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A two-plasmid strategy for engineering a dengue virus type 3 infectious clone from primary Brazilian isolate

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ABSTRACT

Dengue infections represent one of the most prevalent arthropod-borne diseases worldwide, causing a wide spectrum of clinical outcomes. Engineered infectious clone is an important tool to study Dengue virus (DENV) biology. Functional full-length cDNA clones have been constructed for many positive-strand RNA viruses and have provided valuable tools for studying the molecular mechanisms involved in viral genome replication, virion assembly, virus pathogenesis and vaccine development. We report herein the successful development of an infectious clone from a primary Brazilian isolate of dengue virus 3 (DENV3) of the genotype III. Using a two-plasmid strategy, DENV3 genome was divided in two parts and cloned separately into a yeast-bacteria shuttle vector. All plasmids were assembled in yeast by homologous recombination technique and a full-length template for transcription was obtained by *in vitro* ligation of the two parts of the genome. Transcript-derived DENV3 is infectious upon transfection into BHK-21 cells and *in vitro* characterization confirmed its identity. Growth kinetics of transcript-derived DENV3 was indistinguishable from wild type DENV3. This system is a powerful tool that will help shed light on molecular features of DENV biology, as the relationship of specific mutations and DENV pathogenesis.

Key words: reverse genetics, dengue virus, molecular cloning, infectious clone.

INTRODUCTION

Dengue viral infections are a major global health issue with estimates suggesting that 50-100 million individuals are infected annually (Kyle and Harris 2008, Monath 1994). With no vaccine or antiviral therapy available, Dengue virus (DENV) is responsible for a wide spectrum of clinical outcomes, raging from asymptomatic infections to undifferentiated fever, classical dengue fever (DF) or dengue hemorrhagic fever/dengue shock syndrome (DHS/DSS). Four serotypes (DENV1-4) have been identified and are transmitted to humans by *Aedes* spp. mosquitoes (Galler et al. 2011, Halstead 2007, Noble et al. 2010).

DENV belongs to the *Flavivirus* genus in the *Flaviviridae* family of enveloped viruses. Other members in this genus include yellow

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fever virus (YFV), West Nile virus (WNV), tick-born encephalitis virus (TBEV) and Japanese encephalitis virus (JEV). The flavivirus genome is organized as a single-stranded positive sense RNA molecule of approximately 11 kb. It consists of a 5' type 1,7-methyl-guanosine cap structure followed by a 5' untranslated region (5'-UTR), a single open reading frame (ORF) and a non-polyadenilated 3'-UTR (Chambers et al. 1990, Clyde et al. 2006). The virus genomic RNA is translated monocistronically into a polyprotein that is co- and posttranslationally processed by both viral and cellular proteases into three structural (C, prM and E) and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) proteins (Lindenbach et al. 2007).

Many advances in flavivirus biology have been facilitated by the use of reverse genetics, a powerful method that allows directed genetic manipulation of an RNA virus (Alvarez et al. 2008, Laurent-Rolle et al. 2010, Leung et al. 2008, McElroy et al. 2006, Ruggli and Rice 1999). Several infectious cDNA clones have been described for flaviviruses: YFV (Bredenbeek et al. 2003, Rice et al. 1989), WNV (Yamshchikov et al. 2001), JEV (Pu et al. 2011, Sumiyoshi et al. 1992, Yun et al. 2003), DENV1 (Puri et al. 2000, Suzuki et al. 2007), DENV2 (Kapoor et al. 1995, Polo et al. 1997, Pu et al. 2011), DENV3 (Blaney et al. 2004, Messer et al. 2012) and DENV4 (Lai et al. 1991). Subsequently, self-replicating RNAs termed replicons were also developed (Jones et al. 2005, Khromykh and Westaway 1997). Those subgenomic replicons carry a deletion in the structural coding region of the virus genome, making them unable to assemble virion particles on their own while remaining fully replication-competent. Uncoupling RNA replication from virion assembly and maturation allows the direct identification of the role of nonstructural proteins in virus pathogenesis (Hershkovitz et al. 2008, Jones et al. 2005, Khromykh and Westaway 1997). Yet, engineering flavivirus genomes particularly full-length infectious clones remain a difficult challenge.

