Journal of Virological Methods 235 (2016) 158–167

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Targeted full-genome amplification and sequencing of dengue virus types 1–4 from South America

Cristopher D. Cruz[a], Armando Torre[a], Gilda Troncos[a], Louis Lambrechts[b,c], Mariana Leguia[a,*]

[a] U.S. Naval Medical Research Unit No. 6 (NAMRU-6), Av. Venezuela c36s/n, Bellavista, Callao 2, Peru
[b] Insect-Virus Interactions Group, Department of Genomes and Genetics, Institut Pasteur, Paris, France
[c] Centre National de la Recherche Scientifique, Unité de Recherche Associée 3012, Paris, France

Article history:
Received 31 March 2016
Received in revised form 1 June 2016
Accepted 3 June 2016
Available online 19 June 2016

Keywords:
Dengue virus
Full-genome sequencing
Next-generation sequencing
Amplicon sequencing

A B S T R A C T

We report optimized workflows for full-genome sequencing of dengue viruses (DENVs) 1–4. Based on alignments of publicly available complete genomes we modified and expanded existing primers sets to amplify DENV genotypes that were previously difficult or impossible to sequence. We also report improvements to streamline laboratory handling, including a dual amplification strategy for easy and difficult to sequence "high-copy" and "low-copy" templates, respectively, and normalization of PCR cycling conditions across serotypes. High-copy templates can be sequenced following amplification of as few as 5 overlapping segments covering the complete viral genome, whereas low-copy templates can be sequenced following amplification of no more than 10 overlapping segments of smaller size. These changes have been validated using a balanced set of wild-type DENV genomes (11 of DENV1, 14 of DENV2, 13 of DENV3 and 7 of DENV4) derived from human serum samples collected throughout South America over the past 15 years. The changes described enable generation of complete DENV genomes from wild-type samples without the need for viral enrichment via passaging through laboratory cell lines. This should facilitate quick and cost-effective generation of DENV full-genome sequences of the type needed for accurate epidemiological surveillance and thorough evolutionary studies of wild-type DENVs.

1. Introduction

Dengue fever remains a serious public health threat throughout the world. According to the World Health Organization, dengue viruses (DENVs) (family *Flaviviridae*; genus *Flavivirus*) infect ~390 million people each year, result in 0.5 million hospitalizations, and lead to death in 2.5% of those affected (WHO, 2015). Using modeling frameworks to map the global distribution of dengue risk others have estimated the dengue burden to be three times higher than the estimate of the WHO (Bhatt et al., 2013). Despite the recent historic licensing of the first dengue vaccine approved for use in Mexico (Sanofi-Pasteur, 2015), robust countermeasures against DENVs continue to rely on a combination of vector control and surveillance efforts. All four DENV serotypes circulate in South America, where almost all countries are hyper-endemic for DENV transmission (Cafferata et al., 2016). Surveillance efforts in these countries depend heavily on laboratory diagnostics that are sensitive (such as PCR and ELISA based assays) and accurate (such as direct sequencing) in order to track viral evolution and inform public health interventions.

Several phylogenetic studies have documented the evolution of DENVs in South America, highlighting the importance of continuous monitoring of circulating DENV strains that could be linked to severe forms of the disease and/or to clade displacement (Aquino et al., 2008; Cruz et al., 2013; Dettogni and Louro, 2011; Forshey et al., 2009; Kochel et al., 2008; Mendez et al., 2012; Oliveira et al., 2010; Roca et al., 2009; Romano et al., 2010; Uzcategui et al., 2001; Uzcategui et al., 2003). Many of these studies have focused on envelope (*E*) gene sequences generated using traditional Sanger sequencing (Sanger and Coulson, 1975; Sanger et al., 1977). Despite being a major antigenic determinant, the *E* gene (~1484 bp in length) only provides limited phylogenetic information compared to the complete viral genome (~11 kb in length). The development

Abbreviations: DENVs, dengue viruses; NGS, next-generation sequencing; PGM, personal genome machine.

* Corresponding author.
E-mail addresses: cristopher.d.cruz.ln@mail.mil (C.D. Cruz), armando.torre@med.navy.mil (A. Torre), gilda.troncos.fn@mail.mil (G. Troncos), louis.lambrechts@pasteur.fr (L. Lambrechts), mariana.leguia.fn@mail.mil (M. Leguia).

