Generation of Mutant DENV2 Infectious Clones

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Background

Aedes Vector DENV Structure

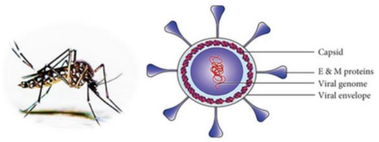

Figure 1. *Aedes Vector and Dengue Viral (DENV) Structure.* Schematic representation of the mosquito vector that transmits DENV as well as the core virion structure of DENV, including the capsid, envelope (E) and membrane (M) proteins, the viral RNA genome, and the viral envelope (Islam et al. 2021).

Dengue fever is a serious rising global health threat, with over 390 million cases every year worldwide (Bhatt et al. 2013, Brady et al. 2012). DENV is an enveloped flavivirus with a positive-sense ssRNA and is the causative agent of dengue fever. There are four distinct serotypes: DENV1-4 (Guzman et al. 2016). Our aim is to clarify the role of RNA structural elements in modulating viral replication-specifically, the Viral Capsid-Coding Structure (VCCS). By introducing silent mutations into an infectious clone of DENV2, we intend to define the functional importance of this putative RNA structural element, which could inform future antiviral strategies that target conserved RNA elements in dengue and other flaviviruses.

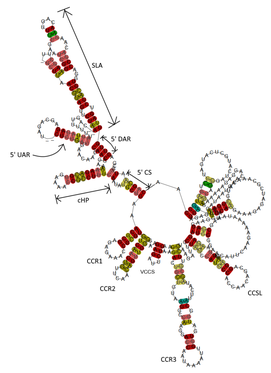

Figure 2. *Consensus RNA Secondary Structure for the 5' Untranslated Region (UTR) and Capsid-Coding Region in the DENV1-4 Serogroup.* The predicted RNA secondary structure for the 5' UTR and capsid-coding region within the DENV1-4 serogroup is shown with annotations depicting key conserved RNA elements. Stem-loop A (SLA) is a conserved RNA structural element essential for viral RNA synthesis (Filomatori et al. 2006). The 5' upstream AUG region (UAR), downstream AUG region (DAR), and conserved sequence (CS) are conserved RNA sequence elements involved in circularizing the ssRNA genome during viral replication (Alvarez et al. 2008, Alvarez et al. 2005, Friebe et al. 2010). cHP (capsid hairpin) is a conserved RNA structural element that determines translational start site selection (Clyde et al. 2006). CCR1 (Conserved Capsid-Coding Region 1) is a conserved RNA structure element within the capsid-coding region that modulates viral assembly (Groat-Carmona et al. 2012). Conserved regions of interest (ROIs) for this study include VCCS and CCSL (Conserved Capsid Stem Loop) though our focus was on disrupting VCCS. Color coding indicates nucleotide conservation within predicted secondary structure for the DENV1-4 serogroup, with red denoting high structural conservation, yellow indicating moderate structural conservation, and green/blue marking variable sites. Dashed lines represent predicted base-pairing (bp) interactions within predicted secondary structures. The consensus secondary structure was generated using RNAalifold.

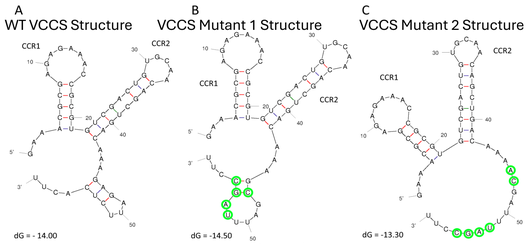

Figure 3. *Wild-Type (WT) vs. Mutant RNA Secondary Structure for VCCS.* Predicted RNA secondary structure in DENV2 for the (A) WT VCCS candidate and two, mutant variants of VCCS: (B) mutant 1, which maintains the predicted WT structure despite the introduced silent mutations, and (C) mutant 2, which highlights how the predicted secondary structure has been eliminated using silent mutations with no change to upstream hairpin structures (CCR1 and CCR2). Free energy values (ΔG) for all structure predictions are indicated with mutated nucleotides circled in green. Structural and sequence changes in the proposed mutants will demonstrate the functional role of VCCS in viral replication. Images generated using mFold.