The main obstacle to the development of these systems is an intrinsic instability of flavivirus full-length cDNA clones propagated in *Escherichia coli* (Polo et al. 1997, Ruggli and Rice 1999). The reasons of this toxicity are not completely understood, but some reports have suggested that the genome sequence at the E/NS1 region or the expression of a deleterious product from it causes instability in *E. coli* (Blaney et al. 2004, Polo et al. 1997). Distinct approaches have been assessed to overcome genome instability, including (i) the cloning of the genome in separate fragments and recovering a full-length infectious clone by *in vitro* ligation (Kapoor et al. 1995, Messer et al. 2012, Sumiyoshi et al. 1992); (ii) the use of low-copy number vectors (Bredenbeek et al. 2003) or (iii) Bacterial Artificial Chromosomes (BACs) (Suzuki et al. 2007); (iv) the use of different *E. coli* strains (Bredenbeek et al. 2003, Sriburi et al. 2001) or (v) the cloning in yeast cells (Polo et al. 1997, Puri et al. 2000).

In the present study, we describe the successful construction of a DENV3 infectious clone from a primary isolate from Recife, Brazil. We overcame the genome instability of DENV3 by splitting the genome in two fragments and cloning them separately in yeast. A full-length DENV3 infectious clone was then restored by *in vitro* ligation.

MATERIALS AND METHODS

Cell Lines, Yeast and DENV3 Strain

BHK-21 cells were propagated at 37 °C in a humidified incubator with 5% CO_2 in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS). Mosquito C6/36 cells were cultured at 30 °C in Leibovitz's medium (L-15) with 5% FBS. Both growth media were supplemented with 1% antibiotic solution (containing penicillin and streptomycin). *Saccharomyces cerevisiae* RFY206 (*MATa trp1Δ::hisG his3Δ200 ura3-52 lys2Δ201 leu2-3*) strain (Finley and

Brent 1994) was grown in YPD media and made competent by lithium acetate treatment (Sambrook and Russell 2001). 95016/BR-PE/02 strain is a DENV3 clinical sample isolated in Recife, Pernambuco, Brazil. Virus stocks were produced in mosquito C6/36 cell cultures and stored at -70 °C.

Plasmid Constructions

DENV3 genotype III genome was split in two parts and cloned separately in a plasmid vector (Fig. 1). All subgenomic fragments were amplified by PCR with overlapping sequences at their extremities from independent isolated cDNA clones using the KlenTaq LA polymerase mix (Clontech), a high fidelity DNA polymerase enzymes mix. The pBSC-IC-DENV3-pA (nt 1-2464) was assembled from a single subgenomic fragment and contained the genome sequence of the 5'-UTR, C, prM, E and NS1 (the first 51 nt). The 5'-end primer used on this PCR reaction included an *Rsr*II restriction site, a T7 promoter recognition

