Full-length infectious clone of a low passage dengue virus serotype 2 from Brazil

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Full-length dengue virus (DENV) cDNA clones are an invaluable tool for many studies, including those on the development of attenuated or chimeric vaccines and on host-virus interactions. Furthermore, the importance of low passage DENV infectious clones should be highlighted, as these may harbour critical and unique strain-specific viral components from field-circulating isolates. The successful construction of a functional Brazilian low passage DENV serotype 2 full-length clone through homologous recombination reported here supports the use of a strategy that has been shown to be highly useful by our group for the development of flavivirus infectious clones and replicons.

Key words: infectious clone - yeast homologous recombination - flavivirus

Dengue is an arthropod-borne disease found in tropical and subtropical regions throughout the world. The incidence of dengue has increased significantly in the past years and a recent study estimates that 390 million people are infected with dengue viruses (DENV) annually (2013). In the Americas, Brazil and Mexico account for most cases (Bhatt et al. 2013). The burden of dengue is due to the mortality rate (which can reach approximately 5% of the severe cases) and to the morbidity rate among symptomatic patients, which involves a broad spectrum of symptoms and may last for several days. In fact, the classification of dengue clinical cases remains a complex and changing subject (Horstick et al. 2015). Although there are currently a few vaccine formulations in clinical trials, there are no licensed vaccines available against DENV (Ishikawa et al. 2014). The complex interactions among hosts (humans and mosquitoes) and different virus strains most likely represent part of the obstacle to the development of an effective vaccine.

DENV (genus Flavivirus, family Flaviviridae) are transmitted to humans by female adult mosquitoes, with *Aedes aegypti* being the most important vector (Nene et al. 2007). There are four recognised DENV serotypes (DENV1-DENV4), with multiple genotypes/lineages identified within each serotype (Chen & Vasilakis 2011). The importance of viral genotype/lineage identification is due to strain-associated features involved in viral repli-

cation and pathogenesis (Rico-Hesse 2007). The genome of DENV consists of positive single-stranded RNA and is approximately 10.7 Kb with a 5' cap structure and a 3' untranslated region that lacks a poly (A) tail. A polyprotein is encoded by a single open reading frame within the genome and it is further cleaved into three structural and seven nonstructural (NS) proteins (NH$_2$-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH) by viral and host proteases (Chambers et al. 1990).

A full-length complementary DNA (cDNA) clone or infectious clone of a virus is a powerful tool that allows for the manipulation of the viral genome for various purposes, providing a homogeneous viral population for research (Ruggli & Rice 1999). Although not all infectious clones rely on in vitro transcription or cloning vectors, to simplify, here we will consider full-length clones that consist of the whole viral genome inserted in a cloning vector downstream of the sequence of an RNA polymerase phage promoter. The construct is then propagated in a bacteria or yeast host, linearised and in vitro transcribed to generate transcripts that resemble the parental virus genome. The transcripts are further used to transfect cells, allowing for the production of mature virions. Full-length flavivirus infectious clones have been reported since 1991 (Lai et al. 1991). However, the major obstacle for these studies is the instability of the viral genome in host systems, especially in *Escherichia coli* (Ruggli & Rice 1999). Different methodologies have been applied to overcome viral genome instability, including the use of low copy bacterial plasmids and bacterial artificial chromosome (BAC) vectors.

