

## **Genetic Stability The Protein Encoding Envelope (E) Genes Dengue Virus Serotype-4 Passaged in Vero Cell As A Candidate Chimera Vaccine Material**

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### **ABSTRACT**

This study aims to analyze genetic stability of the gene encoding the envelope protein (E) dengue virus serotype-4 passaged in vero cells, Denv-4 passaged in vero cells serially then continued with RNA extraction at passage 0, 10, 20, 30, 40, 50, and 60, and then continued with two step PCR and amplification, and sequencing then analyze the nucleotide stability with BLAST and MEGA 5 software. The result shows that there are many variable site in nucleotide and amino acid with high mutation rate 57.4% for nucleotide and 71.9% for amino acid, while the similarity between passages are high ranging from 91% - 98%. The conclusion for this study is Denv-4 after analyzed shows that the gene encoding protein E has many variable site but high in similarity.

**Key words: Dengue virus serotype-4, envelope, passage, stability**

### **INTRODUCTION**

Dengue virus has become one of the viruses that receive high attention in the world because of the lack of a licensed vaccine that can provide protection against all four dengue virus serotypes (WHO, 2009). Dengue virus is spread by Aedes species mosquitoes (*A. aegypti* and *A. albopictus*) in tropical and subtropical areas, the dengue virus is included in the genus *Flavivirus* which has four serotypes, namely Denv-1, Denv-2, Denv-3, and Denv-4, which are single-stranded positive RNA genomes (Coller et al., 2011, Mantel et al., 2011). Cases of disease

caused by Denv-4 are described as sylvatic genotypes, namely dengue viruses that circulate exclusively in non-human primates and can be transmitted by Aedes mosquitoes and in majority cases of dengue hemorrhagic fever are caused by secondary infection of Denv-4 (Klungthong et al. al., 2004).

The wild strain virus type has been successfully patented through several passages in primary dog kidney cells and monkey kidney cells (Chi-Lee et al., 2012). The biggest challenge in developing live-attenuated vaccines is evaluating the genomic stability of the new

vaccines to ensure that the genomic heterogeneity produced by replication does not affect the stability of viral attenuation (Kenney et al., 2011). Protein E mediates the main biological activity that plays a major role in viral infectivity, in addition to several neutralizing epitopes that induce protective antibodies that are also present in protein E, therefore protein E is an antigen that has received high attention in the dengue vaccine

## MATERIALS AND METHODS

### Research Type and Research Design

This research is a laboratory experimental study with a time series research design. Analyzing the genetic stability of the envelope protein coding gene (E) Denv- 4 of the University of

### Research Samples

Virus samples were selected ITD Denv-4 isolates from various regions in Indonesia and had been identified and attached to vero cells,

### Research Variables

The definitions of the variables in this study are as follows: Independent variable: passage. Dependent variables: nucleotide arrangement,

### Primary Design

The primers used in this study were primers designed by the Dengue Institute of Tropical Disease team, Airlangga University based on data from GenBank GQ398256.1, complete

development strategy (Coller, 2011 ; Kuhn et al., 2002).

This study was designed to improve the chimera vaccine based on protein E for a vaccine model that is able to induce cellular and humoral immune responses for a long time without interference, reactogenicity, high safety based on Indonesian local isolates.

Airlangga Institute of Tropical Disease (ITD) isolate paired on vero cells based on the arrangement of nucleotides, amino acids, between passages-0, 10, 20, 30, 40, 50, and 60.

using systematic sampling techniques aimed at 0, 10, 20, 30, 40, 50, and 60 passages.

amino acid composition. Control variables: vero cell culture, temperature, CO2 incubator

genome at positions 939 - 2423 based on the genomic map for dengue virus 4 by Kuiken and Turmond, (2005) such as in Appendix 3, FBSO, Citrogen technologies Pte. Ltd. Singapore.

Table 1. Specific Forward and Reverse primers for E Denv-4 protein

Kode	Sequence
FDen4/Env	5'-AGGAGCAGACACATCAGAAG -3'
RDden4/Env	5'-TTGCACCTCTGTATGTGGAC -3'

### RNA extraction

The vero cell culture that had been inoculated with Denv-4 was a coded dish culture blind passage (BP) 0, 10, 20, 30, 40, 50, and 60 each added trizole 1000  $\mu$ l for lysis of cells and resuspended, then put into a microtube then 200  $\mu$ l of chloroform was added, shake for homogenization and incubated at room temperature for 5 minutes. Centrifugation at 12,000 rpm, 15 minutes, at 4°C. Aquaface was taken and put into a new 1.5 ml microtube and 500  $\mu$ l of isopropanol was added, for RNA

precipitation, shake and incubated at room temperature for 10 minutes. Centrifuged at a speed of 12,000 rpm, 10 minutes, at 4 ° C, then discard the supernatant carefully so that the pellets are not wasted and washed with 1000  $\mu$ l of 75% ethanol each. Centrifugation was performed again at a speed of 9,100 rpm, 7 minutes at 4°C, discarded the supernatant and then let it air dry on laminar flow, then added 10  $\mu$ l of nuclease free water and piped for homogenization followed by RT-PCR for cDNA synthesis.