http://dx.doi.org/10.1016/j.jviromet.2016.06.001

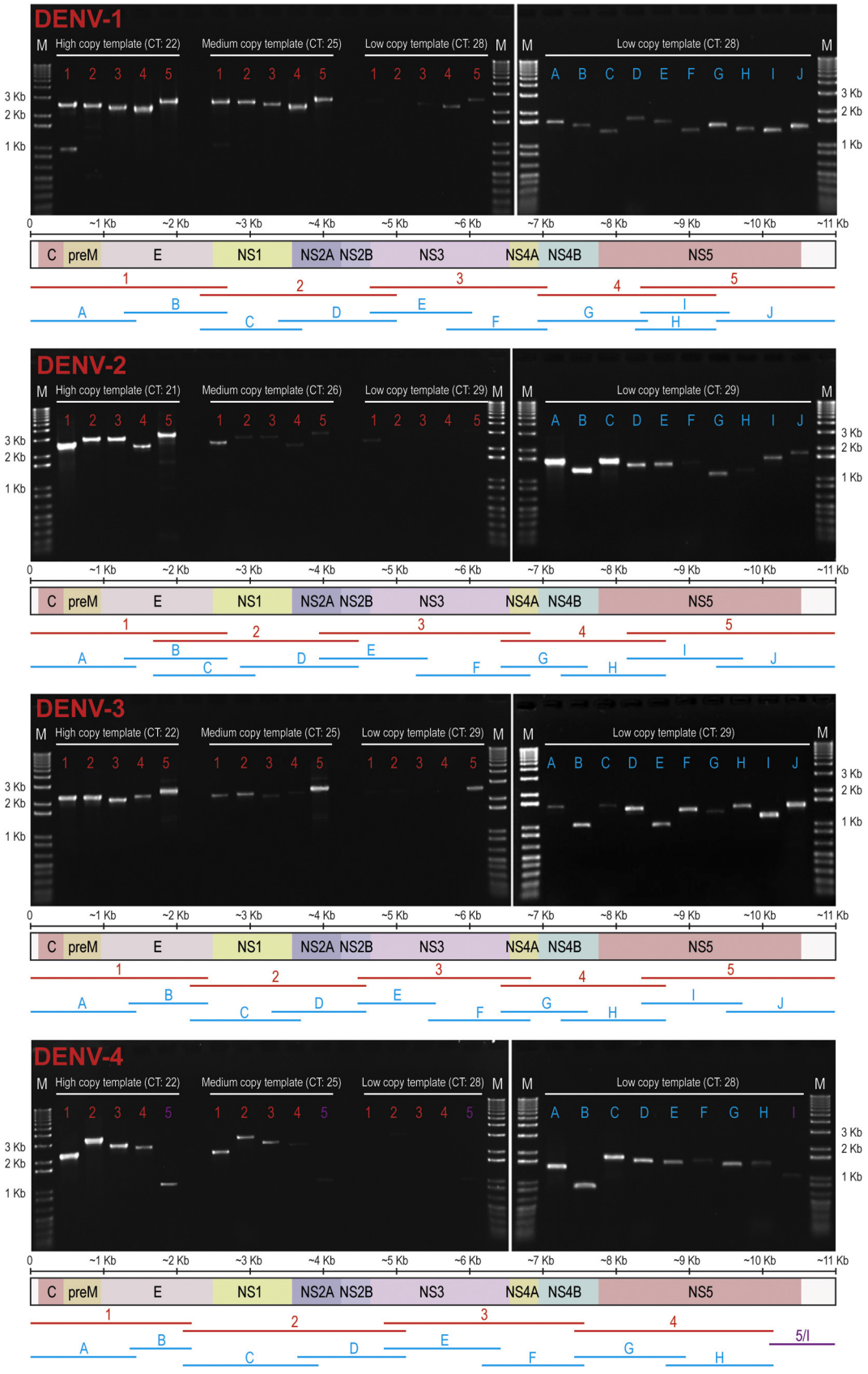

Fig. 1. Dual amplification strategy for DENV1-4.
Gels show representative full-genome amplifications of all DENV serotypes, using both the 5- and 10-overlapping fragment approaches. Amplicons from the 5-fragment approach from three serum samples of different Ct value (representing high-, medium- and low-copy templates) are shown in red, labeled in 1–5. Amplicons from the 10-fragment approach from low-copy templates only are shown in blue, labeled in A–J. M indicates the 1 kb plus DNA ladder.

of robust next-generation sequencing (NGS) platforms has significantly lowered sequencing costs, enabling the use of complete genome sequences to provide greater resolution of viral evolutionary dynamics. As a result, NGS technologies are now increasingly used for epidemiological surveillance and studies of viral genetic diversity (Lequime et al., 2016; Rodriguez-Roche et al., 2016; Sim et al., 2015). Unfortunately, the protocols developed for NGS of DENVs have mainly focused on viral strains that have circulated in Asia (Parameswaran et al., 2012; Sim et al., 2015; Zhao et al., 2014). Given that strains of DENVs circulating in South America can show significant genetic variation (Rodriguez-Roche et al., 2016; Romano et al., 2013), and especially when compared to those from Asia, existing protocols for full-genome amplification of DENVs can fail to generate complete genomes from American samples.

Here we describe changes made to existing sets of primers (Christenbury et al., 2010; Rodriguez-Roche et al., 2016) in order to optimize workflows and reliably generate full genomes of South American DENV strains. Specifically, we have implemented three sets of changes: the first ifs a complete re-design and expansion of primers used for amplicon sequencing of DENV1-4; the second is the development of a dual approach strategy for both high- and low-copy viral templates; the last is the inclusion of an initial viral quantification step that informs whether the high- or low-copy amplification approach should be used. Together, these changes streamline laboratory handling, reduce costs, and enable generation of complete genome data from DENV samples that were previously difficult, or even impossible to sequence.

2. Materials and methods

2.1. Amplicon generation

Nucleic acids were extracted using QIAamp Viral RNA Mini Kit (Qiagen 52906) according to the manufacturer's instructions and converted to cDNA using serotype-specific primers targeting the 3′-ends of viral genomes (Table 1). Briefly, 20-μL reactions containing 10 μL nucleic acids, 1 μL 10 μM gene-specific primer, 1 μL SuperScript™ III (Invitrogen 18080-044), 4 μL 5X RT buffer, 1 μL 0.1 M DTT, 1 μL 10 mM of each dNTPs, 1 μL RNaseOUT™ (Invitrogen 10777-019), and 1 μL nuclease-free water were incubated at 50 °C for 60 min and then terminated at 75 °C for 15 min. Amplicons were generated in 50-μL reactions containing 2 μL DENV cDNA, 1 μL each 10 μM forward and reverse primers, 0.5 μL Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen 11304-029), 5 μL 10X HiFi PCR Buffer, 1.5 μL 50 mM MgSO4, 1 μL 10 mM dNTPs, 10 μL 5X Q solution (supplied withTaq DNA Polymerase, Qiagen 201203), and 28 μL nuclease-free water. The cycling conditions consisted of 40 cycles of denaturation (94 °C for 30 s), annealing (47 °C (DENV1), 53 °C (DENV2), 42 °C (DENV3) and 52 °C (DENV4) for 30 s), and extension (72 °C for 3 min), with a final extension at 72 °C for 5 min. Amplicons were visualized by agarose gel electrophoresis and purified either directly or by gel extraction using QIAquick Gel Extraction Kits (Qiagen 28706).

2.2. Sequencing

Prior to library preparation, overlapping amplicons were quantified by NanoDrop 1000 (Thermo-Fisher) and pooled in equimolar amounts in order to cover the length of each genome. Pools were used to prepare sequencing libraries optimized for both PGM and MiSeq sequencing platforms, according to the manufacturer's instructions. Briefly, PGM libraries were constructed using Bioruptor (Diagenode) fragmented samples, Ion Xpress Barcode Adapters (Thermo-Fisher 4474517) and Ion Plus Fragment Library Kits (Thermo-Fisher 4471252). MiSeq libraries were constructed using enzymatically-fragmented samples, Nextera DNA Indexes (Illumina FC-121-1011) and Nextera DNA Library Preparation Kits (Illumina FC-121-1030). QC was done by Bioanalyzer® (Agilent) using High Sensitivity DNA chips (Agilent 5067-4627) and by Qubit® (Thermo-Fisher) using Qubit®dsDNA HS Assay Kits (Thermo-Fisher Q32854). Prior to sequencing, barcoded libraries were pooled as recommended for each sequencing platform. Briefly, PGM libraries were diluted to 12 pM, amplified by emulsion PCR on the Ion OneTouch™ 2 Instrument (Thermo-Fisher) using Ion PGM™ Template OT2 200 Kits v2 (Thermo-Fisher 4480974), and enriched on the Ion OneTouch™ ES enrichment System (Thermo-Fisher), according to the manufacturer's instructions. PGM libraries were sequenced on the Ion PGM platform (Thermo-Fisher) using Ion PGM™ sequencing 200 kits v2 (Thermo-Fisher 4482006) and Ion 314™ Chips (Thermo-Fisher 4482261). MiSeq libraries were diluted to 12 pM and sequenced on the MiSeq platform (Illumina) using MiSeq v3 600 cycle kits (Illumina MS-102-3003). Bioinformatics processing was carried out using the EDGE platform (Lo, personal communication;) and custom scripts.