Homologous recombination in yeast with linear DNA fragments containing homologous regions in their ends can overcome laborious cloning strategies in bacteria (Gibson 2009). Importantly, with regard to the construction of flavivirus infectious clones, this technique yields more stable plasmid amplification than in bacteria, as it has been shown that the genome of some flaviviruses, such as DENV, may contain cryptic bacterial promoters. These promoters lead to the unwanted production of vi-
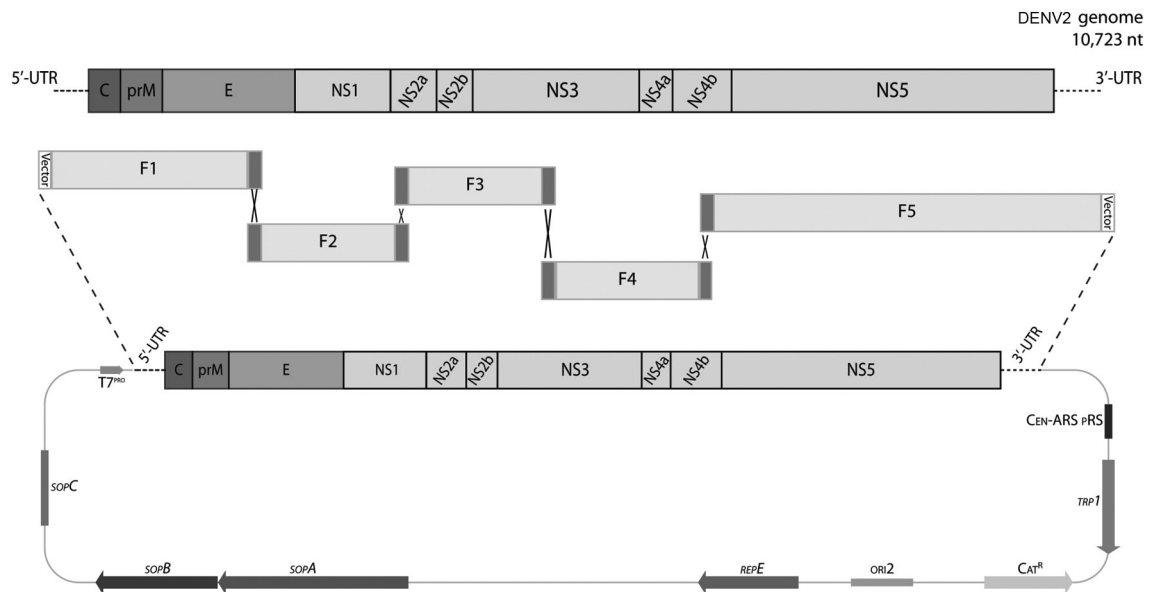
Fig. 1: construction of a low passage full-length dengue virus serotype 2 (DENV2) infectious clone named pSVJS01-DENV2. Five fragments within the DENV2 genome were amplified and inserted into the shuttle vector pSVJS01 through homologous recombination in yeast. E: envelop; F: fragment; NS: nonstructural; UTR: untranslated region.

ral proteins/regions that are toxic to the bacteria (Pu et al. 2011), which results in viral genome modifications (e.g., deletions, insertions) during the amplification process.

In the present paper, we report the construction of a full-length DENV2 infectious clone based on a low passage clinical isolate, through homologous recombination in yeast. The virus DENV2 BR-3808 was isolated from a biological sample collected from a patient presenting with dengue fever during a dengue outbreak in Recife, state of Pernambuco, Brazil, in 1995. An aliquot of the biological sample was inoculated into C6/36 mosquito cells and, after a few days, the flask content was harvested for virus identification through reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for DENV2 (Lanciotti et al. 1992) and indirect immunofluorescence (IFA). The virus was then cultured in C6/36 until passage 3. C6/36 were maintained at 28°C in Leibovitz's medium (L15) containing 5% foetal bovine serum (FBS), 1% antibiotics solution (stock solution at 10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin) and 0.1% Fungizone (stock solution at 250 μg/mL). Next, DENV2 BR-3808 was plaque purified and amplified once in baby hamster kidney (BHK)-21 cells. BHK-21 was maintained at 37°C in a 5% CO_2 incubator in minimum essential medium (MEM) supplemented with 10% FBS, 1% antibiotics solution and 0.1% Fungizone.

Construction of the DENV2 infectious clone was performed using the same strategy described previously by our group for the construction of a DENV3 full-length clone (Santos et al. 2013). Electrocompetent *E. coli* DH10B and *Saccharomyces cerevisiae* YPH252 were used for plasmid amplification. *S. cerevisiae* RFY206 was used for homologous recombination to insert the DENV2 genome into a shuttle vector previously constructed, pSVJS01 (Santos et al. 2013). This plasmid is a modified version of the BAC vector pBeloBAC11, into which a 2 kb fragment of the plasmid pRS414 (that allows replication and selection in yeast), a T7 promoter recognition site and a multiple cloning site was inserted. This construct ensures amplification at low copy number in yeast, a strategy known to minimise infectious clone instability (Bredenbeek et al. 2003, Suzuki et al. 2007). The DENV2 infectious clone was constructed as shown in Fig. 1. Firstly, the viral genome was amplified in five overlapping fragments. Primers sequences and fragment size are depicted in Table I. Primer PSVJS01-F contained an RsrII restriction site, a T7 promoter recognition site and an additional G immediately before the first base of the DENV2 genome. Amplified fragments (F1-F5) were assembled into the NotI-linearised pSVJS01 vector by homologous recombination in the YPH252 yeast strain. The DENV2 full-length infectious clone, named pSVJS01-DENV2, was successfully assembled by this strategy. The full-length PCR amplicon from two pSVJS01-DENV2 clones (3 and 10) was confirmed by gel electrophoresis. Functional clones were maintained in yeast to prevent infectious clone instability that may occur upon amplification in bacteria (Polo et al. 1997, Ruggli & Rice 1999). PCR amplicons derived from pS-VJS01-DENV2 were purified using phenol-chloroform extraction and ethanol precipitation and the purified amplicons were used as a template for full-length in vitro RNA transcription. For transcription, the MEGAScript T7 kit (Ambion) was used with the addition of a 7-methyl-guanosine cap analogue (Ambion), following the manufacturer's instructions. Transcript analysis through formaldehyde-agarose gel electrophoresis showed high-quality products (data now shown). RNA transcripts

