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Mutagenic Analysis of Putative RNA Sequence Elements in the Dengue Virus Genome

TBIOMD 495

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Background

- **Dengue fever** is caused by four related mosquito-borne viruses (DENV 1-4) in the family *Flaviviridae*, which includes other public health pathogens such as Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV) (Clyde and Harris 2006). This disease is also known as **breakbone fever,** named for the intense joint and muscle pain typically associated with the illness.
- Breakbone fever is debilitating and can be deadly. Manifestations of the disease vary from flu-like symptoms, including fever, chills and nausea to severe disease, which includes plasma leakage, coagulopathy, and bleeding. Without treatment severe symptoms can dramatically affect blood pressure, cause circulatory shock, and impair organ function (Guzman et al. 2016).
- Dengue infections are responsible for around **21,000 deaths** worldwide each year, and that number is expected to increase due to climate change. The *Aedes* mosquitoes that carry the virus thrive in crowded tropical urban centers like those in Southeast Asia and the Americas, and increased urbanization in these areas coupled with environmental changes are **quickly expanding** their range.
- There is currently **no treatment** for breakbone fever outside of supportive care. Research into **the genetic mechanisms underlying viral replication** will help us to develop novel antiviral medications.
- Previously, candidate RNA sequence elements within the DENV genome were identified based on **sequence conservation** across DENV 1-4 (as well as other serogroups), implying they may be **important for viral replication** (Groat-Carmona et al. 2012). This work focuses on two of those sequences: Conserved Protease Coding-Region 1 (CPCR-1) and Methyltransferase RNA-Dependent Coding-Region 1 (MRdCR-1).

To figure out what RNA elements do, first you find them...

Table 1. Sequence homology calculations for regions of interest compared to background sequences. Percent nucleotide homology for two putative RNA sequence elements (CPCR-1 and MRdCR-1) compared to the background region of the genome (30 nucleotides upstream and downstream of the region of interest) within the DENV1-4, JEV, and TBEV serogroups. Sequence locations for CPCR-1 and MRdCR-1 within the DENV serogroup were used as a point of reference to locate the target sequences within the other serogroups, though the MRdCR-1 sequence could not be found within the JEV serogroup.

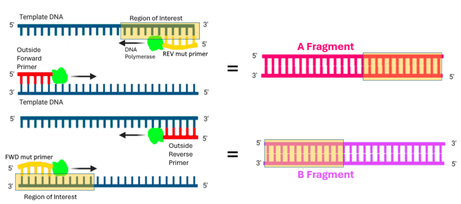
	Flaviviris Serogroup	Region of Interest	Background Sequence (+/-30nts)
CPCR-1	DENV 1-4	86.70%	48.3% (29/60)
	JEV	53.30%	35% (21/60)
	TBEV	60%	58.3% (35/60)
	Flaviviris Serogroup	Region of Interest	Background Sequence (+/-30nts)
MRdCR-1	DENV 1-4	85.20%	48.3% (29/60)
	JEV	--	--
	TBEV	70.40%	63.3% (38/60)

...and then you break them to see what happens.

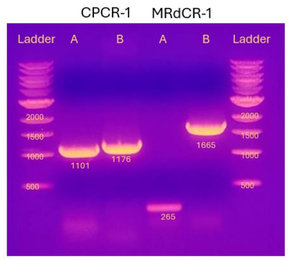
Figure 1. WT vs. mutant CPCR-1 and MRdCR-1 RNA sequence elements. Sequence alignment for regions of interest (underlined) in DENV 1-4 serogroup showing sequence homology (*) for proposed RNA sequence elements, CPCR-1 and MRdCR-1. Sequence alignments were generated using Clustal Omega. Sequence homology in regions of interest decreases after the introduction of proposed silent mutations (lowercase) to DENV2 (highlighted in yellow). Mutagenic primers were generated to introduce these proposed mutant sequences using overlap extension (OE) PCR.

```
                    CPCR-1
DENV4      .tccctttccccagagcaacagc 5513
DENV2      .CCCATTTCCTCAGAGCAATGCA 5511
DENV3      .tgcctttcctcagagcaacgct 5506
DENV1      .ggcctttccacagagcaatgca 5512
             * ***** ********
DENV2
Proposed   .CCCAttCccAcaATCAaaCGCA 5511
Mutant       * ** ** **    **
                    MRdCR-1
DENV4      GGAGTGGAGTGGAAGGGAAGGTCTGCAC 9101
DENV2      TGAGTGGAGTGGAAGGAGAAGGGCTGCAC 9108
DENV3      ACAGTGGAGTAGAAGGAGAAGGACTGCAC 9100
DENV1      TCAGTGGAGTGGAAGGAGAAGGACTCCAC 9109
             ******** ***** ***** ** ***
DENV2
Proposed   TGtcaGGcGTcGAgGGcGAgGGcCTcCAt 9108
Mutant       ** ** ** ** ** ** ** **
```