3. Results

3.1. Primer design

Sequences of all primers used for targeted amplification of DENV1-4 are listed in Table 1. The primers for each serotype were designed to share annealing temperatures so that they could be used interchangeably within each set. The primer sets for DENV1, 2 and 3 consist of 20 primers each with an annealing temperature of 47 °C, 53 °C and 42 °C, respectively; the DENV4 set consists of 18 primers with an annealing temperature of 52 °C. To ensure that each set would amplify South American DENV strains we first downloaded representative full genome sequences for DENVs that have circulated in the region since 2000 (Table 2). Specifically, 81 representative complete genomes (23 of DENV1, 18 of DENV2, 29 of DENV3 and 11 of DENV4) from Argentina, Brazil, Colombia, Paraguay, Peru and Venezuela available from public databases were used as references. These were carefully selected to maximize diversity in terms of serotype, year of isolation, and geographical origin. Previously published sets of DENV primers (Christenbury et al., 2010; Rodriguez-Roche et al., 2016) were also used to guide our design. In some cases, primers included degenerate or semi-degenerate bases. Primers were positioned to generate overlapping amplicons spanning the complete length of each DENV genome in as few steps as possible (Fig. 1). Given that amplification success is linked to target amplicon size and to template concentration, we anticipated that long amplicons from low-copy templates would be harder to generate than short amplicons from high-copy templates. Thus, our design considers both high- and low-copy amplification strategies in order to ensure full-genome amplification in a range of starting template concentrations. High-copy templates are fully covered by as few as 5 overlapping amplicons, whereas low-copy templates are fully covered by no more than 10 amplicons. Overall, target amplicon size ranges between ~1 and ~3 kb.

3.2. Targeted full-genome amplification and sequencing

To test the modifications introduced in our primers, we selected a set of 43 DENV positive samples (9 of DENV1, 14 of DENV2, 13 of DENV3 and 7 of DENV4) consisting of acute-phase sera (Table 3) collected during routine febrile surveillance programs conducted between 2000 and 2015 by investigators from the U.S. Naval Medical Research Unit No.6 in Peru and neighboring countries (Bolivia, Ecuador, Paraguay, Venezuela), with institutional review board approval from all implicated partners (proto-

Table 1
Primer sequences used for full-genome amplification of DENV1-4.
All primers, with the exception of d3a10 (*), are either new or modified for this work. Primers for each serotype share annealing temperatures, which enables interchangeable use within each set. Genome locations are reported with respect to GenBank isolates JX669464 for DENV1, KC294222 for DENV2, JX669501 for DENV3, and JN983813 for DENV4. The last primer of the 3′ end in each set (shown in red) was used for reverse transcription (note that all reverse transcription reactions were carried out at 50 °C regardless of primer annealing temperature). In the 5-fragment amplification strategy individual overlapping amplicons are named 1–5, whereas in the 10-fragment amplification strategy individual overlapping amplicons are named A-J (except for DENV4, where the last overlapping amplicon (named 5 and/or I) is shared by both the 5- and 10-fragment amplification strategies.

Serotype	Primer Name	Sequence 5′-3′	Location	10 Products	5 Products	Annealing Temperature
DENV-1	DEN1 CC AF	ACGTGGACCGACAAGAACAGTTTCG	13–37	A ~1.65Kb	1 ~2.55Kb	47 °C
	DEN1 CC 1R	CTTCCTGTTTCTTTGCATGAGCTGTC	1657–1682			
	DEN1 CC 1F	GAGCATGGAAGCTGCGTCACCAC	1010–1032	B ~1.55Kb		
	DEN1 CC BR	CTTYCCAATGGCTGCTGACAGTCTT	2539–2563			
	DENV1-LAB-5F-CC	CGCATGGGACTTCGGYTCTATAGG	2191–2213	C ~1.40Kb	2 ~2.50Kb	
	DEN1 CC 2R	TGTTCCAGTCATCAGCATCTTTCTAC	3558–3583			
	DEN1 CC 2F	ATTGCGTGACTCCYACACCCAAATG	2930–2953	D ~1.75Kb		
	DEN1 CC DR	CCTGGTGACGTGCCACATTGTGTG	4658–4681			
	DENV1-LAB-8F-CC	CAAAGAGGACTGTTGGGCAGGTCC	4600–4620	E ~1.65Kb	3 ~2.35Kb	
	DEN1 CC 3R	ATCTCCACGTCCATGTTCTCCTCC	6241–6264			
	DEN1 CC 3F	TCATGGAACTCAGGCTACGACTGG	5549–5572	F ~1.40Kb		
	DENV1-LAB-10R-CC	CTGCATAGAGRGTCCAGGCTGAAG	6933–6956			
	DENV1-LAB-12F-CC	CACAAAGAAAGACCTGGGGATTGGC	6850–6874	G ~1.50Kb	4 ~2.25Kb	
	DEN1 CC 4R	GTTCCACTGCCACATGTCTTGTTCC	8351–8375			
	DEN1 CC 4F	GAGAAACAACCAAACATGCAGTGTCG	7716–7741	H ~1.40Kb		
	DENV1-LAB-14R-CC	TTGTGCAGTCCTTCTCCTTCCACTC	9087–9111			
	DENV1-LAB-14F-CC	TACGTGTTCTAAARATGGTGGAACCAT	8058–8084	I	5 ~2.65Kb	
	DEN1 CC 5R	AGCCATAAGTTCCGACCTGTCCAC	9372–9395	~1.35Kb		
	DEN1 CC 5F	ACTGACATCATGGAGCCTGAACATG	9230–9254	J		
	DEN1 CC JR	CGTTCTGTGCCTGGAATGATGCTG	10684–10707	~1.45Kb		
DENV-2	d2s1C—CC	AGTTGTTAGTCTACGTGGACCGACA	1–25	A ~1.50Kb	1 ~2.45Kb	53 °C
	DEN2 CC 1R	AGCACCATCTCATTGAAGTCGAGG	1506–1529			
	DEN2 CC 1F	CAAACACTCCATGGTAGACAGAGG	1212–1235	B ~1.25Kb		
	d2a18-CC	TCCCRCTGCCACATTTCARTTCTTT	2455–2474			
	d2s4-CC	ACGCSAAGAAACAGGATGTYGTTGT	1669–1690	C ~1.55Kb	2 ~2.75Kb	
	DEN2 CC 2R	TCCTGCTGTTTGTGTATGGTAGCC	3196–3219			
	DEN2 CC 2F	CACATGGAAGATGGAGAAAGCCTC	3045–3068	D ~1.35Kb		
	d2a14-CC	GCCGTRATTGGTATYGATACWGGAA	4401–4425			
	d2s8-CC	ATGGGMGTGACTTAYCTTGCCCTA	3686–3704	E ~1.45Kb	3 ~2.80Kb	
	DEN2 CC 3R	CTTCTCTAACTATGGCTGGAAGG	5130–5152			
	DEN2 CC 3F	TGGTGTTGTCACAAGGAGTGGAG	4976–4998	F ~1.50Kb		
	d2a10-CC	TACGCCYTTCCRCCTGCYTCAG	6477–6497			
	d2s13-CC	GCAGACAGAARGTGGTGTTTTGATG	6193–6213	G ~1.15Kb	4 ~2.30Kb	
	DEN2 CC 4R	CATTAATACTTGAGTCACGCAGAGG	7350–7374			
	DEN2 CC 4F	TTCAAGCAAAAGCAACCAGAGAAGC	7199–7223	H ~1.25Kb		
	d2a6-CC	CATGGTAWGCCCAYGTTTTGTATGG	8468–8488			
	d2s16-CC	CAGGAAGTGGATAGAACCYTRGCA	7669–7692	I ~1.65Kb	5 ~3.05Kb	
	DEN2 CC 5R	CATTACTGTGCCTCTTGGTGTTGG	9316–9339			
	DEN2 CC 5F	CTGGTTGACAGGGAAAGAAATCTCC	8870–8893	J ~1.85Kb		
	d2a5B-CC	AGAACCTGTTGATTCAACAGCACCAT	10706–10725			
DENV-3	d3s1C—CC	AGTTGTTAGTCTACGTGGACCG	1–22	A ~1.55Kb	1 ~2.30Kb	42 °C
	DEN3 CC 1R	CATGCTTTGTTCTTCATTGTTAGC	1524–1547			
	DEN3 CC 1F	CAACATAACAACTGACTCAAGATG	1132–1155	B ~1.20Kb		
	DEN3 CC BR	CAAGTCAAGAGAACTCCTATTCC	2300–2322			
	d3s5-CC	CTGAACCTCCTTTTGGGGAAAG	2035–2053	C ~1.55Kb	2 ~2.30Kb	
	DEN3 CC 2R	CTCCATGTTATTTGCCCTGAGA	3599–3621			
	DEN3 CC 2F	AGGTGGAAGATTACGGGTTCG	2874–2894	D ~1.45Kb		
	d3a14-CC	AACTGTGATCATTAARTTGTGGGA	4334–4356			
	DEN3 CC EF	TTCTCTCCTTAGAAATGATGTACC	4180–4203	E ~1.05Kb	3 ~2.15Kb	
	DEN3 CC 3R	TCAATGCTTCTTCCATCTCAGC	5201–5222			
	DEN3 CC 3F	GAGTGGTTACAAAGAATGGTGG	4971–4992	F ~1.35Kb		
	d3a10*	GCYGCAAARTCCTTGAATTCCT	6339–6360			
	d3s13-CC	CCAGCTCTCTTTGARCCAGAAA	6032–6053	G ~1.30Kb	4 ~2.30Kb	
	DEN3 CC 4R	AGCATAACCTGTCCCAGTTGC	7318–7338			
	DEN3 CC 4F	AGATGTGGACTTGCACCCAGC	6904–6924	H ~1.45Kb		
	d3a6-CC2	CGCATTAACATGTCGAGTTCC	8342–8361			
	DEN3 CC IF	TCCACTTTCACGAAACTCCACG	8185–8206	I ~1.25Kb	5 ~2.50Kb	
	DEN3 CC 5R	TTCAGACCATAAGTTCCTACCTG	9368–9390			
	DEN3 CC 5F	GAAGATGACCTGCACAATGAGG	9191–9212	J ~1.50Kb		
	DEN1 CC JR	CGTTCTGTGCCTGGAATGATGCTG	10646–10669			
DENV-4	d4s1C—CC	AGTTGTTAGTCTGTGTGGACCGACAA	1–26	A ~1.45Kb	1 ~2.15Kb	52 °C
	DEN4 CC 1R	GTGACCTGGGAGTTATCGTGGCTG	1420–1443			
	DEN4 CC 1F	GACACCCATGCAGTAGGAAATGACAC	1377–1402	B ~0.80Kb		
	DEN4 CC BR	ACTCAAACATCTTGCCAATGGAACTC	2123–2148			
	d4s5-CC	CTCCGTGTAAAGTCCCCATAGAGATAA	1910–1933	C ~1.75Kb	2 ~3.05Kb	
	DEN4 CC 2R	CATGATGAGGGCTCGTAGCAAGTCC	3632–3656			
	DEN4 CC 2F	AGAAGATGGGTGCTGGTATGGGATG	3398–3422	D ~1.55Kb		
	DEN4 CC DR	ACTCCATTTCCATAGAGTCCGATGAC	4959–4984			
	DEN4 CC EF	GACTGGGAGATTGGAGCCATCTTG	4706–4729	E ~1.50Kb	3 ~2.60Kb	