TABLE I

Primers used to amplify fragments of the dengue virus serotype 2 (DENV2) (strain BR-3808)
genome used to assemble the full-length infectious clone through homologous recombination in yeast

Fragment	Primer	Sequence (5'-3')	Amplicon size (bp)	Primer annealing Position (nt)[a]	Primer annealing Gene region
F1	PSVJS01-F	**cggtccg***taatacgactcactata*<u>G</u>AGTTGTTARTCTACGTGGACCGA	2,045	1-23	5'-UTR
	DENV2-2045-R	TCTGCTTCTATGTTGACTGGG		2,025-2,045	E
F2	DENV2-1810-F	CTACAGCTCAAAGGAATGTCAT	1,760	1,810-1,831	E
	DENV2-3570-R	CATGTTTCGTTCCTACTCGGGTCC		3,548-3,571	NS2A
F3	DENV2-3501-F	TCACTAGGAGTCTTGGGAATGGC	2,058	3,502-3,524	NS2A
	DENV2-5559-R	TCCGTGACCCATTCATGTCC		5,560-5,579	NS3
F4	DENV2-5491-F	CATTTCCTCAGAGCAATGCACCAATC	1,878	5,492-5,517	NS3
	DENV2-7369-R	AATACTTGAGTCACGCAGAGG		7,350-7,370	NS4a
F5	DENV2-7190-F	GCCAGGACTTCAAGCAAAGC	3,533	7,191-7,211	NS4a
	PSVJS01-R	ttcaacatttccgtgtcgcgcggccgcAGAACCRGTTGATTCAACAGCACCATT		10,697-10,723	3'-UTR

a: nucleotide numbering refers to DENV2 full-length genome (GeneBank accession JX669481). RsrI restriction site was marked in bold type. T7 RNA polymerase promoter sequence was marked in italic and a single G for initiation of transcription is underlined. Regions of homology to pSVJS01 vector shown in capitals. E: envelope; F: fragment; NS: nonstructural; UTR: untranslated region.

were then introduced into BHK-21 cells by electroporation using the ECM 830 electro cell manipulator (BTX; Harvard Apparatus). For electroporation, 2×10^6 BHK-21 cells were trypsinised, washed twice with serum-free medium, resuspended in 100 µL cytomix solution (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 25 mM HEPES, pH 7.6, 2.0 mM EGTA, 5.0 mM $MgCl_2$) plus 2.0 mM of ATP and 5.0 mM of glutathione and mixed with 5 µg of the transcript. Cuvettes of 0.2 cm gap were used and electroporation settings were as follows: two pulses of 100 µs at 1,200 V and 1 s interval. As a negative control, BHK-21 cells were transfected under the same conditions with no RNA. After electroporation, cells were allowed to recover for 10 min at room temperature, resuspended in complete growth medium and plated into 25 cm² flasks.

To detect DENV2 production in BHK-21, transfected cells were analysed by IFA. Specifically, three days after electroporation cells were trypsinised from the flask and seeded on glass coverslips. Coverslips were then collected four days later (i.e., 7 days post-infection), rinsed with phosphate-buffered saline (PBS), fixed with 50% cold acetone (v/v) in PBS for 5 min at 4º C and air-dried. Next, fixed cells were incubated with a 1:100 dilution of a polyclonal hyperimmune mouse ascitic fluid (HMAF) as the primary antibody for 1 h at 37ºC. The HMAF reacts against group B flaviviruses, including the four DENV serotypes and yellow fever virus. After incubation, the cells were rinsed twice with PBS and incubated for 1 h at 37ºC with a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) as the secondary antibody. Cells were then rinsed twice with PBS, air-dried and mounted. A DMI4000 B inverted microscope (Leica) was used for analysis. Positive immunofluorescence was observed in infected cells (Fig. 2A) showing DENV2 production in BHK-21 cells transfected with RNA transcripts derived from pSVJS01-DENV2. Transfected, noninfected cells did not show any fluorescence (data not shown).

