Functional Characterization of a Conserved RNA Structural Element in Dengue Virus Replication

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Background

- Dengue virus (DENV) is a mosquito-borne positive-sense virus in the *Flaviviridae* family that is primarily transmitted by *Aedes aegypti* mosquitoes. Four genetically distinct serotypes (DENV1-4) circulate globally and infect hundreds of millions of people annually (Guzman et al. 2016).

- Dengue incidence has increased dramatically in recent decades due to urbanization, climate change, and the expansion of mosquito vectors into new geographic regions (Guzman et al. 2016). Despite widespread transmission and increased infection rates, there are still limited antiviral treatment options available (Guzman et al. 2016). Understanding the molecular mechanisms regulating viral RNA replication may support the development of antiviral therapeutics and more efficacious vaccines.

- Previous studies identified conserved RNA secondary structures (Fig. 1) that perform essential regulatory functions during the viral life cycle, including the capsid-coding hairpin (cHP), which regulates translation initiation during viral replication (Clyde et al. 2006) and capsid-coding region 1 (CCR1), which was shown to influence infectious particle production in both mammalian and mosquito cells (Groat-Carmona et al. 2012).

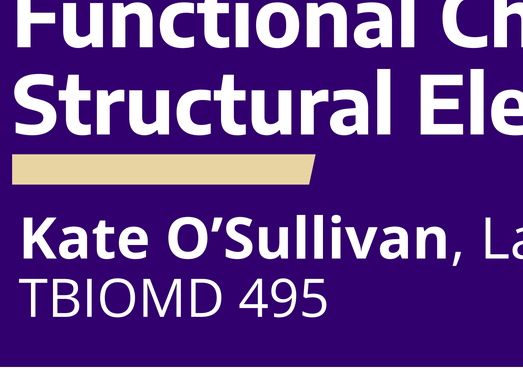

Figure 1. Predicted consensus RNA secondary structure for the 5' untranslated region (UTR) and capsid-coding region of dengue virus serotypes 1-4 (DENV1-4) was generated using Clustal Omega and RNalifold. Conserved structural motifs that are known know to contribute to viral replication are annotated in blue circles, including stem-loop A (SLA), the 5' cyclization sequences (upstream AUG region [UAR], downstream of AUG region (DAR), and complementary sequence [CS]), capsid-coding regions 1 (CCR1), CCR2, CCR3, the capsid hairpin (cHP), and the putative structural element currently under investigation, conserved capsid stem-loop (CCSL).

- The conserved capsid stem-loop (CCSL) was identified in the DENV2 capsid-coding region, acting as one of the candidate RNA structural elements in this investigation (Fig. 2).

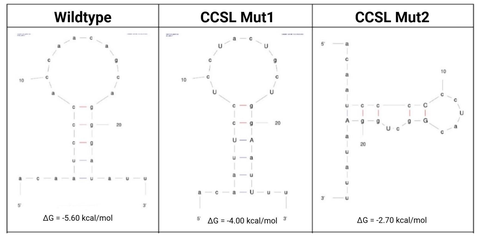

Figure 2. Comparative RNA structure analysis (mFold) of the wildtype (WT) and mutant CCSL variants (Mut1 and Mut2). CCSL Mut1 exhibits sequence-level disruption without affecting proposed secondary structure, whereas CCSL Mut2 demonstrates both sequence- and structure-based disruption. The images illustrate phenotypic structural difference between CCSL Mut1 and 2 relative to the WT control without affecting the protein-coding sequence of DENV2 (relative ΔG values provided).