Table 1 (*Continued*)

Serotype	Primer Name	Sequence 5′-3′	Location	10 Products	5 Products	Annealing Temperature
	DEN4 CC 3R	TCTTTGTAAGAAATGCCAGCAGAAGC	6175–6200			
	DEN4 CC 3F	CTCAAGCCAGTTATCCTAACAGATGG	5809–5834	F ~1.50Kb		
	d4a8-CC	CATGACYTGCCCTAATTGCTTTTCAAA	7289–7311			
	d4s16-CC	GAAAAGRACAGCTGCTGGRATCATGA	7205–7226	G ~1.40Kb	4 ~2.45Kb	
	DEN4 CC 4R	TCTGTCATGGCTAACTGAGTCACCA	8573–8597			
	DEN4 CC 4F	TTCACAACAAGGCATAGGAAACCCAC	8290–8315	H ~1.40Kb		
	d4a3-CC	TGTGRAARTGGTGGGAGCAAAAAGGA	9668–9689			
	d4s21-CC	GAAAGAYATYCCGCAGTGGGAACCA	9610–9631	I ~1.00Kb	5 ~1.00Kb	
	d4a5B-CC	AGAACCTGTTGGATCAACAACACCAAT	10630–10650			

cols NMRCD.2000.0006, NMRCD.2000.0008, NMRCD.2001.0002, NMRCD.2005.0008, NMRCD.2010.0010, NAMRU6.2014.0028, NAMRU6.2015.0010). In two additional instances for DENV1, we also used infected culture supernatants derived from samples. To address the issue of template concentration, DENV genomes were initially quantified using a previously described qRT-PCR protocol that targets a conserved region in all DENV serotypes (McAvin et al., 2005), and thus, can be used to compare DENV-specific RNA concentrations across serotypes. McAvin cycle threshold (Ct) values for the samples tested ranged between 18 and 37. As expected, samples with high concentration of DENV template (Ct values lower than 26) were easily amplified (Fig. 1). In these cases, complete genome coverage was achieved with as few as 5 overlapping amplicons. As the concentration of DENV template decreased (Ct values between 26 and 29), amplification efficiency for large amplicons also decreased. In these cases of medium and low concentration of DENV template, complete genome coverage was achieved by increasing the number of overlapping amplicons up to 10 (Fig. 1). In the case of extremely low concentrations of DENV template (Ct values of 29 and higher), at least some overlapping fragments could be generated using the 10-fragment amplification strategy (not shown). However, we consider that above Ct values of 29, DENV samples are sub-optimal for the purposes of amplicon sequencing by NGS. Although it is possible that further optimization of the PCR conditions could improve the genome coverage in samples of extremely low concentration of DENV template, such efforts were beyond the scope of this work.

Once generated by PCR, amplicons representing the total length of each tested DENV genome were pooled together and sequenced using both Illumina MiSeq and Ion Torrent PGM sequencing platforms at different depths of coverage (Fig. 2). Our targeted amplification strategy results in full-genome coverage of all DENV serotypes for every overlapping fragment in each sample. Sequencing statistics for representative samples are shown in Table 4. Between 93% and 99+% of all reads in each sample corresponded to DENV sequences, indicating this is an effective amplification and sequencing strategy for DENVs that have circulated in South America since the year 2000.

4. Discussion

Amplicon sequencing by NGS is a relatively simple technique, at least in theory. In practice however, the generation of overlapping amplicons that cover the complete length of a particular genome of interest can be complicated by a number of factors, including template concentration, sequence diversity, primer specificity, and PCR amplification efficiency. In the current study we report improvements to existing full-genome amplification strategies for DENVs (Christenbury et al., 2010). These changes should benefit those attempting to generate complete DENV genomes in a quick and cost-effective manner, particularly for DENV strains that have circulated in South America since 2000.