Virus characterisation was performed by *focus*-forming assay in BKH-21 cells and growth curve analysis between both viruses. For the *focus*-forming assay, 4×10^5 cells/per well were plated in six-well plates and 24 h later cell monolayers were incubated for 1 h at 37ºC with 0.8 mL of serial dilutions of parental DENV2 BR-3808 (parental-DENV2) and virus derived from pSVJS01-DENV2. After removing the *inoculum*, cells were covered with 3 mL of MEM overlay medium (containing 2% carboxymethyl cellulose, 5% FBS and 1% antibiotic solution) and incubated at 37ºC with 5% CO_2 for five days. Next, overlay medium was removed and the cells were fixed with 30% cold acetone (v/v) in PBS for 13 min at 4ºC and washed once with PBS. Fixed cells were incubated for 1 h at 37ºC with primary anti-DENV2 monoclonal antibody D1-4G2-4-15 (HB112 - ATCC) diluted 1:2 in ligation buffer (0.5 M NaCl and 0.01% Tween-20 in PBS). Cells were rinsed with wash buffer (0.05% Tween-20 in PBS) and incubated for 1 h at 37ºC with horseradish peroxidase-conjugated recombinant protein G (Invitrogen) diluted 1:500 in ligation buffer. Three additional washing steps were performed and *foci* were developed by adding 3-amino-9-ethylcarbazole substrate buffer. In these assays, *foci* were visible at up to the 10^{-5} dilution for DENV2 derived from pSVJS01-DENV2. Moreover, *foci* of pSVJS01-derived DENV2 virus did not differ in formation or size from parental-DENV2 (Fig. 2B). These results show the production of functional and infectious DENV2 from the infectious clones.
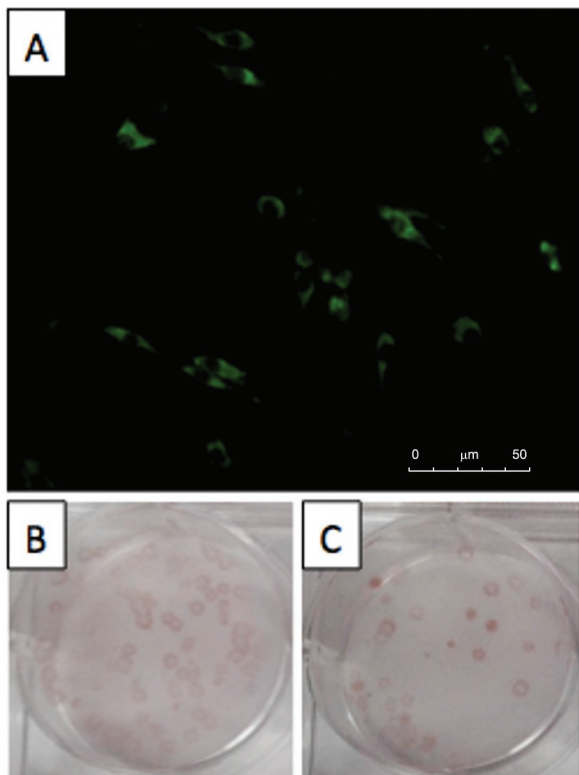
Fig. 2: in vitro characterisation of pSVJS01-DENV2 virus. A: detection of dengue virus serotype 2 (DENV2) in baby hamster kidney (BHK)-21 cells transfected with RNA derived from pSVJS01-DENV2 clone 10 by indirect immunofluorescence assay. Cells were collected at five days post-transfection and labelled with anti-flavivirus group B as the primary antibody and anti-mouse IgG-fluorescein isothiocyanate as the secondary antibody; B, C: plaque phenotypes of the wild type DENV2 (BR-3808) and the virus derived from pSVJS01-DENV2. BHK-21 cells were infected with the different viruses for five days at 37°C. Plaque formation was revealed by immunoperoxidase assay; B: DENV2 BR-3808; C: pSVJS01-DENV2 clone 10.

