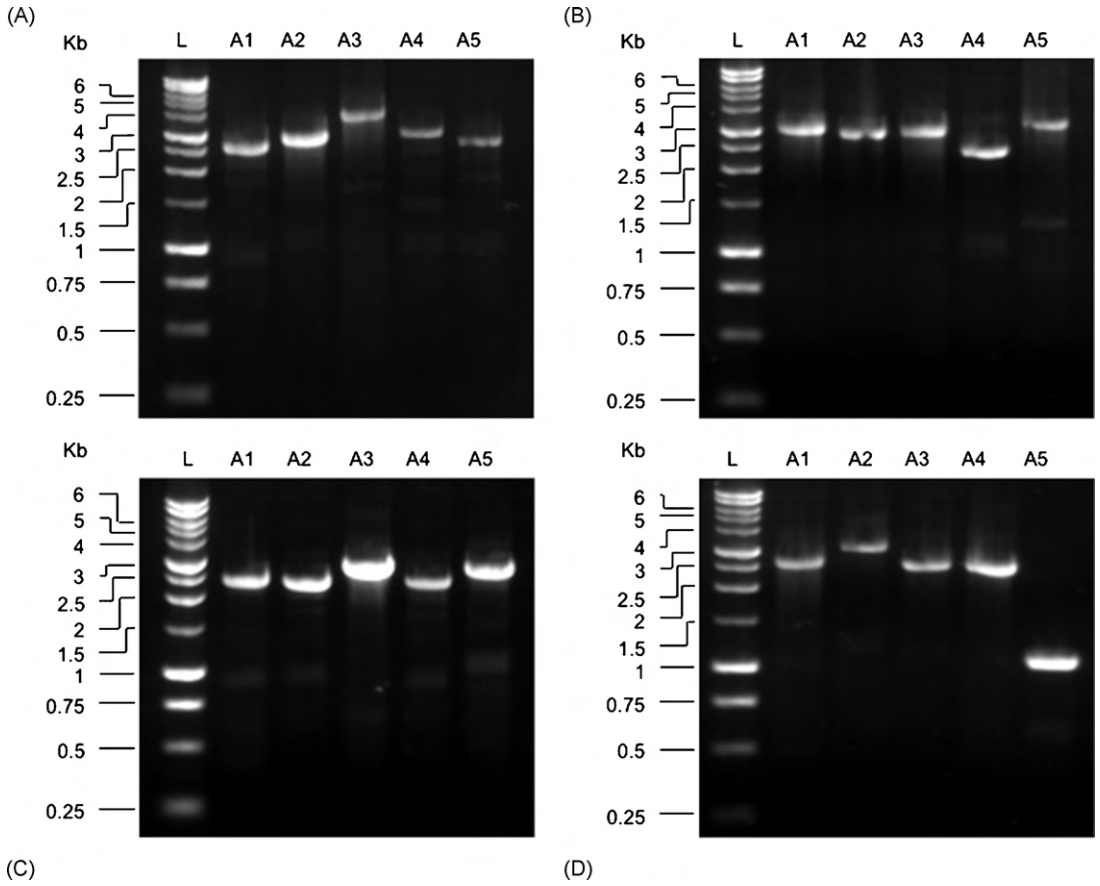

Fig. 2. Full genome amplification results. (A) DENV-1/SG/07K3640DK1/2008, (B) DENV-2/ID/1183DN/1977, (C) DENV-3/SG/05K863DK1/2005, and (D) DENV-4/SG/06K2270DK1/2005. PCR products were separated in 1% agarose and imaged under ultraviolet light with ethidium bromide staining. L indicates the 1 kb DNA ladder.

Another limitation of this study is the predominance of DENV-2 among the clinical isolates we have sequenced. Sequencing a larger panel of DENV-1, -3 and -4 viruses could thus refine the primer sequences reported further. Mutations in the DENV genome may also arise in future and modifications to these primers may thus be required.

While adaptive evolution does not always trigger epidemic transmission, mutations across the entire genome can result in circulation of virus populations with increased epidemic potential when ecological and immunological factors create ideal conditions for an outbreak. The availability of a tool to determine the full sequence of all four serotypes of the DENV could thus be useful not only for virologic research but also for surveillance to guide disease prevention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2010.06.013.

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