The first modification we have implemented is the inclusion of an initial serotype-independent relative quantification of DENV genome copies using qPCR (McAvin et al., 2005). The advantages of using viral load, as measured by qPCR, to guarantee successful amplification of DENVs from acute serum samples has been previously documented (Rodriguez-Roche et al., 2012). Because this assay targets a conserved region in all DENV genomes it can be used to compare DENV-specific genome copies across serotypes. Our results clearly indicate that DENV positive samples showing Ct values of up to 29 are suitable starting material for this amplicon sequencing strategy. In contrast, DENV positive samples with Ct values higher than 29 are not suitable, even for the 10-fragment amplification strategy developed for "low-copy" templates. Despite being DENV positive, such samples contain sub-threshold levels of DENV RNA that render them sub-optimal because they only lead to partial genome amplification. By establishing the relative concentration of starting material at the outset of the amplicon sequencing strategy, the researcher is able to focus on samples that have a high probability of success and avoid potentially costly and time-consuming preparation of samples that are a dead-end. A possible workaround for "low-copy" templates is to passage viruses through an established tissue culture line, like C6/36 cells (Singh and Paul, 1969; Tesh, 1979). However, the drawback to this approach is that viruses may mutate and adapt to the host cell type (Villordo et al., 2015). Thus, it is always preferable to generate amplicon-sequencing data from samples that have not been subjected to laboratory culture (Rodriguez-Roche et al., 2012). This is particularly important in downstream applications that assess viral evolution, since by avoiding passage through a laboratory culture one also avoids the introduction of mutations that are not representative of viruses circulating in the wild. In this study, we have tested a total of 46 samples, representing all four DENV serotypes. All samples reported here were human serum samples, which is significant for two reasons. First, serum samples contain viruses that have not been subject to passage through laboratory cell lines, and thus, they represent wild-type viruses that have actually circulated in nature. Second, serum samples tend to have much lower viral RNA concentrations that those found of cultured samples, thus a methodology that can successfully generate full genomes from wild-type viruses in serum samples is robust. In some instances we have also sequenced cultured samples, indicating the primer sets described can be used both for "easy-to-sequence" cultured samples and for "high-value" wild-type samples.

The second modification we have implemented is a dual approach for high- and low-copy templates. High-copy templates (with Ct values as high as 26) can be easily covered in full by as few as 5 overlapping amplicons, whereas low-copy templates (with Ct values between 26 and 29) can be covered by no more than 10. Given that the relative concentration of starting template in the sample is determined before amplicon sequencing takes place, the researcher again has an added level of control that informs strategy and enables selection of the most appropriate amplification strategy. Perhaps this flexibility is not very significant in the context of a handful of genomes, but when hundreds or thousands of full genomes need to be generated accurately, quickly, and consistently, tailored strategies that increase the possibilities of success

Table 2
DENV complete genome sequences used as references for primer design.
Sequences were carefully selected to maximize diversity in terms of serotype, year of isolation, and country of origin.

Serotype	Accession #	Year	Location	Length (bp)
DENV-1	FJ850070	2000	Brazil	10677
	GU131832	2000	Venezuela	10460
	FJ850073	2001	Brazil	10692
	GU131948	2001	Colombia	10627
	GU131834	2001	Venezuela	10468
	FJ850075	2002	Brazil	10683
	FJ850077	2003	Brazil	10684
	FJ850081	2004	Brazil	10688
	FJ639796	2004	Venezuela	10676
	FJ850084	2005	Brazil	10686
	KJ189304	2005	Colombia	10447
	FJ639812	2005	Venezuela	10640
	FJ850087	2006	Brazil	10690
	GQ868563	2006	Colombia	10661
	HQ332182	2006	Venezuela	10735
	FJ850090	2007	Brazil	10673
	GQ868567	2007	Colombia	10690
	HQ332183	2007	Venezuela	10735
	FJ850093	2008	Brazil	10690
	GQ868570	2008	Colombia	10674
	FJ850104	2008	Venezuela	10690
	KC692513	2010	Argentina	10735
	JX669462	2010	Brazil	10759
DENV-2	FJ898466	2000	Venezuela	10679
	FJ850074	2001	Brazil	10678
	FJ850076	2002	Brazil	10663
	KC294222	2002	Peru	10723
	FJ850078	2003	Brazil	10667
	FJ850082	2004	Brazil	10679
	FJ639788	2004	Venezuela	10678
	FJ850085	2005	Brazil	10677
	FJ850088	2006	Brazil	10667
	FJ850091	2007	Brazil	10678
	GQ868558	2007	Colombia	10679
	KC294200	2007	Peru	10724
	EU482607	2007	Venezuela	10679
	GQ199890	2008	Brazil	10679
	FJ850107	2008	Venezuela	10667
	KC294201	2009	Peru	10723
	KC294223	2010	Peru	10723
	KC294218	2011	Peru	10723
DENV-3	FJ898457	2000	Ecuador	10663
	FJ898468	2000	Venezuela	10663
	FJ913015	2001	Brazil	10663
	GU131950	2001	Colombia	10616
	GQ252678	2001	Venezuela	10641
	GQ868571	2002	Colombia	10663
	FJ898458	2002	Peru	10663
	FJ898471	2002	Venezuela	10663
	FJ898447	2003	Brazil	10663
	GU131952	2003	Colombia	10638
	FJ898473	2003	Venezuela	10663
	FJ850083	2004	Brazil	10663
	GU131953	2004	Colombia	10588
	KJ189266	2004	Peru	10696
	JX669501	2005	Brazil	10707
	KJ189299	2005	Peru	10692
	GU131865	2006	Brazil	10501
	GU131954	2006	Colombia	10525
	KJ189297	2006	Peru	10692
	FJ639825	2006	Venezuela	10648
	FJ850092	2007	Brazil	10648
	GQ868578	2007	Colombia	10659
	HQ235027	2007	Paraguay	10707
	KJ643590	2007	Peru	10692
	FJ850111	2007	Venezuela	10663
	FJ850094	2008	Brazil	10662
	KJ189301	2008	Peru	10693
	FJ639827	2008	Venezuela	10635
	JF808120	2009	Brazil	10707
DENV-4	FJ639748	2000	Venezuela	10551
	GQ868582	2001	Colombia	10606
	FJ639773	2001	Venezuela	10606
	GQ868584	2004	Colombia	10552
	GQ868585	2005	Colombia	10606

Table 2 (*Continued*)

Serotype	Accession #	Year	Location	Length (bp)
	JN819406	2006	Venezuela	10357
	GQ868643	2007	Venezuela	10593
	FJ882592	2008	Venezuela	10606
	JN983813	2010	Brazil	10649
	JQ513335	2011	Brazil	10604
	KJ596671	2012	Brazil	10649

Table 3
Samples used for protocol validation.
Most samples are human serum containing wild-type DENVs. In two instances for DENV1, supernatants from cultured serum samples were also used.

