Infectious clone construction of dengue virus type 2, strain Jamaican 1409, and characterization of a conditional E6 mutation

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A full-length infectious cDNA clone (iC) was constructed from the genome of the dengue virus type 2 (DENV-2) Jamaican 1409 strain, pBAC1409ic, by using a bacterial artificial chromosome plasmid system. Infectious virus was generated and characterized for growth in cell culture and for infection in Aedes aegypti mosquitoes. During construction, an isoleucine to methionine (Ile→Met) change was found at position 6 in the envelope glycoprotein sequence between low- and high-passage DENV-2 1409 strains. In vitro-transcribed genomic RNA of 1409ic with E6-Ile produced infectious virions following electroporation in mosquito cells, but not mammalian cells, while 1409ic RNA with an E6-Met mutation produced virus in both cell types. Moreover, DENV-2 1409 with the E6-Ile residue produced syncytia in C6/36 cell culture, whereas viruses with E6-Met did not. However, in vitro cell culture-derived growth–curve data and in vivo mosquito-infection rates revealed that none of the analysed DENV-2 strains differed from each other.

The four dengue virus (DENV) serotypes are mosquito-borne members of the genus Flavivirus (family Flaviviridae) and are endemic in the tropical and subtropical regions of the world. Transmission to humans is by peridomestic Aedes spp. mosquitoes, primarily Aedes aegypti. Manifestations of a DENV infection range from subclinical to a self-limited fever and rash [dengue fever (DF)] to a severe and sometimes deadly illness characterized by capillary leakage, thrombocytopenia and hypovolaemic shock [dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)]. Taken together, these viruses are estimated each year to cause 100 million cases of DF, 500 000 cases of DHF/DSS and 25 000 deaths, and 40% of the world population (i.e. 2·5 billion people) is at risk of infection (Monath, 1994).

Epidemiological studies have identified dengue virus type 2 (DENV-2) as the most important dengue virus serotype worldwide. The DENV-2 genome is a single-stranded, positive-sense RNA molecule of approximately 10 700 nt that contains a type I 5’-cap structure, but lacks a 3’ poly(A) tail. The genome is translated directly as a single polyprotein containing the three structural genes (NH2–Capsid–Tail) and are endemic in the tropical and subtropical regions of the world. Transmission to humans is by peridomestic Aedes aegypti mosquitoes. Manifestations of a DENV infection range from subclinical to a self-limited fever and rash [dengue fever (DF)] to a severe and sometimes deadly illness characterized by capillary leakage, thrombocytopenia and hypovolaemic shock [dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)]. Taken together, these viruses are estimated each year to cause 100 million cases of DF, 500 000 cases of DHF/DSS and 25 000 deaths, and 40% of the world population (i.e. 2·5 billion people) is at risk of infection (Monath, 1994).

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routinely (>25 passages) in C6/36 cells, were used (Bennett et al., 2002).

For genome sequencing and infectious clone (ic) construction, five cDNA fragments (f1–f5) representing the entire 1409hp genome were generated by RT-PCR (Titan RT-PCR kit; Roche Biochemicals), each of the fragments was inserted into a modified pBR322 plasmid (Kinney et al., 1997) and the five plasmids (p1–p5; Fig. 1a) were transfected by electroporation into competent Escherichia coli XL-1 Blue cells (Stratagene). Three individual bacterial colonies for each cloned fragment were cultured, plasmids were isolated and sequenced and the data were aligned against previously published DENV-2 1409 (1409pub) data (GenBank accession no. M20558). A significant nucleotide change in the genome between 1409hp and the 1409pub sequences was identified as a nucleotide substitution that occurred at the same position in all three colonies. In no cases were variations found in only two of three colonies. Nucleotide substitutions that occurred in only one of three colonies are not reported here.