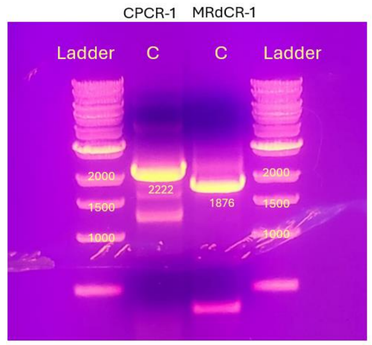
Figure 2. Making DENV2 infectious clones.



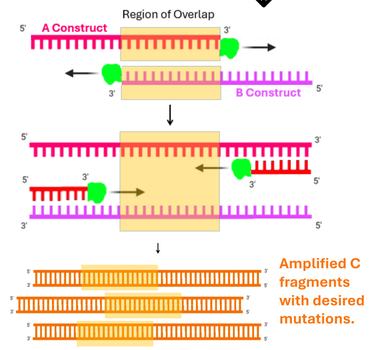
An initial round of OE-PCR was used to incorporate silent mutations into CPCR-1 and MRdCR-1, generating A and B amplicons for each region of interest.



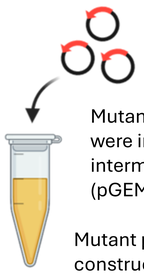
Generation of A and B fragments was confirmed using agarose gel electrophoresis. Expected band sizes (shown in base pairs, bp) for the CPCR-1 A and B fragments and the MRdCR-1 A and B fragments matched expected band sizes when compared to the 1kb ladder. Mutant A and B fragments were isolated from the gel.



Generation of C fragments was confirmed using agarose gel electrophoresis. Expected band sizes for the CPCR-1 and MRdCR-1 C fragments are shown (bp). Bands matched expected fragment sizes when compared to the 1kb DNA ladder. Mutant C fragments were isolated from the gel.

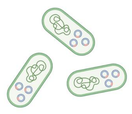


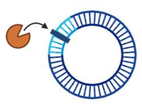
OE-PCR was used to combine the A and B fragments and create C fragments with desired mutations in regions of interest (CPCR-1 and MRdCR-1).



Mutant C fragments were inserted into an intermediate vector (pGEM-T).

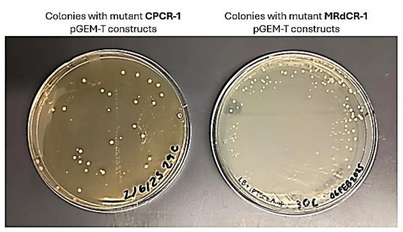
Competent *Escherichia coli* (strain DH5α) were generated by Dr. Groat-Carmona with a demonstrated 10^6 CFU/μg transformation efficiency. This strain is commonly used for cloning and propagating plasmid DNA.





Mutant pGEM-T constructs were propagated in competent *E. coli* cells.

A diagnostic restriction digest and agarose gel electrophoresis analysis was performed, but we were unable to verify plasmid isolation or the presence of our mutant amplicons. Troubleshooting steps were performed.



Transformed *E. coli* cells were plated on LB/AMP agar and incubated at room temperature (RT) for 3-4 days. Colonies were selected for plasmid isolation.