Serotype	Sample Code	Year	Location	Sample Type	Ct value
DENV-1	OBS9842	2000	Peru	culture	22.03
	FSB2837	2009	Bolivia	culture	12.63
	FCB0983	2009	Bolivia	serum	37.27
	FSE7098	2010	Ecuador	serum	32.39
	FPA2544	2010	Paraguay	serum	31.59
	FSP1646	2010	Peru	serum	26.44
	FYA00055	2011	Paraguay	serum	28.61
	FEG00002	2012	Ecuador	serum	35.07
	FPJ00740	2014	Peru	serum	24.68
	FYC00721	2015	Paraguay	serum	22.93
	FPJ00811	2015	Peru	serum	21.45
DENV-2	FVB2118	2011	Bolivia	serum	20.95
	FVB2121	2011	Bolivia	serum	26.11
	FPI00403	2011	Peru	serum	22.1
	FEG00005	2012	Ecuador	serum	32.34
	FPI04256	2012	Peru	serum	23.30
	FYA00328	2013	Paraguay	serum	23.61
	FPI06921	2013	Peru	serum	22.60
	FPY00498	2014	Peru	serum	20.69
	FPU00873	2014	Peru	serum	32.32
	FPI10982	2014	Peru	serum	25.54
	FPM00818	2014	Peru	serum	28.64
	FPI13322	2015	Peru	serum	22.41
	FPP02381	2015	Peru	serum	23.43
	FVM00186	2015	Venezuela	serum	26.17
DENV-3	IQD3765	2002	Peru	serum	20.99
	FSB0417	2003	Bolivia	serum	25.28
	FSL1212	2003	Peru	serum	19.35
	FSE1036	2004	Ecuador	serum	22.36
	FSB0879	2005	Bolivia	serum	28.56
	FSB0931	2005	Bolivia	serum	22.87
	IQE2562	2005	Peru	serum	18.64
	IQE3021	2006	Peru	serum	18.53
	FCB0589	2007	Bolivia	serum	23.82
	FPA0171	2007	Paraguay	serum	28.58
	FPA0313	2007	Paraguay	serum	23.16
	FPI13521	2015	Peru	serum	20.89
	FPP02377	2015	Peru	serum	18.36
DENV-4	FSL3797	2008	Peru	serum	26.73
	FSL3887	2009	Peru	serum	26.37
	FSP1830	2010	Peru	serum	27.81
	FMD3542	2010	Peru	serum	27.32
	FPM00691	2013	Peru	serum	28.24
	FPM00697	2013	Peru	serum	28.03
	FVM00212	2015	Venezuela	serum	21.55

Table 4
Sequencing statistics for sequenced DENV samples.
Following PCR amplification, overlapping fragments were pooled in roughly equimolar amounts in order to cover the length of each genome. Pools were prepared into both Ion and Illumina libraries and sequenced using PGM and MiSeq platforms, respectively, at different depths of coverage.

		Ion Torrent Data				MiSeq Data			
Sample	Serotype	Total Reads	Total Bases	DENV Reads	% DENV Reads	Total Reads	Total Bases	DENV Reads	%DENV Reads
1	DENV-1	581	90,570	548	94.32%	582,328	174,440,938	581,361	99.83%
2	DENV-1	4,136	702,875	3,960	95.74%	841,056	252,934,514	815,656	96.98%
3	DENV-2	500	54,731	466	93.20%	444,294	293,192,662	424,307	95.50%
4	DENV-2	6,179	908,554	5,944	96.20%	1,353,824	405,714,890	1,343,717	99.25%
5	DENV-2	82,997	11,132,821	79,689	96.01%	946,264	283,873,702	938,124	99.14%
6	DENV-3	3,603	560,731	3,545	98.39%	926,635	277,773,636	909,683	98.17%
7	DENV-4	3,592	568,281	3,555	98.97%	901,131	269,778,474	898,848	99.75%

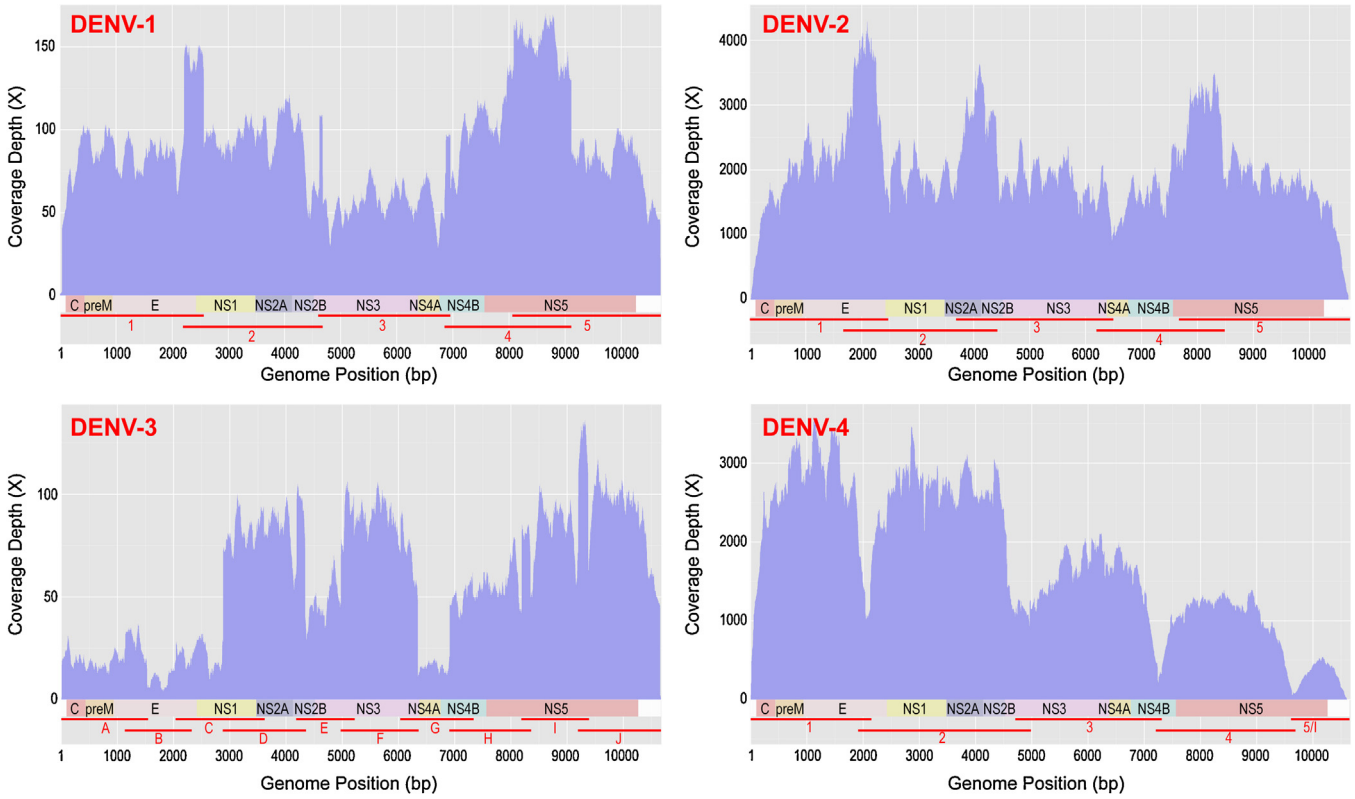

Fig. 2. Representative depth of coverage plots for DENV1-4.
DENV1 and 3 were sequenced on the Ion Torrent platform at a low depth of coverage (50X on average), whereas DENV2 and 4 were sequenced on the MiSeq platform at a high depth of coverage (1,500X on average). DENV1, 2 and 4 coverage plots were derived from 5 "long" overlapping fragments (shown in red with labels 1–5), whereas the DENV3 coverage plot was derived from 10 "short" overlapping fragments (labels A–J). In the case of DENV4, the last overlapping fragment is the same in both the high and low copy amplification strategies (label 5/I).

while reducing costs, time and handling, can have a very significant positive impact.