Thirteen significant nucleotide differences between 1409hp and 1409pub were detected (Table 1). Genomic 1409lp was then sequenced at these specific positions to corroborate the published sequence, and seven nucleotide variations between the 1409pub sequence and the 1409lp were subsequently identified: 3934–3935, 7086–7087, 8241–8242 and 10089 (Table 1). Nucleotide substitutions specific to our 1409hp laboratory strain and the 1409lp strain occurred at six positions: 954, 1101, 4107, 4596, 8562 and 10403. This resulted in only one amino acid change, located at envelope protein position 6 (E6) (Ile→Met), and one untranslated region (UTR) change, located at 3′ UTR-143 (genome position 10403) (T→G), between 1409lp and 1409hp (Table 1). As our goal was to produce an ic representing the early 1409 isolate, we included these two 1409lp sequence sites in our ic. Thus, the 1409ic has an Ile at E6 and a thymine at 3′ UTR-143. Engineering of the clone also required an intentional silent nucleotide substitution of t/6651/c in the 1409ic genome via site-directed mutagenesis PCR to produce an NheI restriction site used in full-length clone construction. Additionally, unique PCR primers attached the T7 RNA polymerase recognition sequence and the linearization SphI restriction site, each of which are required for the A-cap analogue-dependent in vitro transcription of the clone (Fig. 1b).

For ic construction, subclones p1–2–3 (nt 1–6650) and p4–5 (6651–10724) were first constructed (Fig. 1a). Next, f4–5 (extracted from p4–5) was ligated to p1–2–3 to generate p1–2–3–4–5 (i.e. p1409ic). Unfortunately, a pBR322 plasmid containing the full-length 1409 cDNA was never recovered from electroporated (BTX ECM 630; 25 kV, 25 μF and 200 Ω) XL-1 Blue E. coli cells. Attempts to modify growth temperature, medium type, vector construction and E. coli
cell type all failed to yield full-length p1409ic. We observed that transformants containing either p1–2–3 or p4–5 produced a small colony size in *E. coli* host cells on standard YT plates [8 g tryptone, 5 g yeast, 5 g NaCl, 15 g agar (1 H2O)]

![Image of table](https://via.placeholder.com/150)

Table 1. Nucleotide and amino acid variation among DENV-2 1409 genomes

<table>
<thead>
<tr>
<th>Genome nucleotide position</th>
<th>Nucleotide</th>
<th>Amino acid</th>
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</tr>
<tr>
<td>10403</td>
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*pub, lp, hp and ic represent the DEN2 published, low-passage, high-passage and infectious clone residues, respectively.
†Unique sequence identifier for DEN2 1409ic.

Dengue virus type 2 1409 infectious clone construction

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The mosquito midgut-infection rate (MIR) and dissemination rate (MDR) of DENV-2 were determined by oral challenge of IVs in an infectious blood meal (6–5–6–9 p.f.u. ml−1) to A. aegypti strain Rex D mosquitoes (RexD; Rexville D, Puerto Rico, obtained from the CDC) and after an 8 day extrinsic incubation period as described previously (Bosio et al., 1998; Myles et al., 2004). Of the mosquitoes ingesting a blood meal (n = 24, two replicates) containing the 1409ic virus, 66–6 % had DENV-2 envelope antigen (Ab 4G2) in their midguts (i.e. MIR = 66–6) 8 days post-infection. The MIRs observed after mosquitoes ingested 1409lp, 1409hp or 1409-I/E6/M were 58–3, 72–9 and 75–0, respectively, none of which differed significantly from each other or from the value for 1409ic-infected mosquitoes (Fisher’s exact test, α of 0–05). The MDR, as determined by the number of anti-DENV-2 envelope IFA-positive heads over the number of IFA-positive midguts from the MIR group, for all of these viruses ranged between 78–6 and 88–5, none of which differed significantly from each other.

Clearly, the E6 residue of DENV-2 conditions viral propagation in cell-culture experiments and mutational pressure may be directed at this site. As our 1409hp stock had been cultured repeatedly in mosquito cells (> 25 times), cell-adapted selection may have yielded the observed mutation. Previous work has found that repeated passage of DENV-2 (strain Jamaica) in C6/36 cells also resulted in an Ile→Met variation at E6 (Guirakhoo et al., 1993). These authors noted that the DENV-2 with E6-Met was coded in the genome of a fusion-mutant strain of DENV-2, although a direct link between a specific amino acid residue and the fusion phenotype was not established.

