Mutagenesis of a Dengue virus replicon by introducing stop codons within NS5
Abbreviation

Abstract

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Abbreviations

A  Adenine
bp base pairs
C  Cytosine
Ca Capsid
DENV Dengue Virus
DF Dengue fever
DHF Dengue hemorrhagic fever
DNA Deoxyribonucleic acid
dNTP Deoxyribonucleosidetriphosphate
DSS Dengue Shock Syndrome
E Envelope
EtOH Ethanol
G Guanine
KB Kilo base
KDa kilo Dalton
LB Luria-Bertani
Mtase Methyltransferase
nMole nano mole
NS nonstructural protein
PBS Phosphate buffer saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PrM</td>
<td>Memberan precursor</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tick-borne encephalitis virus</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WNV</td>
<td>West-Nile virus</td>
</tr>
</tbody>
</table>
Abstract

Dengue Virus (DENV) is a positive single stranded RNA virus presented in four serotypes, DEN1V, DEN2V, DEN3V and DEN4V. Its structure is composed of three structural proteins, capsid, precursor membrane (prM) and envelope, and seven non structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS5 has a vital role in the replication of flavivirus. In this project, four different experimental procedures based on standard protocols, were used to create mutations in NS5 of DEN2V. There was no growth in original plates after overnight observation in experiment 1 and experiment 2, competent cells were streaked on LB ampicillin plates but overgrowth reported in the result after overnight observation. Experience 3 the result of sequencing demonstrated that there was no change in the sequence of NS5 (DEN2V) as compared to the original sequence. Out of all the experimental procedures, optimized mutational results in experiment 4 i.e., insertion of stop codon TAA and TGA were achieved in NS5 of DEN2V Replicon. It is clear from the sequencing analysis of NS5 of DEN2V Replicon after site directed mutagenesis that two stop codons (TAA & TGA) are inserted instead of AAA & AGA in the original SN5 sequence of DEN2V.
1. Introduction

1.1 Dengue virus

Dengue Virus (DENV) is mainly transmitted to humans by the mosquitoes Aedes aegypti and A. albopictus. DENV belongs to the family Flaviviridae having a genomic structure of positive single stranded RNA (Medin et al., 2005). It causes a spectrum of fatal diseases called DHF and DSS (Holden et al., 2006). In the recent time it is clear that DHF and DSS are burdened with world health and more than 50 million people per year are infected in the Western Pacific, South Asia, Central and south America. It is a sharp threat to global health as there are yet no antiviral drugs and vaccine present. It is important for the public health organizations throughout the world that this fatal problem needs much more concentration by developing vaccines and antiviral drugs because and vaccines (Perera et al., 2008).

1.2 Structure of Dengue virus

On the basis of antigenic properties, there are four serotypes of DENV (DEN1V, DEN2V, DEN3V and DEN4V). All these four serotypes of DENV have similar epidemiological and genetical features. The length of dengue genome is about 11000 bases (11kb) which encodes large polyproteins (Pankhong et al., 2009). The diameter of a lipid membrane envelop surrounding the DENV genome is 50nm (Weaver et al., 2009).

The viral polypeptide chain is cut by proteases (both cellular and viral encoded) into short polypeptide proteins. There are three structural proteins, Capsid (Ca), precursor Membrane (prM) and envelope (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Blaney et al., 2007).

1.3 Dengue Replicon and Replication

The dengue Replicon is representing Dengue genomic structure with noninfectious and self replicating properties (Ng et al., 2007).

Complex of NS5 with NS3 is responsible for replication of DENV, this complex has a significant role in RNA replication by attaching two important enzymes which are N-terminal, RNA-cap MTase and C-terminal RdRp domains, so it is clear that NS5 and NS3 are important non-structural protein for replication of RNA (Wigerius et al., 2010).
There are new investigation tools developed to study the life cycle of the virus more properly and to strengthen the novel antiviral strategies against DENV.

The main task is to know the molecular mechanism of dengue virus replication. This will help us to design antiviral strategies to stop dengue infections. After viral infection, the single stranded RNA genome start to synthesize proteins. After synthesizing dengue viral proteins there begins copying of viral RNA, which contributes to the production of a complementary negative stranded RNA. The complementary negative stranded RNA further transcribes to another new form of RNA (positive strand molecule) and this process is catalysed by cellular factors with a virus-encoded RdRp (Iglesias et al., 2011).

