

## Cloning of RNA-dependent RNA Polymerase (RdRp) Gene from Genotype Dengue Type-2 (New Guinea-C Strain)

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### ABSTRACT

Dengue virus causes febrile disease in human. Dengue infection causes dengue fever that is not life threatening. However, a severe form of the disease called dengue hemorrhagic fever (DH) or dengue shock syndrome (DSS), proven to be fatal. A positive single stranded RNA virus genome encodes for a single polyprotein precursor and is arranged in the order of NH<sub>2</sub>-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. The purpose of this study was to clone NS5 gene that encodes for the RNA-dependent RNA polymerase (RdRp). This enzyme plays an important role in viral RNA replication. The RdRp associated by cofactors produce minus-strand single stranded RNA, which in turn, serves as a template for the production of new plus-strand single stranded genome. The virus RNA was extracted from *Aedes albopictus* cell line C6/36 that was infected with dengue virus type 2. Then, the extracted virus RNA was used as the template for RT-PCR. A 2.7 kb DNA fragment, representing the RNA-dependent RNA polymerase gene, was successfully amplified using specific primers. The PCR product was then cloned into cloning vector (pGEM-T) and transformed into *E. coli* JM109.

*Keywords:*

### INTRODUCTION

Dengue fever (DF) is an acute febrile viral disease frequently presenting with headaches, bone or joint and muscular pain, rash and leucopenia as symptoms caused by dengue virus infection. Dengue hemorrhagic fever (DHF) is characterized by four major clinical manifestations: high fever, hemorrhagic phenomena, often with hepatomegaly and in severe cases, sign of circulatory failure. Such patient may develop hypovolaemic shock resulting from plasma leakage. This is called dengue shock syndrome (DSS) and can be fatal. DHF/DSS is a leading cause of hospitalization and death among children in many southeast-asia countries (Gubler, 1998; Monath, 1994). Dengue viruses are members of the *Flavivirus* family of positive-strand RNA viruses. The genome of the New Guinea-C strain of DEN2 is 10,723 nucleotides long and contains a type 1 cap structure at the 5' terminus but lacks a poly(A) tail at the 3' end (Irie *et al.*, 1989; Chambers *et al.*, 1990). The genome encodes a single poly-protein, NH<sub>2</sub>-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH, which is processed within the endoplasmic reticulum (ER), producing the capsid (C), precursor membrane (prM) protein, and envelope protein (E) through cotranslational processing by the ER-resident signal peptidase (Markoff, 1989; Nowak *et al.*, 1989; Svitkin *et al.*, 1984). The C-terminal portion of the polyprotein, NS1 to NS5, is processed into at least seven nonstructural proteins in the ER by both the host protease(s) and the virally encoded serine protease, NS2B/NS3, of the trypsin family. NS5, the largest of the

DEN2 structural proteins, contains conserved motifs consistent with those of RNA-dependent RNA polymerases (RdRP) encoded by several positive-strand RNA viruses (O'Reilly *et al.*, 1998; Poch *et al.*, 1989.). One step in dengue virus life cycle is replication of the genomic viral RNA. In this step, the RdRp associated by cofactors, produces minus-strand single stranded RNA which in turn serves as template for the production of new plus-strand single stranded genomes. The newly made viral genomes are encapsidated into the nucleocapsid proteins. The synthesis of this RdRp would involve the interaction between the RdRp protein itself and the nucleic acid (RNA). Thus, cloning of the RdRp gene of dengue virus is the beginning for the expression step and for the future experiments.

### MATERIALS AND METHODS

Dengue virus type 2 (New Guinea-C Strain) and *Aedes albopictus* cell line C6/36 were used in this study. Extraction of virus RNA was done using QIAamp® Viral RNA Mini Kit (Qiagen, Inc.). 3 sets of primers were designed based on published nucleotide sequence of Dengue virus type 2 (New Guinea-C Strain) (Accession No. M29095) to amplify the 2.7 kb RdRp gene. PCR was carried out to amplify 3 fragments i.e. 239 bp (fragment A1), 1673 bp (fragment A2) and 915 bp (fragment B) in size. The sequence of the primers used are as follow:

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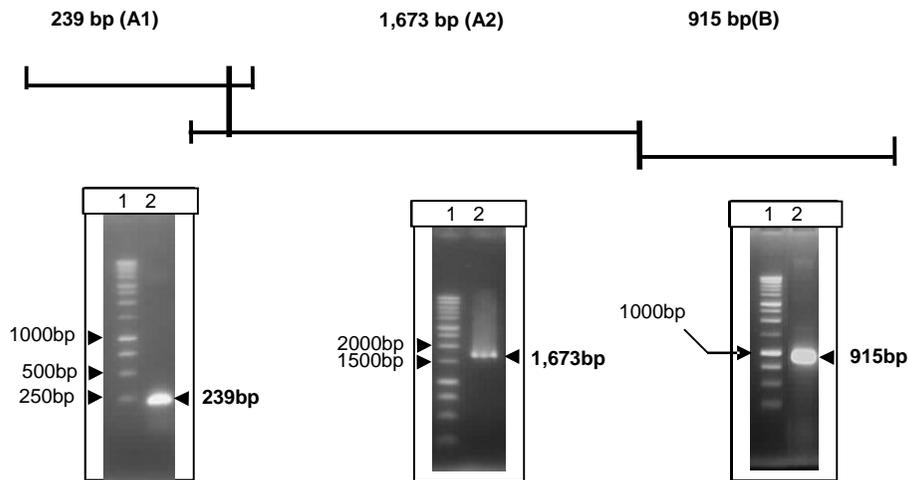
- (1) Forward : 5'-CCA-ACA-CGC-ATA-TGG-GAA-CTG-G-3'  
Reverse : 5'-CCC-TTC-TGG-CGC-GCC-CAT-ATT-TC-3'
- (2) Forward : 5'-CGC-TGT-GTC-GCG-AGG-CTC-AG-3'  
Reverse : 5'-TAT-TGA-GTC-CAT-AGG-TAC-CAA-CTT-GTC-CAC-3'
- (3) Forward : 5'-TGG-GAC-AAG-TTG-GTA-CCT-ATG-GAC-TCA-ATA-3'  
Reverse : 5'-GTT-AGT-GGA-TCC-TTC-TAC-CAC-3'

All purified fragments (PCR products) were ligated to pGEM®-T vector system (Promega, Inc.) and transformed into *E.coli* JM 109. Plasmid containing fragment A1 and A2 was subjected to restriction enzyme digestion using *ScaI* and *NruI*. Purified A1 and A2 fragment were then ligated and transformed. Plasmid of positive clone which contain ligated fragment A1 and A2 as well as the plasmid that contain fragment B were then introduced to digestion with *ScaI* and *KpnI*. These two fragments were then purified, ligated and transformed into *E.coli* JM 109 to obtain a 2.76 kb of complete RdRp gene. The plasmid of the right clone was subjected to digestion with *BamHI* and *EcoRI*, and further verified using PCR and DNA sequencing using SP6 and T7 primers.

## RESULT

Fragments with sizes of 239 bp (Figure 1a), 1673 bp (Figure 1b), and 915 bp (Figure 1c), representing A1, A2, and B respectively, were successfully amplified and ligated into PCR vectors. These individual fragments are parts of the RdRp gene based on results from DNA sequencing (data not shown).