Experimental Road Map

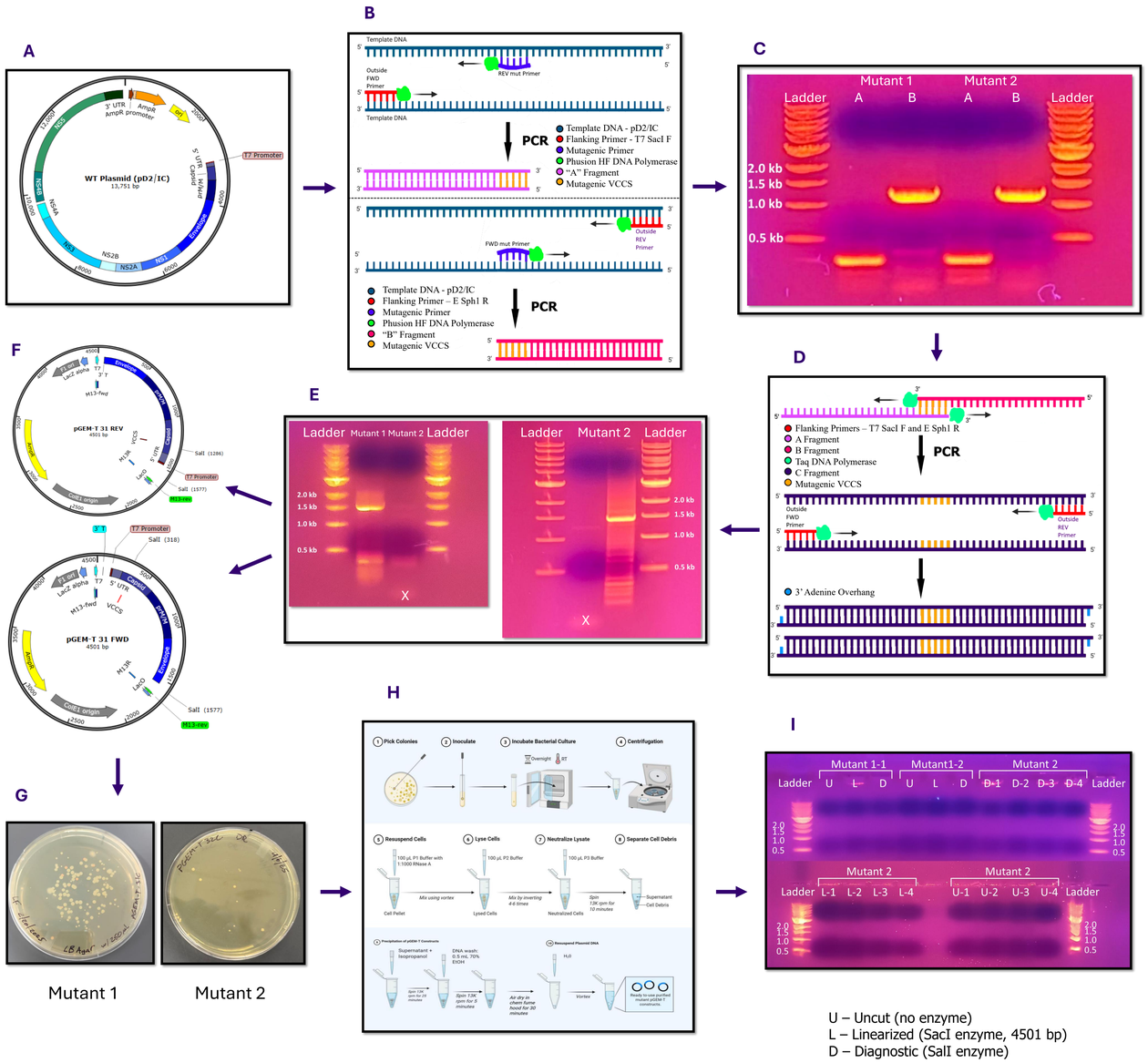

Figure 4. *Experimental Road Map.* (A) Generating mutant DENV2 infectious clones involves targeted mutagenesis to introduce silent mutations into the WT plasmid (pD2/IC). (B) We created A and B fragments for both mutant variants of VCCS using flanking and mutagenic primers targeting the ROI in DENV2 during the first step of OE-PCR (overlap extension). (C) A and B fragments for VCCS mutant 1 and mutant 2 were analyzed using agarose gel electrophoresis and mutant amplicons of the appropriate size were isolated. Expected band sizes for fragments A and B from both VCCS mutants were 300 bp and 1221 bp, respectively. (D) Another round of OE-PCR was performed to create a C fragment that integrated the A/B fragments into a single amplicon. (E) Agarose gel electrophoresis was used to analyze and later isolate the C fragments. Expected band size for the C fragments (mutant 1 and mutant 2) was 1486 bp. (F) Potential orientation of integrated C fragments within the intermediate vector (pGEM-T). 3' adenine overhangs were added to the C fragments using Taq DNA polymerase, which matched with the 5' thymine overhangs within the linearized pGEM-T vector to aid ligation. (G) Competent *Escherichia coli*, presumably transformed with our mutant pGEM-T constructs, were plated on LB/ampicillin agar and incubated at room temperature for 3-4 days to allow for colony growth. (H) Selected colonies, presumed to contain the desired mutant pGEM-T constructs, were chosen for propagation and plasmid isolation. (I) A diagnostic restriction digest was done to confirm the creation of the mutant pGEM-T constructs and analyzed using agarose gel electrophoresis to see if the mutant amplicons were integrated in the forward (FWD) or reverse (REV) direction based on the expected band sizes. Predicted fragment sizes for the diagnostic digests (SalI) are 1259 and 3242 bp for the fragments inserted into pGEM-T in the FWD direction, and 291 and 4210 bp for the C fragments inserted in the REV direction. (J) Assuming the digest pattern was as expected, we would then send the mutant pGEM-T plasmids to be sequenced. Following this would be a subcloning restriction digest to isolate our mutant amplicons before ligation into pD2/IC. Transformation of competent *E. coli* would allow for the propagation of the mutant pD2/IC constructs. Image generated using SnapGene and BioRender.

Conclusion

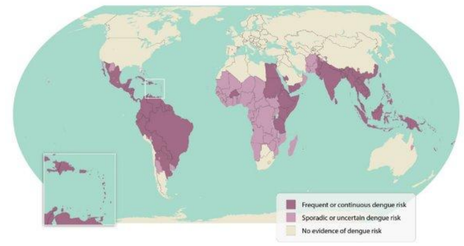