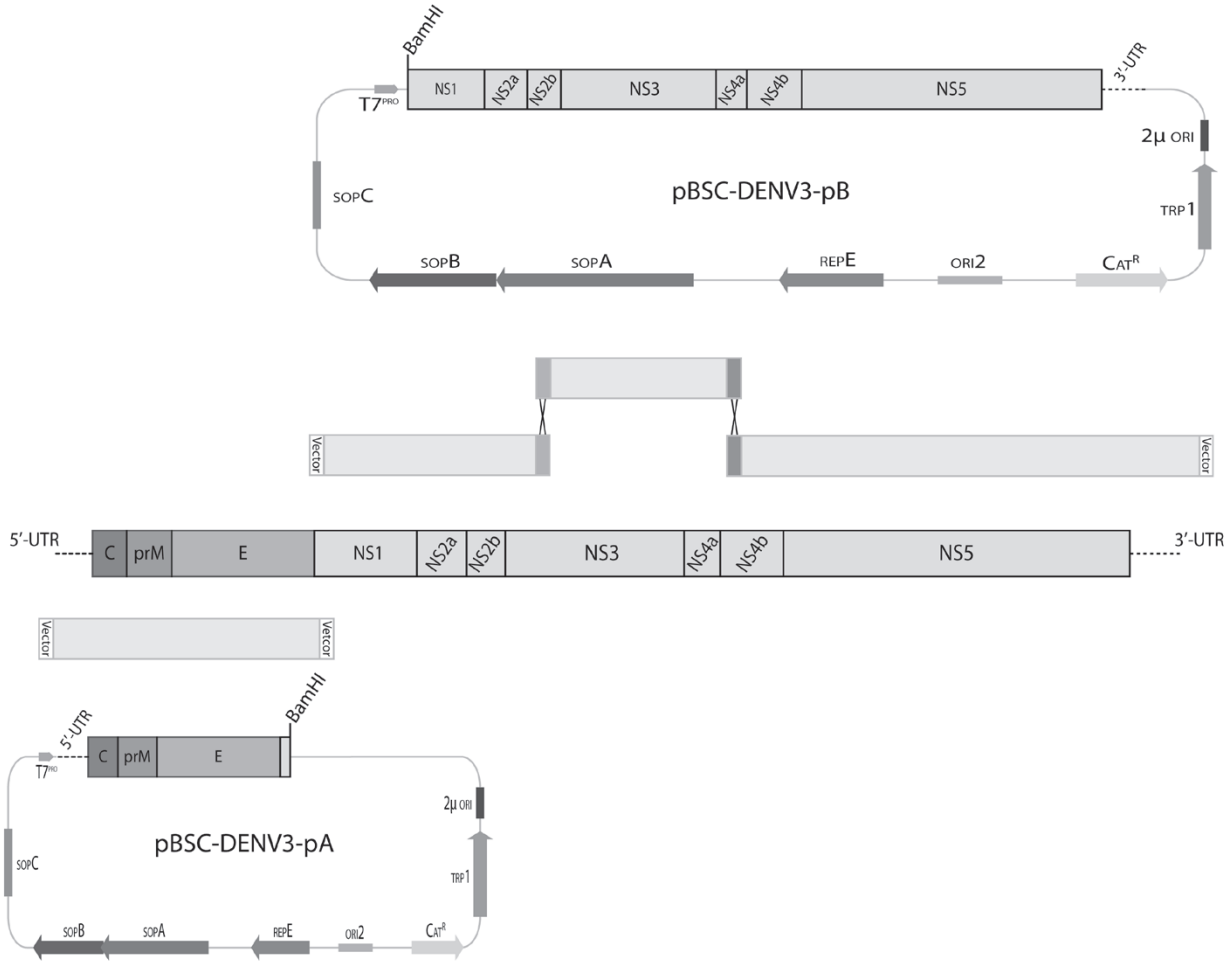

Figure 1 - Schematic diagram for the construction of a DENV3 infectious clone using a two-plasmid strategy. DENV3 genome was divided and cloned as two separate fragments with the yeast shuttle vector pBSC by homologous recombination in yeast cells. All subgenomic fragments were amplified by PCR with overlapping sequences. The pBSC-IC-DENV3-pA was assembled from a single subgenomic fragment and contains the genome sequence encoding the 5'-UTR, C, prM, E and NS1 (the first 51 nt). The pBSC-IC-DENV3-pB was generated from three overlapping subgenomic fragments (F1, F2 and F3). It contains the rest of DENV3 genome, spanning from the remaining sequence of NS1 to the 3'-UTR. Both constructs have a common *Bam*HI restriction site at nt 2459. Not drawn to scale.

site and an additional G immediately before the first base of DENV3 genome. The pBSC-IC-DENV3-pB was generated from three overlapping subgenomic fragments (F1, F2 and F3, see Table I). It contained the rest of DENV3 genome, spanning from the remaining sequence of NS1 to the 3'-UTR. Both constructs had a common *Bam*HI restriction site at nt 2459. A list of oligonucleotide primers used on the construction of the infectious clone is shown in Table I. Oligonucleotides primers were designed at highly conserved regions in the virus genome. Those regions were identified by aligning multiple, related sequences of dengue virus 3 available on the GenBank. The two constructs were assembled into the yeast-bacteria pBSC shuttle vector through recombination among fragments at homologous regions in yeast (Gibson 2009, Polo et al. 1997), and recombinant clones were selected in drop-out YNB medium lacking tryptophan. Individual clones were screened by PCR and sequence analyses, and positive clones were propagated only in yeast to prevent any possible instability of their inserts in bacteria. Plasmid sequences were deposited in the GenBank under the accession numbers KC425217 and KC425218.

TABLE I
Primer set for construction of the DENV3 infectious clone.