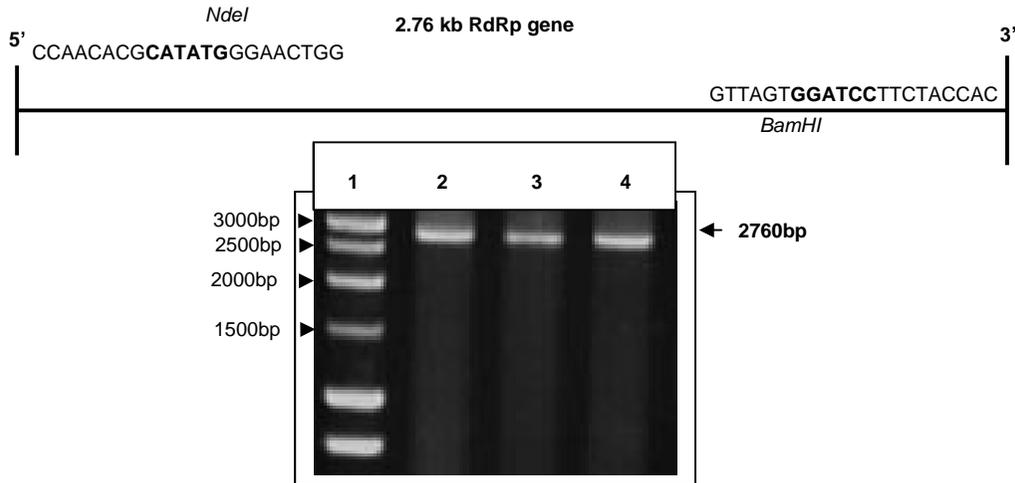
The presence of overlapping restriction enzyme sites allowed for the assembly of all the fragments to produce a full-length RdRp gene. A 2.76 kb fragment was successfully assembled to construct the full-length RdRp gene as shown in Figure 2, after PCR screening using the sequence specific primer pair (forward A1/reverse B).



**Figure 1a:** A successfully amplified PCR product of 239bp in size named as fragment **A1**.  
Lane 1: 1kb DNA ladder  
Lane 2: PCR product (239bp).  
Observed on 0.8 % agarose

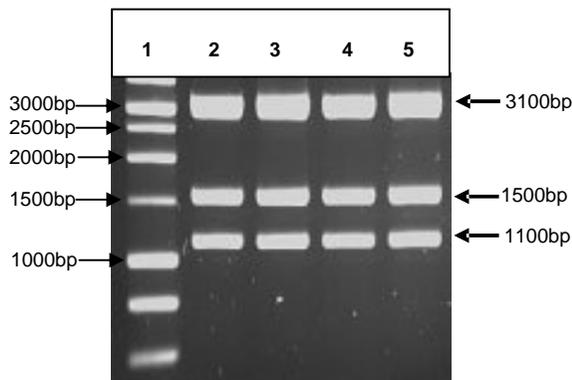
**Figure 1b:** A successfully amplified PCR product of 1,673bp in size named as fragment **A2**.  
Lane 1: 1kb DNA ladder  
Lane 2: PCR product (1,673bp)  
Observed on 0.8% agarose gel.

**Figure 1c:** A successfully amplified PCR product of 915bp in size named as fragment **B**.  
Lane 1: 1kb DNA ladder  
Lane 2: PCR product (915bp)  
Observed on 0.8% agarose gel.



**Figure 2:** Bands with the sizes of 2,760 bp, representing complete RdRp gene.  
Lane 1: 1kb DNA ladder.  
Lane 2, 3 and 4: A product of PCR at 2.7kb (full-length RdRp gene) using constructed plasmid as a template and specific primers (forward 1/reverse 3)  
Observed on 0.8% agarose gel.

Restriction enzymes analysis was performed to further confirm the suspected clones. Clones carrying the full-length RdRp gene would produce three bands with the sizes of 3kb, 1.5kb, and 1.1kb when digested with enzymes *Bam*HI and *Eco*RI (Figure 3).



**Figure 3:** Restriction enzyme digestion of clones carrying the full-length RdRp gene using *Bam*HI and *Eco*RI.  
Lane1: 1 kb DNA ladder  
Lane 2, 3, 4, and 5: Clones carrying the full-length RdRp gene digested with *Bam*HI and *Eco*RI.

Sequence analysis of the full length RdRp gene cloned using BLAST programme showed 99% similarity with the dengue virus type 2 complete genome (Gene Bank Accession No: M29095) (Figure 4).

## DISCUSSION

An attempt to amplify a 2.7kb of RdRp gene in one shot was failed, probably due to a strong secondary structure present on viral RNA. Subsequently, three sets of primers were designed to amplify the gene of interest into three overlapping fragments. All three fragments were digested and sub-cloned to obtain a full-length RdRp gene. *Nde*I site was introduced using the forward primer at the 5' end of the interest gene while *Bam*HI site was introduced using the reverse primer at the 3' end of the gene for subsequent cloning purposes. A full-length RdRp gene with the size of 2.76kb was successfully constructed. This complete RdRp gene was assembled from three overlapping fragments of RdRp gene. Restriction enzyme analysis, PCR screening using SP6/T7 primer pair and gene specific primer, and sequence analysis using BLAST programme (Figure 4), confirmed the identity of the constructed clone. Hence, the RdRp gene of dengue virus type 2 (New Guinea C-Strain) has been successfully cloned into *E. coli*.