The final modification we have implemented is a complete redesign of primer sequences with the dual purpose of ensuring successful amplification of DENV strains that have circulated in South America, and of normalizing annealing temperatures for all primers within each serotype set. South American DENV strains are often poorly amplified, if amplified at all, by existing primer sets (Christenbury et al., 2010; Rodriguez-Roche et al., 2016) because those primers have been developed for DENV strains that have primarily circulated in Asia. In addition, some of these primer sets have been developed using clinical isolates where specific serotypes predominated over others (introducing bias towards the over-represented serotype(s)), or using viruses that have been passaged through laboratory cell lines (introducing bias towards laboratory-adjusted strains). The primer sets described here have been validated using balanced samples sets composed of diverse wild-type DENV genomes derived from human serum samples representing all four DENV serotypes and a variety of geographical regions in South America over a 15-year period. These primer sets have the added benefit of sharing annealing temperatures, which should streamline handling by enabling interchangeable use at a single annealing temperature within each set. Once again, the aim has been to simplify workflows as much as possible in order to increase processing output while maintaining or improving PCR amplification efficiency for South American strains that were previously difficult or impossible to sequence. This added efficiency should be particularly relevant during outbreaks, when a laboratory may be required to process increased numbers of samples in a short period of time.

DENVs are known for their genetic variation within serotype groups and geographical regions (Rico-Hesse, 2003; Rodriguez-Roche et al., 2012; Weaver and Vasilakis, 2009). Our targeted amplicon-sequencing strategy aims to normalize and facilitate workflows that can be cumbersome, expensive, and time-consuming, particularly if they are attempted on samples with sub-optimal concentrations of viral genomes that fail to yield complete genome sequences. We have shown that the modifications implemented result in successful generation of overlapping amplicons that can be processed using different library constructions approaches and NGS platforms. Specifically, we have processed a diverse group of wild-type DENV sequences derived from serum using both mechanical and enzymatic library construction approaches, two separate sequencing platforms (Ion Torrent and MiSeq), and two different depths of coverage (DENV1 and DENV3 at a low depth of 50X on average, and DENV2 and DENV4 at a high depth of 1,500X on average). Nevertheless, we have avoided a detailed discussion of sequencing statistics, including depth of coverage and error rates for example, in order to highlight the flexibility of this approach, which allows the researcher to control different aspects of sequencing in order to suit a variety of applications that may require dramatically different levels of depth of coverage. The main advantage of this sequencing strategy is that the majority of sequencing reads obtained (97% on average) correspond to DENVs. This has the added benefit of simplifying downstream bioinformatics processing because a reduced number of "contaminating" reads is an advantage over non-targeted methods like shotgun sequencing, where a large number of host-specific reads often impede adequate representation of the complete viral genome of interest (Yozwiak et al., 2012). We believe this methodology has a number of

downstream applications, including epidemiological surveillance, studies of quasi-species and dominant genomes, and studies of DENV evolution where measurements of intra-host diversity can be used to identify evolutionary forces (e.g.: natural selection vs. genetic drift) that act on viral populations, particularly in the case of DENV strains like the ones that have circulated in South America over the last 15 years.

5. Disclaimers

The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. We are employees of the U.S. Government. The work was prepared as part of our official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines U.S. Government work as a work prepared by military service members or employees of the U.S. Government as part of that person's official duties. This work was supported by an award to ML (work unit number 847705.82000.25GB.B0016, Promis ID# MLeguia-PO166-14, for FY2014) from the Global Emerging Infections Surveillance and Response System (GEIS) of the Armed Forces Health Surveillance Center (AFHSC). LL received support from the French Government's Investissement d'Avenir program Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases grant ANR-10-LABX-62-IBEID, the City of Paris Emergence(s) program in Biomedical Research, and National Institutes of Health grant 1P01AI098670-01A1. The study protocol was approved by NAMRU-6's IRB in compliance with all applicable Federal regulations governing the protection of human subjects.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We are deeply grateful to Dr. Julia Sonia Ampuero for enabling access to DENV-positive human serum samples gathered throughout Latin America. We also thank local and international collaborators who generously provide samples as part of joint, ongoing surveillance efforts, in particular the Instituto Nacional de Salud (INS) – Ministerio de Salud del Perú; Dr. Yelin Roca Sánchez at the Centro Nacional de Enfermedades Tropicales (CENETROP) in Bolivia; Dr. Nicolás Aguayo at ONG Rayos de Sol – Ministerio de Salud Pública y Binenestar Social del Paraguay; Dr. Guillermo Comach at Laboratorio Regional de Diagnóstico e Investigación del Dengue y otras Enfermedades Virales (LARDIDEV) – Instituto de Investigaciones Biomédicas de la Universidad de Carabobo (BIOMED-UC) in Venezuela. Finally, we are grateful to Dr. Rosmari Rodríguez-Roche for providing some of the modified primer sequences and Sebastian Lequime for assistance with primer redesign.

References

Aquino, J.D., Tang, W.F., Ishii, R., Ono, T., Eshita, Y., Aono, H., Makino, Y., 2008. Molecular epidemiology of dengue virus serotypes 2 and 3 in Paraguay during 2001–2006: the association of viral clade introductions with shifting serotype dominance. Virus Res. 137, 266–270.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, T., Wint, G.R., Simmons, C.P., Scott, T.W., Farrar, J.J., Hay, S.I., 2013. The global distribution and burden of dengue. Nature 496, 504–507.

Cafferata, M.L., Bardach, A., Rey-Ares, L., Alcaraz, A., Cormick, G., Gibbons, L., Romano, M., Cesaroni, S., Ruvinsky, S., 2016. Dengue epidemiology and burden of disease in Latin America and the Caribbean: a systematic review of the literature and meta-analysis. Value in Health Regional Issues 2, 347–356.

Christenbury, J.G., Aw, P.P., Ong, S.H., Schreiber, M.J., Chow, A., Gubler, D.J., Vasudevan, S.G., Ooi, E.E., Hibberd, M.L., 2010. A method for full genome sequencing of all four serotypes of the dengue virus. J. Virol. Methods 169, 202–206.

Cruz, C.D., Forshey, B.M., Juarez, D.S., Guevara, C., Leguia, M., Kochel, T.J., Halsey, E.S., 2013. Molecular epidemiology of American/Asian genotype DENV-2 in Peru. Infect. Genet. Evol. 18, 220–228.

Dettogni, R.S., Louro, I.D., 2011. Phylogenetic characterization of dengue virus type 2 in Espirito Santo, Brazil. Mol. Biol. Rep. 39, 71–80.