Image generated using BioRender

Troubleshooting and Next Steps

Table 2. Expected agarose gel electrophoresis results for diagnostic restriction digests. These results would demonstrate successful insertion of our mutant C fragments for CPCR-1 and MRdCR-1 into the intermediate vector (pGEM-T) and establish their directionality (forward or reverse).

	Digest	Expected Band Pattern
CPCR-1	Uncut	Single band (supercoiled DNA) at 4882bp.
	Linearized	Single band at 4882bp.
	Diagnostic: Forward Inserstion	Three bands at 2542bp, 1449bp, and 1246bp.
	Diagnostic: Reverse Inserstion	Three bands at 2317bp, 1674bp, and 1246bp.
MRdCR-1	Digest	Expected Band Pattern
	Uncut	Single band (supercoiled DNA) at 4882bp.
	Linearized	Single band at 4882bp.
	Diagnostic: Forward Inserstion	Two bands at 3153 bp and 1729bp.
	Diagnostic: Reverse Inserstion	Two bands at 4646bp and 236bp .

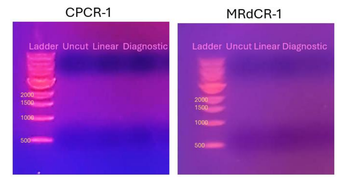


Figure 3. Agarose gel electrophoresis results (representative) from diagnostic restriction digest showed no bands. Gel results from the diagnostic digest would have determined the directionality of our CPCR-1 and MRdCR-1 amplicons in pGEM-T. No bands were visible except for the 1kb DNA ladder. Digest conditions included: uncut (no enzyme), linearized (KpnI for CPCR-1 and XbaI for MRdPCR-1), and the diagnostic digest (ApaLI for CPCR-1 and ApaI for MRdCR-1).

Troubleshooting: Adjustments were made to the plasmid isolation procedure and restriction digest protocols after initial agarose gel electrophoresis analyses did not verify the presence of mutant pGEM-T plasmids. Troubleshooting efforts are ongoing.

Adjustments to Plasmid Isolation:

- Additional centrifugation time after addition of neutralization buffer to maximize amount of plasmid-containing supernatant.
- Additional incubation after addition of isopropanol to maximize precipitation of plasmid DNA.

Adjustments to Restriction Digest:

- Addition of sodium acetate and increased volume of ethanol in precipitation reaction to "clean up" plasmid DNA and reduce salt content.
- Incubation time and temperature for digestion reaction was adjusted to increase efficiency.

- Once the presence of the desired mutations in pGEM-T is confirmed using a diagnostic restriction digest and gel electrophoresis analysis, mutant amplicons will be subcloned into a final DENV2 infectious cloning vector (pD2/IC).

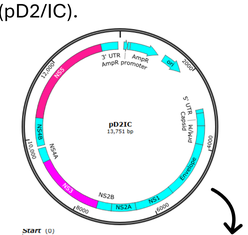
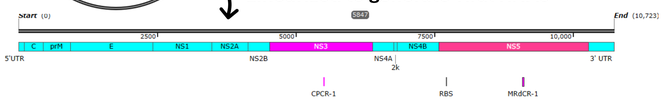


Figure 4. Annotated DENV2 genome within final pD2/IC vector. CPCR-1 and MRdCR-1 regions are shown in the context of the entire DENV2 genome, located within NS3- and NS5-coding regions, respectively. The final vector (pD2/IC) will contain the entire DENV2 genome with our mutant regions incorporated separately. Annotated genome generated using SpapGene.

Linearized to generate viral RNAs



- Mutant pD2/IC constructs will be used to generate mutant viral RNAs that can be analyzed in functional assays to determine the effect of these mutations on viral replication and infectivity.
- This understanding will be foundational for the development of novel antiviral medications to combat dengue fever.

Acknowledgements

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Thank you to Dr. Groat-Carmona for being a diligent, encouraging, and fascinating mentor.

And thank you to all my loved ones who do not know or care what PCR is and got to hear about it anyway.

Citations