Syncytium formation is the result of fusion of cellular membranes leading to the development of a polykaryocyte. DENV-2 fusion-from-without syncytium development has been demonstrated after 20 min incubation following infection with a high m.o.i. (Summers et al., 1989). Additionally, low-pH cell-culture media have been shown to induce DENV-associated syncytium progression (Randolph & Stollar, 1990). These reports are consistent with our findings that a high m.o.i. and/or a low-pH medium resulted in enhanced syncytium development in C6/36 cells (data not shown).

The flavivirus envelope glycoprotein is considered a class II type fusion protein and has a putative fusion peptide found at the tip of domain II (Allison et al., 2001; Rey et al., 1995). Under natural physiological states, the tip region, including the fusion peptide, is thought to be seated in a protected, hydrophobic pocket between domains I and III of an opposing envelope monomer. As a result of exposure to low-pH conditions in the endocytic vesicles, the entire envelope protein undergoes a conformational change, resulting in the exposure of the fusion peptide to membrane surfaces (Bressanelli et al., 2004; Modis et al., 2004). By analysing previous findings on envelope glycoprotein structure, we determined that the E6 site appears to be located within the domain I–III interface pocket in very close proximity to the fusion peptide (Allison et al., 2001; Modis et al., 2003; Rey et al., 1995). We speculate that the E6 Ile residue alters the domain I–III pocket conformation, which then becomes more sensitized to pH conditions and affects the envelope protein’s function, leading to syncytium formation in C6/36 cells. Potentially, this alteration could cause the premature
exposure of the fusion peptide, thereby activating fusion between neighbouring cell membranes. This effect may be mosquito cell-type dependent, as we did not observe syncitia in DENV-2 infected A. aegypti midgut cells.

The specific inhibitory effect of E6-Ile on IV production after electroporation of mammalian cells with in vitro-transcribed mRNA points to the critical role of E6 in translation, replication, packaging, release and/or reinfec-
tion of the virus. This was an unexpected result and may be a unique aspect of DENV-2 1409 replication that warrants more investigation. We acknowledge that this may be an effect of in vitro-derived RNA and that this effect may not occur in nature. However, previous studies have found that low-passage strains of DENV-2 show functional differences in their ability to infect human cells productively (Diamond et al., 2000a, b; Edgil et al., 2003). The low-passage DENV-2 strain Thai CO477 failed to produce any infectious viral particles in human foreskin fibroblast cells at an m.o.i. of <0.1. The differences were attributed to low translational efficiency of input viral RNA. Electroporation of in vitro-transcribed 1409ic RNA may mimic a low m.o.i. and an inefficient translational process might prohibit virus pro-
duction. It should be noted that published reports on other DENV-2 ic constructs have an Ile at E6, but were not inhib-
ited in production of IVs in mammalian cells after electro-
poration (Blaney et al., 2004; Kapoo et al., 1995; Kinney et al., 1997). However, DENV-2 IV production has also been shown previously to be influenced by the unique genetic make-up of the viral genome: specifically, a nucleotide alteration in the envelope gene resulting in an amino acid variation at E-390, together with variations in the 3’ UTR, influenced DENV-2 replication in human cells (Cologna & Rico-Hesse, 2003). Therefore, the overall genetic make-up of the 1409 genome, and not just the E6 locus, may have an influence on translational efficiency and IV production.

The impact of quasispecies generation on IV production from transfected RNA also cannot be excluded. While our P-0 C6/36-generated 1409ic had no unexpected sequence variations, we did find that C6/36 P-3 1409ic virus had a nucleotide change in the genome that produced an Asn→His mutation at E-155 that resulted in the loss of a conserved glycosylation site. The loss of this glycosylation site in dengue viruses due to repeated passage in C6/36 cells has also been described by others (Guirakhoo et al., 1993; Lee et al., 1997). Moreover, these data raise questions about the role and impact of host-cell defences to viral infection (e.g. RNA interference) in mammalian versus insect cells. More analysis of the attributes of the 1409 virus in pro-
ductive infection of host cells will probably provide insight into viral pathogenicity, host-cell response to DENV-2 infection and genetic determinants of efficient replication.

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References