For the growth curve analysis, BHK-21 cells were seeded in 24-well plates at 6 x 10^4 cells/well and were infected 24 h later with both viruses at a multiplicity of infection of 0.1. The supernatant was collected from the wells at the following time-points: 0 h, 24 h, 48 h and 72 h. Viral RNA was extracted from the material with the QIAamp Viral RNA Mini Kit (Qiagen). To quantify the viral genome in this material, quantitative real-time PCR was performed with the QuantiTect SYBR Green RT-PCR Kit (Qiagen), as described by Carvalho-Leandro et al. (2012). Briefly, primers that amplify a 104 bp region of the DENV NS5 according to Kong et al. (2006) were used. PCR reactions were performed with a fixed amount of RNA and 0.4 µM of each primer. Reaction conditions were as follows: 50°C for 30 min, to allow reverse transcription, 95°C for 15 min, to activate Taq, and 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The amount of viral RNA in each sample was calculated by cycle threshold values from the standard curve in-

cluded in each PCR plate. This curve consisted of known concentrations of purified NS5 transcript, as described in Kong et al. (2006). The negative controls consisted of one reaction with no RNA template and one reaction with RNA extracted from noninfected cells. The positive control was RNA extracted from a known DENV2 infected-culture. Reactions were performed in duplicate using the Applied Biosystems 7500 fast and Real-Time PCR System. Similar results were observed between samples of cultures infected with either DENV2 BR-3808 or the infectious clone-derived DENV2 at the different time-points assayed. The amounts of viral RNA at 0 h, 24 h, 48 h and 72 h were 15.6, 16.8, 17.5 and 18.0, respectively, for the BR-3808, and 15.6, 17.1, 17.1 and 17.7 RNA molecules/µL ($\log_{10}$) for the pSVJS01-derived DENV2 (Fig. 3). These results also show the similar phenotype between the viruses.

Finally, the gene coding for the envelope protein, which is approximately 1,500 bp, was sequenced from both viruses using the Sanger method. The results showed that the sequences were 100% identical (data not shown), demonstrating that at least in this part of the genome no mutations arose from the strategy described here. In addition, any mutation in other genome regions that may have occurred was not enough to cause significant phenotypic differences among the parental and infectious clone-derived viruses.

The availability of DENV full-length cDNA clones that correspond to the original DENV genome is an invaluable tool for several types of study, including those on the development of attenuated or chimeric vaccines and on host-virus interactions. Furthermore, the importance of low passage DENV infectious clones should be highlighted, as these may harbour critical and unique strain-specific viral components from field-circulating isolates; hence, the importance of reporting the construction and availability of such clones.

Full-length clones have been reported for all four DENV serotypes. However, only a remarkably small amount of these are based on low passage isolates (Table II). Although there is a higher number of available
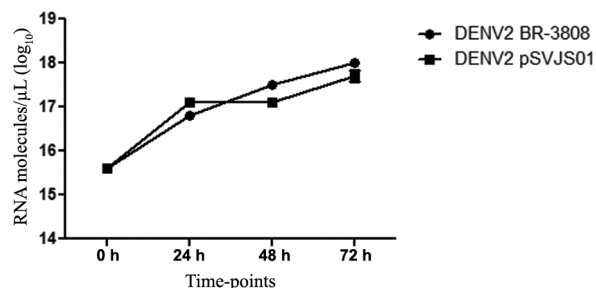

Fig. 3: growth curve analysis of parental (BR-3808) and infectious clone-derived dengue virus serotype 2 (DENV2) (pSVJS01) assessed by quantitative real-time polymerase chain reaction. Viral RNA was quantified in baby hamster kidney-21 cells infected with either viruses at 0 h, 24 h, 48 h and 72 h post-infection.

TABLE II

Full-length infectious clones of dengue virus (DENV) serotypes 1-4 and their passage history[a]