1.4 Historical Epidemiology of Dengue

During the Chin dynasty (Common Era 265-420) dengue like complication was first identified in China as early as in the 3rd century and it was notified in 7th & 10th century that similar clinical complication occurred again (Common Era 610) (Hammon et al., 1964). On the other side In Northern Sung dynasty (Common Era 992), there was again Dengue like clinical complication occurred during 7th & 10th century respectively (Murray et al., 2013). The above Dengue like clinical conditions were considered to be transmitted through polluted water (Sundaravadivelan et al., 2013). After a long absence period of seven centuries, again the outbreak of similar clinical condition like Dengue occurred in Panama 1635 and in French West Indies 1699 (Murray et al., 2013). From 1779 to 1788, there were several reports of Dengue from Seville, Spain, Philadelphia, Jakarta and Cadiz (Sundaravadivelan et al., 2013). These reports demonstrate the widespread geographical distribution of Dengue in the 18th century. Which was considered to be the first Dengue pandemic (Hammon et al., 1964). The second series of Dengue pandemic is considered to have happened during 1823 to 1916 (Murray et al., 2013). The second dengue pandemic badly affected the World (from Africa to India to Oceania to the Americas) (Hammon et al., 1964). It is assumed that the same stereotype was involved in all this Dengue pandemic. In 1927-1928, a Greek dengue epidemic caused approximately 1000 deaths and one million cases reported (Murray et al., 2013). Dengue was a common public health problem until the 1940s, during the 20th century, in South Asia the demographics as well as ecological changes created suitable conditions for the evolution of Dengue hemorrhagic fever (Hammon et al., 1964).
During 1960s and 1970s a dengue epidemic was reported in America and the report demonstrated that the single serotype involved in this outbreak, but on the other side in 1960s dengue activity was at peak in most of tropical areas of the world. In 1981 the transfer of dengue genotypes from South East Asia to America occurred (Hammon et al., 1964).

Severity of dengue infection is based on incidence rate in hyper endemic regions, which may cause epidemic and pathogenic severity. About 100 million dengue infection reported per year, which comprising 20,000 deaths and 500,000 cases of DHF (Hammon et al., 1964).

1.5 Why NS5 is important in replication

NS5 is a gene which has a vital role in the replication of flavivirus RNA (Khromykh et al., 1999). NS5 play its role in the translation and in RNA replication (Baleotti et al., 2003). During replication the full-length KUN RNAs starts deletions of nucleotides at 3’-terminal of NS5 gene, which results into a larger RNA portion deletion (Khromykh et al., 1999). Due to this deletion the Chimeric KUN genomic RNA replaced with NS5 gene is unable to replicate in normal BHK cells. NS5 gene encodes Mtase and RdRp, structurally RdRp domains of flavivirus has two cavities A and B which are located in a sub-domain called thumb and provides improvement targets for allosteric inhibitors and can be use for antiviral development (Khromykh et al., 1999).
2. Aims and Objective

The aim of the current project was to create an insertion of two stop codons i.e., TAA and TGA in the genome of the DEN2V Replicon at the NS5 position, comparing four different protocols with troubleshooting.
3. Materials and Methods

3.1 Designing of primers

In the current study mutagenic primers were designed using web tool (Primer-BLAST). Mutagenic primers were used for the amplification of DEN2V with the introduction of a stop codon in NS5. The original sequence of the selected part of NS5 (DEN2 Replicon) is given below.

Original sequence (DEN2 Replicon):

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>5’ AAAGAAGGCATTTAAAGAGGAGAAACGGAC 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Primer</td>
<td>3’ TTTCTTCCGTAATTTTCTCCTTTGCTTG 5’</td>
</tr>
</tbody>
</table>

Mutated Primers of Original Primers as above:

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>5’AAAGAAGGCAATTTAATGAGGAGAAACGGAC 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Primer</td>
<td>3’ GTCCGTTTCTCCTCATTAAATGCCTTCTTT 5’</td>
</tr>
</tbody>
</table>

Two codons AAA and AGA were mutated to TAA and TGA respectively.

(Ref: order ID: 2414680, customer ID: 98618, Order Date: 15l04l2011, Lab No: 5007).

Dilution of mutated primers

335µl of double distal water was added to the forward primer while 324µl of double distal water was added to reverse primer in order to get the yield of 100pmol per µl. These diluted primers (Forward and reverse) were mixed by vortexing samples properly.