PCR fragment	Primer ID	Primer sequence (5'-3')	Primer annealing	
			Position (nt)[a]	Gene region
pA	pBSC-RsrII-T7-5'DENV3	CAAGCATGTAAATATCGTTTGAGTTCGGTCCGTAAT ACGACTCACTATAGAGTTGTTARTCTACGTGGACCGAC	1-23	5'-UTR
	DENV3-IC-BamHI-pA-R	AGTACCGAGAAACTAGAGCGGCC GCGGATCCACATTTGAGTTCTTTGCC	2441-2465	NS1
pB F1	DENV-3-IC-BamHI-pB-F	CGGTCCGTAATACGACTCACTATAGGGATCC GGAATCTTCGTCACCAACGAG	2459-2485	NS1
	DENV3-4570-R	ttccagttctgctttctgtgtc	4549-4570	NS3
pB F2	DENV3-4370-F	acaatgagaataaaagatgatgaga	4370-4394	NS2b
	DENV3-6700-R	gactatagccgacgcgatccat	6679-6700	NS4a
pB F3	DENV3-6521-F	acaatggaaacactcctactct	6521-6542	NS4a
	pBSC-DENV3-3'UTR-R	TCCTCCTTCGGATGCCCAGGTCGGACAGAA CCRGTTGATTCAACAGCACCATT	10681-10707	3'-UTR

[a]Nucleotide numbering refers to DENV3 full-length genome.

Full-Length Genome Assembly and *in vitro* Transcription

After plasmid DNA purification from yeast cells, both parts of DENV3 genome were amplified by PCR using the KlenTaq LA polymerase mix (Clontech). The 5' portion of virus genome was amplified from pBSC-IC-DENV3-pA with the same set of primers used on the construction of the plasmid. The 3' portion was amplified from pBSC-IC-DENV3-pB. To produce a 3'-terminus identical to the virus genome, the 3'-end primer was designed to flank exactly at the end of DENV3 genome, leaving no extraneous bases in the final RNA transcript. PCR products were purified, digested with restriction endonuclease *Bam*HI and re-purified. Purified PCR products were joined together at a molar ratio of 1:1 with T4 DNA ligase to yield a full-length DENV3 cDNA infectious clone, L-IC-DENV3 L42. The *in vitro* ligation mixture was purified by phenol-chloroform extraction, followed by ethanol precipitation. Full-length RNA transcripts were *in vitro* synthesized using the MEGAScript T7 kit (Ambion) with the addition of a 7-methyl-guanosine cap analog (Ambion), following manufacturer's instructions. An aliquot from the reaction was analyzed by formaldehyde-agarose gel electrophoresis and RNA transcripts were used without further processing.

RNA Transfection and Immunofluorescence Assay (IFA)

RNA transcripts were introduced into BHK-21 cells by electroporation using the ECM 830 electro cell manipulator (BTX Harvard Apparatus). 2 x 10^6 BHK-21 cells were trypsinized and washed twice with serum-free medium, resuspended in 100 µl Cytomix solution (Ansari et al. 2004), and mixed with 5 µg of transcribed RNA. Cells were transferred to an ice-cold electroporation cuvette with 0.2-cm gap. Electroporation was performed with 2 pulses of 100 µs at 1200 V separated by 1 s interval. As negative control, BHK-21 cells were transfected under the same conditions except no RNA was added (mock). After electroporation, cells were allowed to recover for 10 min at room temperature and resuspended in complete growth medium. For immunofluorescence analyses, transfected cells were seeded onto glass coverslips. At different time points, RNA-transfected cells in glass coverslips were rinsed with PBS, fixed with 50% cold acetone (v/v) in PBS for 5 min at 4 °C and air-dried. Fixed cells were incubated with a 1:100 dilution of a polyclonal hyperimmune mouse ascitic fluid (HMAF) for 1 h at 37 °C. This HMFA was raised against group B flaviviruses, including YFV and the four DENV serotypes. After incubation with primary antibody, cells were rinsed twice with PBS and incubated for 1 h at 37 °C with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma-Aldrich), developed in goat. Then, cells were rinsed twice with PBS and the nuclei stained with a Hoechst 33258 solution (Sigma-Aldrich). Prior to visualization by fluorescent microscopy, cell were washed twice with ddH_20, air-dried and mounted. Immunofluorescence analyses were carried out on a DMI 4000B inverted microscope (Leica).