Figure 5: *Global Risk Distribution for DENV1-4.* World map where countries are shaded based on the degree of dengue transmission. Dark purple indicates frequent or continuous risk, light purple indicates sporadic risk or degree of risk has yet to be determined, and cream coloring indicates no current evidence for risk of dengue transmission (CDC 2024). This graphic highlights the need for global dengue research and reaffirms the importance of continued investigation into conserved viral RNA elements, such as VCCS.

What We Accomplished:

- Successfully started engineering two mutant DENV2 infectious clones (VCCS mutant 1 and mutant 2) that contain silent mutations to disrupt the structure or sequence for the candidate RNA structure element.
- Verified fragment assembly from two rounds of OE-PCR using agarose gel electrophoresis analyses, producing expected bands for the A, B, and C fragments for VCCS mutant 1 and mutant 2.
- Subcloned mutant C fragments into the intermediate pGEM-T vector and performed diagnostic restriction digests to verify direction of C fragment insertion.

Limitations:

- Variable diagnostic digest results (aberrant banding patterns) suggests possible recombination or loss of mutant C fragments during pGEM-T cloning step.
- Desalting using an ethanol precipitation protocol was attempted, but was ultimately unsuccessful in improving the outcome for the diagnostic digest.
- Low transformation efficiency and poor DNA quality likely hampered attempts at plasmid isolation from transformed *E. coli*.

Next Steps:

- Subclone mutant VCCS amplicons into pGEM-T intermediate vector.
- Sequence mutant pGEM-T constructs to check whether VCCS sequence has been disrupted.
- Construction of mutant pD2/IC plasmids using restriction digest and ligation.
- Use mutant pD2/IC constructs to generate DENV2 vRNA with desired mutations in VCCS.
- Test mutant vRNAs using functional assays in mammalian cells to establish the effect of VCCS mutations on viral replication and fitness.
- Long-Term Goal: Explore the possibility of targeting conserved RNA structures in antiviral drug design.

Significance

Our research provides new insights into the role of conserved RNA elements in regulating DENV replication. By introducing silent mutations into VCCS, we are seeking to determine if this candidate RNA secondary structure is necessary for viral replication. The two mutants we generated (mutant 1 and mutant 2) will allow us to experimentally distinguish whether the primary sequence of VCCS is important or if its structure is vital for viral replication. Identification of regulatory RNA elements could lead to the design of novel antiviral drugs that target RNA secondary structures, which could reduce the potential for resistance development. While our research is ongoing, these findings set the ground for future research investigating the molecular mechanisms regulating DENV replication.

References

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