Both primers were diluted 10 times (10µl primer solution + 90µl ddH2O). QuickChange® II XL Site-Directed Mutagenesis protocol (Quick change, 2011) was followed.
3.2 Experimental Procedure

3.2.1 Polymerase Chain Reaction: Mutant Strand synthesis Reaction (Thermal Cycling)

The Components found in table 1 were used in the designing of mutagenic primers.

Table 1: Shows the components for the designing of mutagenic primers

<table>
<thead>
<tr>
<th>S.No</th>
<th>Material provided</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Supplied with 10X reaction buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>2</td>
<td>dsDNA template (DENV2 Replicon): 70ng/µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>3</td>
<td>Oligonucleotide primer (Forward primer)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>4</td>
<td>Oligonucleotide primer (Reverse primer)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>5</td>
<td>dNTP mix</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>6</td>
<td>Quick solution</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>7</td>
<td>ddH2O (final volume to be 50 µl)</td>
<td>37.0 µl</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>

dsDNA template (DENV2 Replicone) was already present at the lab. Components of the mutagenic primers were mixed gently and 1µl of Pfu Ultra HF DNA polymerase (2.5U/µl) was added to the reaction mixture.

Denaturation of DNA template was performed at 95°C followed by annealing. Annealing of PCR reaction was carried out at temperature 60°C. The annealed product was extended at temperature 68°C. All the PCR reactions were performed in UNO II (Biometra PCR system).

Cycling parameters for PCR QuickChange® II XL Method

Table 2: Table two Shows the PCR cycles

<table>
<thead>
<tr>
<th>Segments</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>25 minutes</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68°C</td>
<td>25 minutes</td>
</tr>
</tbody>
</table>
3.2.2 Dpn 1 Digestion of Amplification Products

The product was digested with 1µl of Dpn 1 restriction enzyme (10 U/µl), that only cleaves at methylated sites. Samples were incubated at 37°C for 1½ hours according to manufacturer's instructions (Quick Change, 2011).

3.2.3 Transformation of XL10-Gold Ultra competent Cells in First attempt

Transformation of XL10-Gold Ultra competent cells was carried out by the heat shock method. XL10-Gold Ultra competent Cells were thawed and 45µl of XL10-Gold Ultra competent Cells were added to prechilled 14ml BD Falcon polypropylene round bottom tube. 2µl of β-mercaptoethanol (β-ME mix provided in the Kit) was added to the prechilled tube containing XL10-Gold Ultra competent Cells and incubated for 10 minutes on ice. Dpn 1 digested product was added to the tube and the reaction was incubated on ice for 30 minutes. 0.5ml of preheated LB broth was added to the reaction tube and this mixture was then incubated for 1 hour with shaking speed of 225-250 RPM at 37 °C.

3.2.4 Streaking on LB Plates

Competent cells were streaked on LB ampicillin plates and incubated overnight at 37 °C. After overnight incubation no growth was seen and thus the experimental procedure 1 was stoped.

3.3 Experimental Procedure 2.

The same concentrations of forward and reverse primers were used in experiment 1 was used in experimental procedure 2.

3.3.1 Polymerase Chain Reaction

PCR reaction mixture for the current experimental procedure was prepared according to the conditions shown in Table3.
Table 3: The quantity of materials used in the current experiment

<table>
<thead>
<tr>
<th>S.No</th>
<th>Material provided</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>5</td>
<td>dNTP mix</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>6</td>
<td>Quick solution</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>7</td>
<td>ddH2O (final volume to be 50 µl)</td>
<td>31.0 µl</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>50.0 µl</strong></td>
</tr>
</tbody>
</table>

Table 4: The experimental cycles used for the PCR reaction in experiment 2 is described in table 4.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>95°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>35 minutes</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68°C</td>
<td>35 minutes</td>
</tr>
</tbody>
</table>

PCR operation was stopped and PfuUltra DNA polymerase was added for optimization of experimental procedure.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>25 minutes</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68°C</td>
<td>25 minutes</td>
</tr>
</tbody>
</table>

3.3.2 Dpn 1 Digestion of Amplified Products

In this experiment 1µl of Dpn 1 restriction enzyme (10 U/µl) was used to digest the template. Dpn1 digested product was incubated at 37°C for 1½ hour.