DENV3 Infectious Clone Identity Confirmation

After the third blind passage in mosquito C6/36 cells, virus RNA from both wild type DENV3 (WT-DENV3) and transcript-derived DENV3 was extracted from culture supernatants using the QIAamp Viral RNA mini kit (Qiagen), according to manufacturer's instructions. RNA derived from noninfected C6/36 culture supernatant was used as negative control. Purified RNA preparations served as templates to reverse transcription (RT) using SuperScript III reverse transcriptase (Invitrogen) with reverse primer DENV3-3840-R (5'-gccatttgttcaatgtcctctg-3'), following the manufacturer's protocol. Standard PCR amplifications were carried out using RT products as templates and specific primers: DENV3-1141-F (5'-aactgactcaagatgtcctacc-3') and DENV3-3521-R (5'-ggattgccaaacacaagacac-3'). PCR products were purified, digested with restriction endonuclease *Bam*HI and restriction patterns were analyzed on agarose gel electrophoresis.

Virus Titration and Growth Curves

Virus titration was determined by *focus*-forming assay in mosquito C6/36 cells, as described previously (Das et al. 2007) with modifications. Briefly, 2 x 10^5 cells per well were seeded in 24-well cell culture dishes 48 h prior to conducting the assay. Cell monolayers were incubated at 30 °C with 0.2 mL of serial dilutions of each virus. After removing the inoculum, cells were covered with 1 mL of L-15 overlay medium, containing 1% carboxymethyl cellulose (CMC), 2% FBS and 1% antibiotic solution, and incubated at 30 °C. On day 5, overlaid medium was removed. Cells were fixed with 30% cold acetone (v/v) in PBS for 13 min at room temperature and washed once with PBS. Fixed cells were incubated for 1 h at 37 °C with a HMAF diluted 1:100 in ligation buffer. Cells were rinsed with wash buffer and incubated with a 1:500 dilution in ligation buffer of a horseradish peroxidase (HRP)-conjugated recombinant protein G (Invitrogen) for 1 h at 37 °C. Cells were rinsed with wash buffer and *foci* developed by adding AEC substrate buffer. *Foci* were counted and virus titers, expressed in *focus*-forming unit (FFU) per mL, were determined

by standard methods. For growth curves, 5 x 10^5 mosquito C6/36 cells per well in a 24-well cell culture dishes were infected in duplicate with WT-DENV3 and transcript-derived DENV3 at multiplicity of infection (MOI) of 0.1. Cells were incubated at 30 °C for 1 h. After removal of inoculum, cells were washed twice with PBS and 1 mL of growth medium was added to each well. Aliquots of the supernatant of infected cells were removed daily and stored at -70 °C. DENV3 titers in each sample were determined by titration in mosquito C6/36 cells.

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism 4 for Macintosh (GraphPad Software, Inc). Growth kinetics differences were analyzed by a two-way analysis of variance (ANOVA) with a mixed linear model for repeated measures, followed by a Bonferonni's corrected *post hoc* test for pairwise comparisons at each time point. The differences were considered statistically significant at a P level of ≤ 0.05.

RESULTS

The shuttle plasmid pBSC was used as a vector for the construction of DENV3 infectious clone. To prevent genome instability, DENV3 genome was divided at the E/NS1 region and cloned as two separate fragments by homologous recombination in yeast cells, generating plasmids pBSC-IC-DENV3-pA and pBSC-IC-DENV3-pB (Fig. 1). Successful construction of the plasmids was confirmed by amplification and DNA sequencing. No mutation was identified in plasmid pBSC-IC-DENV3-pA. In the plasmid pBSC-IC-DENV3-pB, sequence analyses showed one mutation at position 7878 (A7878G) leading to a change in NS5 amino acid sequence (K105R).

To assembly the full-length DENV3 cDNA infectious clone *in vitro*, genome fragments cloned into plasmids pBSC-IC-DENV3-pA and pBSC-IC-DENV3-pB were amplified by PCR, digested at *Bam*HI site, and mixed together in a molar ratio of 1:1. *In vitro* ligation mixtures examined on agarose gel electrophoresis showed an expected band, corresponding to the full-length L-IC-DENV3 L42 cDNA. Even though ligation reactions were optimized to produce high yields of full-length DENV3 cDNA, other undesired ligation products and non-ligated fragments were also observed (data not shown).