Serotype	Strain	Passage history	Plasmid (type)	Reference
DENV4	814669 (Dominica, 1981)	Not available[b]	pBR322 (*Escherichia coli*)	Lai et al. (1991)
DENV2	16681 (Thailand, 1964)	BS-C-1 cells (several times); LLC-MK2 cells (6 times); rhesus macaque monkey (once); *Toxorhynchites amboinensis* mosquitoes (twice); primary green monkey kidney cells (once); LLC-MK2 cells (twice); C6/36 (4 times)	pBRUC-139S (derived from pBR322 and pUC19) (*E. coli*)	Kinney et al. (1997)[c]
DENV2	New Guinea C (New Guinea, 1944)	suckling mouse brain (38 times); C6/36 cells (several times)	pRML2 (yeast artificial chromosome) and pRS424 (yest-*E. coli* shuttle vector)	Polo et al. (1997)
DENV2	New Guinea C	suckling mice (24 times); EK cells (5 times); C6/36 (twice)[d]	pWSK29 (*E. coli*)	Gualano et al. (1998)
DENV1	Western Pacific'74 (Nauru Island, 1974)	Not available	pRS424	Puri et al. (2000)
DENV2	16681	C6/36 (several times; virus obtained from another lab, previous history not informed)	pBluescript II KS (*E. coli*)	Sriburi et al. (2001)
DENV3	Sleman/78 (Indonesia, 1978)	Vero cells (passage number not informed; virus obtained from another lab, previous history not informed)	pBR322	Blaney et al. (2004a)
DENV2	Tonga/74 (Tonga, 1974)	*Aedes albopictus* mosquitoes (once); C6/36 cells (passage number not informed)	pBR322	Blaney et al. (2004b)
DENV1	NIID02-20 (Japan, 2002)	Vero cells (passage number not informed)	pMW119 (*E. coli*)	Tajima et al. (2006)
DENV2	1409 (Jamaica, 1983)	LLC-MK2 cells (once); C6/36 (several times)	pBeloBac11 (BAC)	Pierro et al. (2006)
DENV1	BR/90 (Brazil, 1990)	C6/36 (4 times; virus obtained from another lab, previous history not informed)	pBACDV1poly (derived from pBeloBAC11)	Suzuki et al. (2007)
DENV2	43 (China, 1987)	Not informed	pWSK29	Zhu et al. (2007)
DENV4	341750 (Colombia, 1982)	Mosquito (once; species not informed); primary green monkey cells (5 times); FRhL cells (4 times); PDK cells (20 times); FRhL cells (4 times)	pRS424	Kelly et al. (2010)
DENV2	PL046 (Taiwan, 2008)	C6/36 (passage number not informed)	pRS313 (yest-*E. coli* shuttle vector)	Pu et al. (2011)
DENV2	TSV01 (Australia, 1993)	C6/36 cells (5 times)	pACYC177 (*E. coli*)	Zou et al. (2011)

Serotype	Strain	Passage history	Plasmid (type)	Reference
DENV2	SI6803 (Thailand, 1974)	Mosquito (once; species not informed); primary green monkey cells (4 times); PDK cells (50 times); FRhL cells (3 times)	pRS424	Kelly et al. (2011)
DENV3	95016/BR-PE/02 (Brazil, 2002)	C6/36 cells (3 times)	pSVJS01 (shuttle vector derived from pRS414 and pBeloBAC11)	Santos et al. (2013)
DENV2	New Guinea C	C6/36 (passage number not informed)	pBeloBAC11	Usme-Ciro et al. (2014)
DENV2	BR-3808 (Brazil, 1995)	C6/36 cells (3 times); BHK-21 cells (twice)	pSVJS01	Santos et al. (present paper)

a: only articles in English were analysed in PubMed [search terms were: (i) infectious clone, dengue; (ii) cDNA clone, dengue; (iii) full-length clone, dengue]; *b*: full-length clone was obtained from a cDNA library of DENV4 814669; *c*: full-length clones of DENV2 16681 with a different passage history were also constructed, however, only the infectious clone based on the parental virus is cited here; *d*: this is the original passage history cited by Gruenberg et al. (1988), from whom the virus stock was obtained additional passages in C6/36 (derived from larval tissue of *Ae. albopictus* mosquitoes) were performed by Gualano et al. (1998), however the passage number was not informed; BAC: bacterial artificial chromosome; BHK: baby hamster kidney; BS-C-1: grivet monkey kidney; EK: equine kidney; FRhL: foetal rhesus lung; LLC-MK2: rhesus monkey kidney; PDK: primary dog kidney; Vero: African green monkey kidney.

full-length DENV2 clones compared to the other three serotypes, out of the 12 full-length clones found in the literature search performed here, only a few provide the passage history of the virus and only one is reported to have been passaged under five times (Table II). The importance of full-length infectious clones based on low passage viruses is a result of mutations that arise upon sequential passages in biological systems (Lee et al. 1997, Vasilakis et al. 2009), where only low passage viruses most closely resemble the original genotypic and phenotypic features of the parental virus population.

The successful construction of a Brazilian low passage DENV2 full-length clone through homologous recombination reported here supports the use of a strategy that has been shown to be highly useful by our group for the development of flavivirus infectious clones and replicons (Queiroz et al. 2013, Santos et al. 2013).

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