To increase the concentration of DNA in the sample reaction by using “sodium acetate/Ethanol precipitation method” method in this 5.3µl of 3M Sodium acetate (PH 5.2)
was added to 159µl (3times of the DNA solution) of cold EtOH 99% to the total solution of DNA which was 53µl. The mixture was incubated at -20°C for 2 hours. The incubated solution was centrifuged at 13000RPM for 15minutes at 4°C.

Supernatant (EtOH) was removed and the pellet was rinsed with 50µl of 70% EtOH. The washed pellet was centrifuged at 4°C for 10 minutes at 13000 RPM. Supernatant was removed and the pellet was air dried and then pop spined. ddH₂O was added to the pellet.

3.3.3 Transformation of XL10-Gold Ultra competent Cells

The similar methodology which used in first attempt was followed for the transformation process.

3.3.4 Streaking on LB Plates

Competent cells were streaked on LB ampicillin plates and incubated overnight at 37°C. An overnight incubation was carried out for the LB ampicillin plate, and a master plate was prepared. The master plate was divided into 5 sections. Sterile conditions were used for streaking and inoculation of master plate and one falcon tube containing 10ml of LB ampicillin respectively. Section 1 was utilized for falcon tube 1, section 2 for falcon tube 2 and so on. All five inoculated falcon tubes and the master plates were incubated overnight at 37 °C in shaking incubator.

3.4 Experimental Procedure 3.

In experiment 3 the mutagenic primers were diluted 1:10 times compared to the concentrations used in 1st and 2nd experimental procedures.

3.4.1 Polymerase Chain Reaction

The same procedure was used as previously mentioned in experimental procedure 2 and details are tabulated in table 3 and 4.

3.4.2 Dpn 1 Digestion of Amplification Products

A similar procedure was followed as mentioned previously for the Dpn1 digestion of amplification product.
3.4.3 Transformation of Stbl3 competent Cells

In this experiment Stbl3 competent cells were used for transformation. Stbl3 competent cells were thawed on ice. DNA solution containing DNA pellets was mixed with 200µl of Stbl3 competent cells. The reaction tube was incubated for 40 seconds at 40°C followed by incubation of the reaction tube on ice for 2 minutes. The content of the reaction tube was added to 500µl of LB-Broth (preheated at 42°C) and mixed thoroughly, the mixed product was incubated at 37°C for one hour on a shaker with a speed of 225-250 RPM.

3.4.4 Streaking on LB Plates

After one hour, 50µl of reaction product was streaked on Original LB ampicillin and the remaining quantity of reaction sample was centrifuged. The removed supernatant was streaked on another original plate 2. These two Original plates were placed in an incubator for growth at 37°C for an overnight incubation.

Growth occurred in plate 1 after an overnight incubation, plate 1 was used as an original plate for further processing. The next step of streaking and inoculation was done by using one master plate divided into three sections and three falcon tubes respectively. Falcon tubes were used again because it is a fundamental concept of cross checking for growth with the corresponding part of master plate. A new loop was used for each divided part. To check the growth of the remaining parts of the master plate, the same procedure was conducted as for part 1 of the master plate. All three falcon tubes and the master plate were incubated overnight for growth at 37 °C in shaking incubator.

3.4.5 Preparation of Mini Prep from Selected Falcon tube containing Growth

QIA prep Spin Mini prep Kit was used for the purification and isolation of plasmid DNA. The following steps carried out at room temperature for Mini Prep.

i. Falcon tube was centrifuged for 12 minutes. Supernatant was discarded. 250µl of buffer P1 was added to the separated DNA pellets followed by resuspension of the pellets in a falcon tube.

ii. 300µl of buffer P2 was added to the tube, containing the product from step I, the color became blue and mixed thoroughly.
iii. Add 350µl of the buffer N3 to the tube. Mix immediately and gently by inverting the tube 6 times until the color becomes whiter.

iv. The reaction tubes were centrifuged at 13000 RPM for ten minutes.

v. Supernatant was collected and applied to a QIA prep spin column followed by centrifugation for 50 seconds and the flow-through is discarded.

vi. Add 0.5ml buffer PB to QIA prep spin column and centrifug 30 to 60 seconds and again discard the flow-through. Then add 0.75ml buffer PE and centrifuged for 50 second then discarded the flow-through and centrifuged again for an additional minute to discard residual washed buffer.

vii. 50µl of EB buffer was added at the center of QIA prep followed by centrifugation for a minute. The DNA is eluted.

viii. The eluted DNA product was sent for sequencing.