Assembled full-length DENV3 templates were *in vitro* transcribed and resulting full-length DENV3 transcripts were introduced into BHK-21 cells by electroporation. Using immunofluorescence assay (IFA), DENV3 antigens were detected in cells 4 days after electroporation with transcript-derived DENV3. On day 5 (Fig. 2), more antigen-positive cells were observed indicating RNA transcripts derived from L-IC-DENV3 L42 infectious clone were infectious and replicated in BHK-21 cells. The percentage of DENV3 antigen-positive cells increased over time until the first signs of cytopathic effect (CPE) appeared on day 8. As expected, no antigen was detectable in the negative control.

After assembling the full-length DENV3 cDNA by *in vitro* ligation, a unique *Bam*HI site was inserted in the genome and used to distinguish transcript-derived DENV3 from WT-DENV3. On the third blind passage in mosquito C6/36 cells, a 2381-bp fragment was amplified by RT-PCR using virus RNA extracted from either WT-DENV3 (Fig. 3, lane 2) or transcript-derived (Fig. 3, lane 4) culture supernatants. As expected, restriction endonuclease *Bam*HI was unable to cleave the PCR product derived from WT-DENV3 (Fig. 3, lane 3). On the other hand, the PCR product from transcript-derived DENV3 was cleaved by *Bam*HI producing fragments of 1318-bp and 1063-bp (Fig. 3, lane 5). No amplification was observed in the negative control (Fig. 3, lane 6). These results confirm that the rescued virus was indeed derived from the reverse genetics strategy described here.

In order to further characterize the rescued virus, growth kinetics of transcript-derived DENV3 stocks were compared to WT-DENV3 stocks

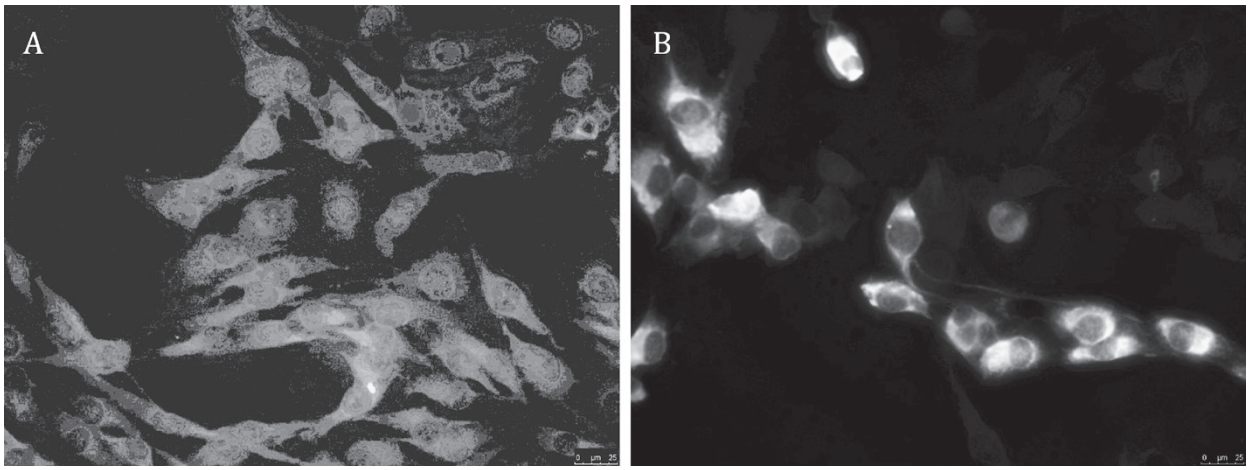

Figure 2 - Transcript-derived L-IC-DENV3 L42 protein expression in BHK-21 cells analyzed by immunofluorescence assay (IFA). BHK-21 cells were electroporated with *in vitro* transcribed RNA from L-IC-DENV3 L42 infectious clone or were mock electroporated. On day 5 after electroporation, cells were fixed and incubated with a polyclonal hyperimmune mouse ascitic fluid (HMAF) raised against group B flaviviruses, followed by an anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC). Cell nuclei were stained with a Hoechst 33258 solution. Images were captured in a DMI 4000B inverted microscope (Leica) with a 400X magnification factor. (A) Mock-electroporated BHK-21 cells. (B) BHK-21 cells electroporated with transcript-derived L-IC-DENV3 L42.