Forshey, B.M., Morrison, A.C., Cruz, C., Rocha, C., Vilcarromero, S., Guevara, C., Camacho, D.E., Alava, A., Madrid, C., Beingolea, L., Suarez, V., Comach, G., Kochel, T.J., 2009. Dengue virus serotype 4, Northeastern Peru, 2008. Emerg. Infect. Dis. 15, 1815–1818.

Kochel, T., Aguilar, P., Felices, V., Comach, G., Cruz, C., Alava, A., Vargas, J., Olson, J., Blair, P., 2008. Molecular epidemiology of dengue virus type 3 in Northern South America: 2000–2005. Infect. Genet. Evol. 8, 682–688.

Lequime, S., Fontaine, A., Ar Gouilh, M., Moltini-Conclois, I., Lambrechts, L., 2016. Genetic drift, purifying selection and vector genotype shape dengue virus intra-host genetic diversity in mosquitoes. PLoS Genet. 12 (6), http://dx.doi.org/10.1371/journal.pgen.1006111, e1006111.

McAvin, J.C., Escamilla, E.M., Blow, J.A., Turell, M.J., Quintana, M., Bowles, D.E., Swaby, J.A., Barnes, W.J., Huff, W.B., Lohman, K.L., Atchley, D.H., Hickman, J.R., Niemeyer, D.M., 2005. Rapid identification of dengue virus by reverse transcription-polymerase chain reaction using field-deployable instrumentation. Mil. Med. 170, 1053–1059.

Mendez, J.A., Usme-Ciro, J.A., Domingo, C., Rey, G.J., Sanchez, J.A., Tenorio, A., Gallego-Gomez, J.C., 2012. Phylogenetic reconstruction of dengue virus type 2 in Colombia. Virol. J. 9, 64.

Oliveira, M.F., Galvao Araujo, J.M., Ferreira Jr., O.C., Ferreira, D.F., Lima, D.B., Santos, F.B., Schatzmayr, H.G., Tanuri, A., Ribeiro Nogueira, R.M., 2010. Two lineages of dengue virus type 2, Brazil. Emerg. Infect. Dis. 16, 576–578.

Parameswaran, P., Charlebois, P., Tellez, Y., Nunez, A., Ryan, E.M., Malboeuf, C.M., Levin, J.Z., Lennon, N.J., Balmaseda, A., Harris, E., Henn, M.R., 2012. Genome-wide patterns of intrahuman dengue virus diversity reveal associations with viral phylogenetic clade and interhost diversity. J. Virol. 86, 8546–8558.

Rico-Hesse, R., 2003. Microevolution and virulence of dengue viruses. Adv. Virus Res. 59, 315–341.

Roca, Y., Baronti, C., Revollo, R.J., Cook, S., Loayza, R., Ninove, L., Fernandez, R.T., Flores, J.V., Herve, J.P., de Lamballerie, X., 2009. Molecular epidemiological analysis of dengue fever in Bolivia from 1998 to 2008. Vector Borne Zoonotic Dis. 9, 337–344.

Rodriguez-Roche, R., Villegas, E., Cook, S., Poh Kim, P.A., Hinojosa, Y., Rosario, D., Villalobos, I., Bendezu, H., Hibberd, M.L., Guzman, M.G., 2012. Population structure of the dengue viruses, Aragua, Venezuela, 2006–2007: insights into dengue evolution under hyperendemic transmission. Infect. Genet. Evol. 12, 332–344.

Rodriguez-Roche, R., Blanc, H., Borderia, A.V., Diaz, G., Henningsson, R., Gonzalez, D., Santana, E., Alvarez, M., Castro, O., Fontes, M., Vignuzzi, M., Guzman, M.G., 2016. Increasing clinical severity during a dengue virus type 3 Cuban epidemic: deep sequencing of evolving viral populations. J. Virol. 90, 4320–4333.

Romano, C.M., de Matos, A.M., Araujo, E.S., Villas-Boas, L.S., da Silva, W.C., Oliveira, O.M., Carvalho, K.I., de Souza, A.C., Rodrigues, C.L., Levi, J.E., Kallas, E.G., Pannuti, C.S., 2010. Characterization of dengue virus type 2: new insights on the 2010 Brazilian epidemic. PLoS One 5, e11811.

Romano, C.M., Lauck, M., Salvador, F.S., Lima, C.R., Villas-Boas, L.S., Araujo, E.S., Levi, J.E., Pannuti, C.S., O'Connor, D., Kallas, E.G., 2013. Inter- and intra-host viral diversity in a large seasonal DENV2 outbreak. PLoS One 8, e70318.

Sanger, F., Coulson, A.R., 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. 94, 441–448.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

Sanofi-Pasteur, 2015. DENGVAXIA®, World's first dengue vaccine, Approved In Mexico, accessed March 2016 http://www.sanofipasteur.com/en/articles/dengvaxia-world-s-first-dengue-vaccine-approved-in-mexico.aspx.

Sim, S., Aw, P.P., Wilm, A., Teoh, G., Hue, K.D., Nguyen, N.M., Nagarajan, N., Simmons, C.P., Hibberd, M.L., 2015. Tracking dengue virus intra-host genetic diversity during human-to-mosquito transmission. PLoS Negl. Trop. Dis. 9, e0004052.

Singh, K.R., Paul, S.D., 1969. Isolation of dengue viruses in *Aedes albopictus* cell cultures. Bull. World Health Organ. 40, 982–983.

Tesh, R.B., 1979. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am. J. Trop. Med. Hyg. 28, 1053–1059.

Uzcategui, N.Y., Camacho, D., Comach, G., Cuello de Uzcategui, R., Holmes, E.C., Gould, E.A., 2001. Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. J. Gen. Virol. 82, 2945–2953.

Uzcategui, N.Y., Comach, G., Camacho, D., Salcedo, M., Cabello de Quintana, M., Jimenez, M., Sierra, G., Cuello de Uzcategui, R., James, W.S., Turner, S., Holmes, E.C., Gould, E.A., 2003. Molecular epidemiology of dengue virus type 3 in Venezuela. J. Gen. Virol. 84, 1569–1575.

Villordo, S.M., Filomatori, C.V., Sanchez-Vargas, I., Blair, C.D., Gamarnik, A.V., 2015. dengue virus RNA structure specialization facilitates host adaptation. PLoS Pathog. 11, e1004604.

WHO, 2015. Dengue and severe dengue, Last accessed March 2016 http://www.who.int/mediacentre/factsheets/fs117/en/.
Weaver, S.C., Vasilakis, N., 2009. Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect. Genet. Evol. 9, 523–540.
Yozwiak, N.L., Skewes-Cox, P., Stenglein, M.D., Balmaseda, A., Harris, E., DeRisi, J.L., 2012. Virus identification in unknown tropical febrile illness cases using deep sequencing. PLoS Negl. Trop. Dis. 6, e1485.Zhao, H., Zhao, L., Jiang, T., Li, X., Fan, H., Hong, W., Zhang, Y., Zhu, Q., Ye, Q., Tong, Y., Cao, W., Zhang, F., Qin, C., 2014. Isolation and characterization of dengue virus serotype 2 from the large dengue outbreak in Guangdong, China in 2014. Sci. China Life Sci. 57, 1149–1155.