Experimental Setup

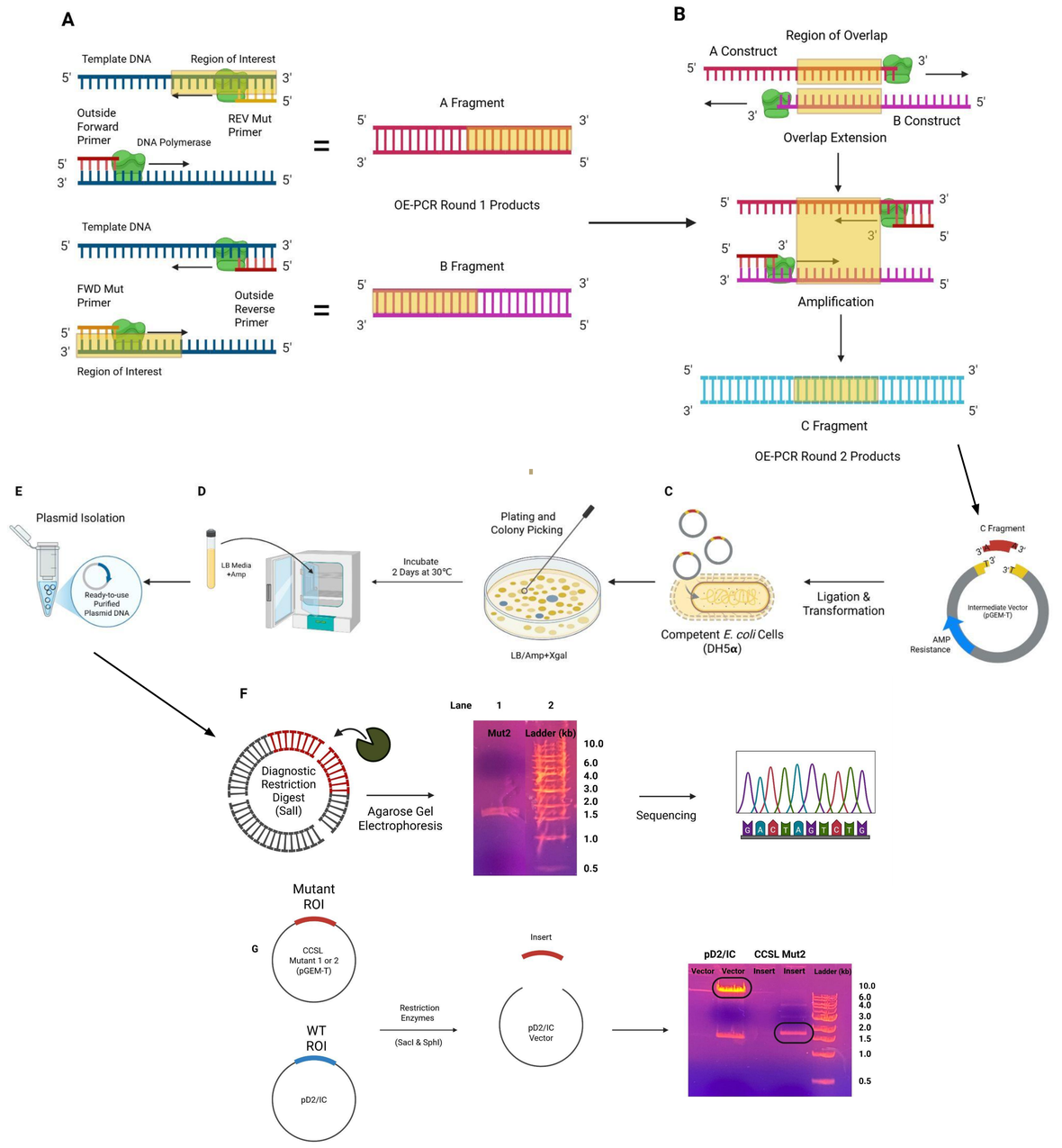

Figure 3. Experimental Layout. Overlap extension (OE)-PCR mutagenesis was previously carried out using the DENV2 infectious clone plasmid (pD2/IC) as the DNA template. The first round of OE-PCR was done using one outside primer and one mutagenic primer to produce the overlapping A and B fragments (Panel A). The A and B fragments were then used as the DNA template for the second round of OE-PCR. The area of overlap between the A and B fragments allows them to come together and be extended during the second round. The resulting C fragment is amplified in the presence of the outside primers and now contains silent mutations in the region of interest (ROI) (Panel B). C fragments (CCSLMut1 and CCSLMut2) were inserted into the pGEM-T intermediate vector and transformed into competent *Escherichia coli* cells (DH5α, Panel C). Resulting bacteria were plated on LB agar with X-gal and ampicillin (Amp) before incubation overnight at 30℃. Colonies were used to set up overnight cultures using LB media with Amp, shaking at 250 rpm at 30℃ for two days (Panel D). A plasmid DNA miniprep was conducted to isolate the mutant pGEM-T constructs from transgenic *E. coli* cultures (Panel E). A diagnostic restriction digest (SalI) was done to determine the directionality of our ROIs in the pGEM-T vector. Using agarose gel electrophoresis, we were able to analyze the results (forward direction bands expected at 3242 bp and 1259 bp; reverse direction bands expected at 4210 bp and 291 bp) (Panel F). Some mutant samples displayed a band near 1.6 kilobases (kb), indicating super-coiled DNA that was unable to be cut by the restriction enzyme. CCSL Mut2 samples with expected banding patterns were sent for sequencing, but identity of the construct is still ongoing. A subcloning restriction digest (SacI and SphI) was performed (Panel G). Agarose gel electrophoresis was performed to analyze results (cut pD2/IC vector expected band 12,338 bp; insert expected band 1,413 bp; pGEM-T vector expected band 2,931 bp) and confirmed the presence of the desired vector and insert. Both vector and insert fragments (CCSL Mut2) were excised from the agarose gel but ligation products are still being screened. Troubleshooting steps were performed throughout the cloning process as needed. Image generated using BioRender.

Future Directions

- Troubleshooting: Adjustments were made to the cloning procedure, specifically the plasmid isolation steps since initial agarose gel analyses did not verify presence of mutant pGEM-T constructs.

- Troubleshooting efforts are still ongoing, but include the following:

Adjustments to Cloning Process
- New batch of competent *E. coli* cells.
- New tube of T4 DNA ligase.
- Cloning process was redesigned to generate mutant pD2/IC infectious clone constructs directly.

Adjustments to Plasmid Isolation Procedure
- Added "dry spin" to Promega miniprep kit.
- Elution volume (water) and final centrifugation length was adjusted to increase plasmid DNA yields.

- Validated mutant pD2/IC infectious clone constructs will be used to generate mutant viral RNAs for functional characterization of the CCSL element.

- Mutant viral RNAs will be introduced into baby hamster kidney (BHK) cells to evaluate the effects of CCSL-disrupting mutations on DENV2 replication efficacy relative to WT virus using plaque assays.

Significance

- Understanding how conserved RNA structural elements regulate DENV replication may reveal new mechanisms controlling the flaviviral lifecycle.

- Since conserved RNA elements are often critical for viral fitness, they may represent potential targets for future antiviral therapeutics or help inform vaccine design strategies.

Acknowledgements

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Citations