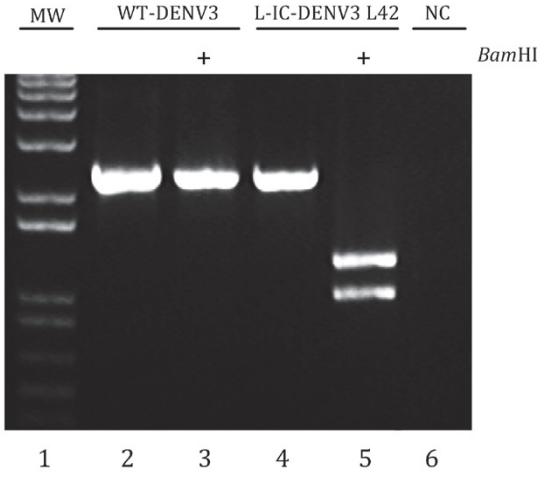

Figure 3 - Transcript-derived L-IC-DENV3 L42 identity confirmation. A unique *Bam*HI site was created during full-length DENV3 cDNA assembly by *in vitro* ligation and used as a genetic marker. A 2381-bp fragment spanning the *Bam*HI site was amplified from WT-DENV3 (lane 2) and transcript-derived L-IC-DENV3 L42 (lane 4). RT-PCR products were digested with *Bam*HI and analyzed on agarose gel electrophoresis. No cleavage occurred in the RT-PCR product derived from WT-DENV3 (lane 3), while the RT-PCR product from transcript-derived DENV3 was digested generating fragments of 1318-bp and 1063-bp (lane 5). As expected, no amplification was observed in the negative control (lane 6). A 1Kb plus DNA ladder (Invitrogen) was loaded on lane 1.

at MOI of 0.1 in mosquito C6/36 cells (Fig. 4). Transcript-derived virus L-IC-DENV3 L42 grew at a similar rate as the WT-DENV3 (P>0.05 at each time point). Both viruses also reached peak virus titer on day 4. Additionally, these results show evidence that the mutation at position 7878 (A7878G) has no effect on transcript-derived virus L-IC-DENV3 L42 growth kinetics in C6/36 cells.

DISCUSSION

Dengue infections represent one of the most prevalent arthropod-borne diseases worldwide, affecting millions of individuals annually in tropical and subtropical areas. Without effective prophylaxis, those numbers are expected to increase over the years as a consequence of climate warming impact in DENV transmission (Jetten and Focks 1997, Sutherst 2004).

Despite enormous progress in unraveling DENV replication cycle and virus-host interaction, many molecular features are not yet clearly understood. The infectious clone methodology is an important asset of the molecular approach in experimental virology, contributing to the study

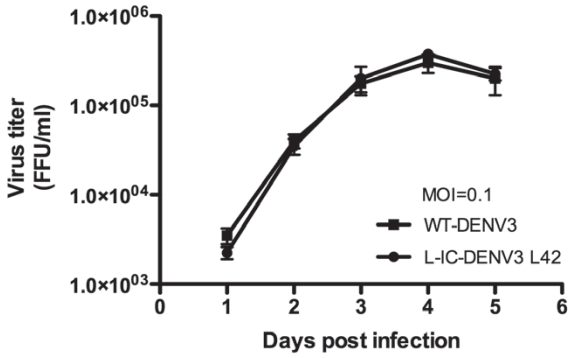

Figure 4 - Transcript-derived L-IC-DENV3 L42 and WT-DENV3 growth kinetics in mosquito C6/36 cells. Mosquito C6/36 cells were infected in duplicate at MOI of 0.1 with WT-DENV3 or transcript-derived DENV3. A sample of the supernatant of infected cells was collected daily and virus titers were determined by titration in mosquito C6/36 cells. No statistical difference in virus titer (P>0.05) was found between WT-DENV3 growth curve and transcript-derived L-IC-DENV3 L42 growth curve in mosquito C6/36 cells.

of genome replication, virus pathogenesis and vaccine development. The first DENV infectious clone has been described for DENV4 (Lai et al. 1991). Subsequently, infectious clones have been developed for DENV2 (Kapoor et al. 1995), DENV1 (Puri et al. 2000) and DENV3 (Blaney et al. 2004). Unfortunately, flavivirus genome instability in bacteria is still a tremendous impediment to the development of these infectious clones. Since there is no standard with regard to the best approach to construct reverse genetics systems for flaviviruses, different strategies aiming to bypass genome toxicity in *E. coli* have arisen. Homologous recombination in yeast has been successfully used in the development of a full-length reverse genetics system for bovine viral diarrhea virus (BVDV) (Gil et al. 2006a, b). Based on this approach, we were able to generate a stable reverse genetics system for YFV (Queiroz et al. 2013). However, this system did not tolerate dengue virus genome and we were unable to rescue a functional infectious clone for dengue virus. We tested distinct dengue virus serotypes in this system. We also attempted to couple this initial strategy with the insertion of a "stabilizing linker" described previously (Blaney et al. 2004) without success. Thereafter, we shifted our efforts to the two-plasmid strategy.