3.5 Experimental Procedure 4.

The next and final experimental procedure was almost the same as the experimental procedure 1 except for the increase in the concentration of DNA in sample reaction. Sodium acetate/Ethanol precipitation method was used for the concentration of DNA before the transformation of Dpn I treated DNA from sample reaction to XL10-Gold Ultra competent Cells. Streaking of the colonies was carried out on the original LB plates. The growth was satisfactory and ideal on the original. A single isolated colony was selected from the original plate and streaked on master plates and inoculated into falcon tubes. After an incubation period there was growth on master plates and in falcon tubes.

Mini prep was prepared from selected falcon tubes. Similar steps of mini prep were followed given in the procedure. 2 (see heading 3.4.5). Sequence analysis was carried out on collecting DNA sample.

4. Results

The result of the sequence analysis expresses a successful site direct mutation by insertion of stop codons. Between 89 to 92 numbers of nucleotides of NS5 sequence of DEN2V Replicon.

It is important to design a primer by mutation of the NS5 (DEN2V Replicon) and it is necessary to introduce stop codons at the beginning of NS5 to stop the production of the protein. Under consideration of 5’ to 3’ the selected part of the NS5 of primer design is from
76 (number of nucleotides in NS5) to 106 (number of nucleotides in NS5) and 89 to 94, respectively. There are two Codons selected for Mutagenesis because some time one fails. That is why we insert two stop codons at adjacent positions for maximum chance of success. There are three Adenine nucleotides (AAA) in first codon from 91 to 93. In the mutant the first adenine is replaced by Thymine (T) and in next codon there are AGA (adenine, guanine, and adenine) and in that case the first adenine (A) is replaced by thymine (T). The sequencing analysis of first three experiments proved that there was no change in the sequence of NS5 (DEN2V Replicon) as compared to the original sequence. While the sequencing results of fourth experiment confirmed that mutations occurred in NS5 (DEN2V Replicon) gene as compared to the original sequence and both stop codons were successfully introduced in the beginning of NS5 (DEN2V Replicon).

Figure 1. Sequencing of Mutated NS5 of DEN2V. This figure demonstrates the desire positive mutation by insertion of two stop codons at 89 and 92 respectively.

Figure 2. Enlarged section from figure 1

It is clear that first stop codon TAA has replaced AAA (Lysine) and second stop codon TGA has replaced AGA (Arginine) (Figure 1).

5. Discussion

Four experimental approaches were conducted to achieve mutagenesis in NS5 of the DEN2V Replicon. Only one successful attempt was achieved which is mentioned in the result section. Experimental procedure 1 was conducted on standard protocol without any troubleshooting but no positive result was found. In experimental procedure 2, the quantity of dsDNA
template and quick solution were increased to 4µl and 2µl respectively. The PCR cycles were also increased (divided into two continuous programs). We also added 1µl of Pfu Ultra DNA polymerase at the root of both programs for best result to enhance the activity of polymerase chain reaction. In experiment 2 the unsatisfactory growth on the master plates was the main cause of failure. Then experimental procedure 3 was performed to achieve required result, in this Stbl3 competent cells were used for transformation instead of XL10-Gold Ultra competent Cells. In the sequence analysis of experiment 3 there was no mutation in NS5 of DEN2V. Here the main cause of failure may be the use of Stbl3 competent cells instead of XL10-Gold Ultra competent Cells because Gold cells are the best option for this mutagenesis. After the failure of experimental procedure 3 the experimental procedure 4 was performed in which the mutation occurred successfully by introducing two stop codons at the start of the NS5 gene (Figure 3). Due to successful mutation the replicon become disabled to replicate and become a non-functional Replicon which can be used as control e.g. in studies searching for allosteric inhibitors of DENV replication using functional replicons.

![Diagrammatic presentation of Wild type and Mutated NS5 gene of DEN2V.](image)

**Figure 3.** Diagrammatic presentation of Wild type and Mutated NS5 gene of DEN2V.
6. Acknowledgments

I dedicate this MS research work to my Parents for their immense love, encouragements and full support at any stage. I would like to give my heartfelt appreciation to my wife for her verbal support and also Arhan khan (MY SON) whose innocent love makes me be able to achieve this MS qualification.

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Special thanks to my friends and my colleagues in Pakistan as well as in rest of World.
7. References