## CONCLUSION

The RNA-dependent RNA polymerase (RdRp) gene of dengue virus type-2 (New Guinea-C Strain) was successfully amplified and cloned from the isolated viral RNA genome.

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Clone:      46  gggaaactggcaacataggagagacgcttggagagaaatggaaaagccgattgaacgcatt 105
           |||
Published: 7569 gggaaactggcaacataggagagacgcttggagagaaatggaaaagccgattgaacgcatt 7628

Clone:      106 agggaaaagtgaattccagatctacaagaaaagtggatccaggaagtggatagaacctt 165
           |||
Published: 7629 agggaaaagtgaattccagatctacaagaaaagtggatccaggaagtggatagaacctt 7688

Clone:      166  agcaaaaagaaggcattaaaagaggagaaacggaccatcacgctgtgtcgcgaggctcagc 225
           |||
Published: 7689 agcaaaaagaaggcattaaaagaggagaaacggaccatcacgctgtgtcgcgaggctcagc 7748

Clone:      226  aaaactgagatggatcgtcgagagaaatattggtcacaccagaagggaaagttagtgacct 285
           |||
Published: 7749 aaaactgagatggatcgtcgagagaaatattggtcacaccagaagggaaagttagtgacct 7808

Clone:      286  cggttgcccagaggaggctggtcatactattgtggggactaaagaatgtaagagaagt 345
           |||
Published: 7809 cggttgcccagaggaggctggtcatactattgtggggactaaagaatgtaagagaagt 7868

Clone:      346  caaaggcctaacaaaaggaggaccaggacatgaagaacccatccccatgtcaacatatgg 405
           |||
Published: 7869 caaaggcctaacaaaaggaggaccaggacatgaagaacccatccccatgtcaacatatgg 7928

Clone:      406  gtggaatctagtagctcttcaaagtggagttgacgttttcttctactccgccagaaaagtg 465
           |||
Published: 7929 gtggaatctagtagctcttcaaagtggagttgacgttttcttctactccgccagaaaagtg 7988

Clone:      466  tgacacattgtgtgtgacataggggagtcgtcaccaaatcccacggtagaagcaggacg 525
           |||
Published: 7989 tgacacattgtgtgtgacataggggagtcgtcaccaaatcccacggtagaagcaggacg 8048

Clone:      526  aaacctcagagtccttaacttagtgaaaaattggttgaacaacaacaccaattttgcat 585
           |||
Published: 8049 aaacctcagagtccttaacttagtgaaaaattggttgaacaacaacaccaattttgcat 8108

Clone:      586  aaaggtttctcaaccatacatgccctcagtcataagaaaaatggaagcactacaaaggaa 645
           |||
Published: 8109 aaaggtttctcaaccatacatgccctcagtcataagaaaaatggaagcactacaaaggaa 8168

Clone:      646  atatggaggagccttagtgaggaatccactctcacgaaactccacacatgagatgtactg 705
           |||
Published: 8169 atatggaggagccttagtgaggaatccactctcacgaaactccacacatgagatgtactg 8228

Clone:      706  ggtatccaatgcctccgggaacatagtgtcatcagtgaacatgatttccaggatggtgat 765
           |||
Published: 8229 ggtatccaatgcctccgggaacatagtgtcatcagtgaacatgatttcaaggatggtgat 8288

Clone:      766  caacagattcacaatgagacacaagaaagccacttacgagccagatgtagacctcggaag 825
           |||
Published: 8289 caacagattcacaatgagacacaagaaagccacttacgagccagatgtagacctcggaag 8348

Clone:      826  cggaaccgcaacatcggaattgaaaggagatac 860
           |||
Published: 8349 cggaaccgcaacatcggaattgaaaggagatac 8383
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**Figure 4:** Sequence alignment between the full length RdRp gene cloned and the dengue virus type 2 complete genome (Accession No: M29095)

#### ACKNOWLEDGEMENT

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