Here, we sought to develop an infectious clone derived from a Brazilian DENV3 clinical isolate. By successfully cloning the full-length genome in two separate fragments in yeast, we were able to surpass genome instability. Recently, our group has employed a different strategy to achieve stability of the cloned virus genome based on the same DENV3 isolate used here. These findings have been published elsewhere (Santos et al. 2013).

It is probable that yeast is more tolerant than bacteria to the toxicity of unstable sequences commonly found in flavivirus genome, making it a very convenient host for the development of reverse genetics systems (Polo et al. 1997, Pu et al. 2011, Puri et al. 2000). Homologous recombination in yeast is a simple and robust technique with higher cloning efficiency when compared to standard cloning procedures in *E. coli* (Gibson 2009, Shanks et al. 2009). It is also a high fidelity cloning strategy, successfully employed in the assembly of virus and bacterial genomes, making the emergence of spurious mutation very unlikely. Even though the *in vitro* ligation strategy derived from two plasmids is not widely used, our results reinforce the effectiveness of this approach to circumvent genome instability.

RNA transcripts derived from L-IC-DENV3 L42 are infectious in BHK-21 cells and antigen-positive cells were readily detected 4 days after transfection. Many factors contribute to RNA infectivity in susceptible cells such as cell type, transfection method, virus serotype/genotype and RNA preparation, but similar results have been described for DENV2 in LLC-MK2 cells (Polo et al. 1997).

The identified mutation did not seem to carry any deleterious effect on transcript-derived DENV3 infectivity in either BHK-21 or mosquito C6/36 cells. This claim is supported by the successful

rescue of DENV3 in BHK-21 cells and growth kinetics experiments in which transcript-derived DENV3 growth curve was comparable to WT-DENV3 in C6/36 cells.

In conclusion, we present an alternative approach to obtain a functional infectious clone for DENV3 while working with unstable sequences. A similar strategy could be engineered for other DENV serotypes or flaviviruses. This infectious clone will be a valuable tool in molecular virology research for better understanding DENV biology and pathogenesis.

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RESUMO

As infecções causadas por dengue representam uma das mais prevalentes doenças transmitidas por artrópodes mundialmente, causando um amplo espectro de manifestações clínicas. A tecnologia de clones infecciosos é uma importante ferramenta para o entendimento da biologia do vírus da dengue (DENV). Clones de cDNA infeccioso têm sido construídos para muitos vírus de RNA com cadeia positiva e são ferramentas valiosas para o estudo dos mecanismos moleculares envolvidos na replicação do genoma viral, montagem da partícula viral, patogênese viral, e desenvolvimento de vacinas. No presente trabalho, nós descrevemos o desenvolvimento de um clone infeccioso baseado num isolado primário brasileiro do vírus Dengue sorotipo 3 (DENV3), genótipo III. Usando uma estratégia de dois plasmídeos, o genoma viral foi dividido em duas partes e os fragmentos gerados foram clonados separadamente num vetor *shuttle* levedura-bactéria. Os plasmídeos foram construídos pela técnica de recombinação homóloga em levedura e a transcrição do genoma completo foi realizada a partir ligação *in vitro* das duas partes do genoma. O transcrito de DENV3 se mostrou infeccioso quando transfectado em células BHK-21 e a identidade do clone infeccioso foi confirmada por caracterização *in vitro*. A cinética de crescimento do DENV3 gerado neste sistema foi indistinguível do vírus parental. Este sistema representa uma poderosa ferramenta que ajudará na elucidação de aspectos moleculares da biologia do DENV bem como no estudo de mutações associadas com patogênese do DENV.

Palavras-chave: genética reversa, vírus dengue, clonagem molecular, clone infeccioso.

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