### cDNA SYNTHESIS

Reverse Transcriptase Polymerase Chain Reaction was carried out in two steps using invitrogen's ThermoScript reagent. The first was to make a master mix, for one reaction in a sterile 0.5 ml microcentrifuge tube, mix 7  $\mu$ l of NFW; 2  $\mu$ l 10 mM dNTP Mix; 2  $\mu$ l of RNA; and 1 $\mu$ l of Env reverse primer. Denv-4 (containing 20 pmol /  $\mu$ l), then incubated in a thermal cycler at a temperature of 65 ° C for 5 minutes, after

incubating place it on ice for 5 minutes, and adding a second master mix consisting of 2  $\mu$ l NFW; 4  $\mu$ l 5xcDNA Synthesis Buffer; 1  $\mu$ l 0.1 DTT; 0.5  $\mu$ l RNase OUT (40 U /  $\mu$ l); and 0.5  $\mu$ l of ThermoScript, incubated in a thermal cycler at 50 ° C for 45 minutes and followed by a temperature of 85 ° C for 5 minutes, then added with 1  $\mu$ l of RNase H and incubated at 37 ° C for 20 minutes.

### PCR amplification

PCR amplification was done by making master mix in a sterile 0.5 ml microcentrifuge tube with a component of 2  $\mu$ l 10X PCR buffer minus Mg ++; 0.6  $\mu$ l 50 mM MgCl<sub>2</sub>; 0.4  $\mu$ l 10 mM dNTP mix, 0.5  $\mu$ l of forward Env primers. Denv-4; 0.5  $\mu$ l of Env reverse primer. Denv-4; 2  $\mu$ l cDNA template; 0.2  $\mu$ l Taq DNA Polymerase; and 13.8

$\mu$ l of NFW for a total of 20 $\mu$ l. Incubate the tube in a thermal cycler with a Lid setting of 95 ° C, predenaturation 94°C, 10 minutes; denaturation 94°C, 1minute; annealing 55°C, 1 minute; extension 72°C, 2 minutes; the cycle is repeated at the denaturation stage for 40 times; extension prolongation 72°C, 10 minutes; and keep at 4°C.

### Electrophoresis

A total of 4  $\mu$ l of PCR results and 1  $\mu$ l of Blue Juice were added to 1% LE agarose well

containing 1  $\mu$ l / 50 ml Ethidium Bromide for running process. The marker used as a reference

was a 100 bp DNA ladder consisting of 15 fragments between 100 bp and 1,500 bp and an additional 2072 bp fragment. The plates were run under 100 V, 400 mA conditions in 30-45

### SEQUENCING

Sequencing is carried out in several stages, the first is purification of PCR products with QIAquick Gel Extraction Kit then labeled with Big Dye Terminator and loading on the sequencing machine This reaction is based on

### RESULT AND DISCUSSION

The results of the electrophoresis of the E Denv-4 protein coding gene isolate ITD passages 10, 20, 30, 40, 50, and 60 using 1% agarose gel and

minutes. Then the electrophoretic gel was read on the Gel Documentation / Transluminator UVP and documented.

fluorescent labeled material which will give the appropriate color change after passing the laser light on the automatic machine ABI Prism™ 310 Genetic analyzer Perkin Elmer.

100 bp DNA ladder as markers can see that the ribbon is parallel to the marker standard at the 562 bp position.

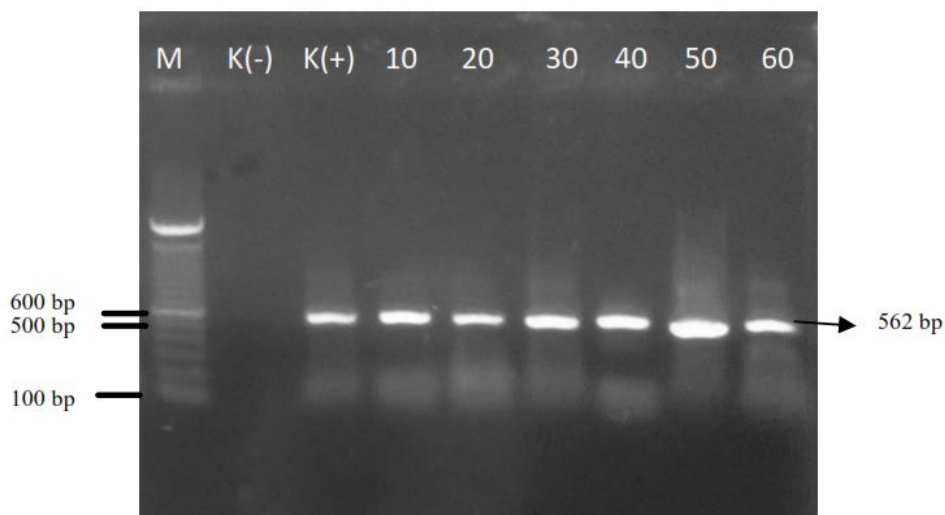


Figure 1 Denv-4 Electrophoresis Results  
Note: M = Marker, K (-) = negative control, K (+) = positive control

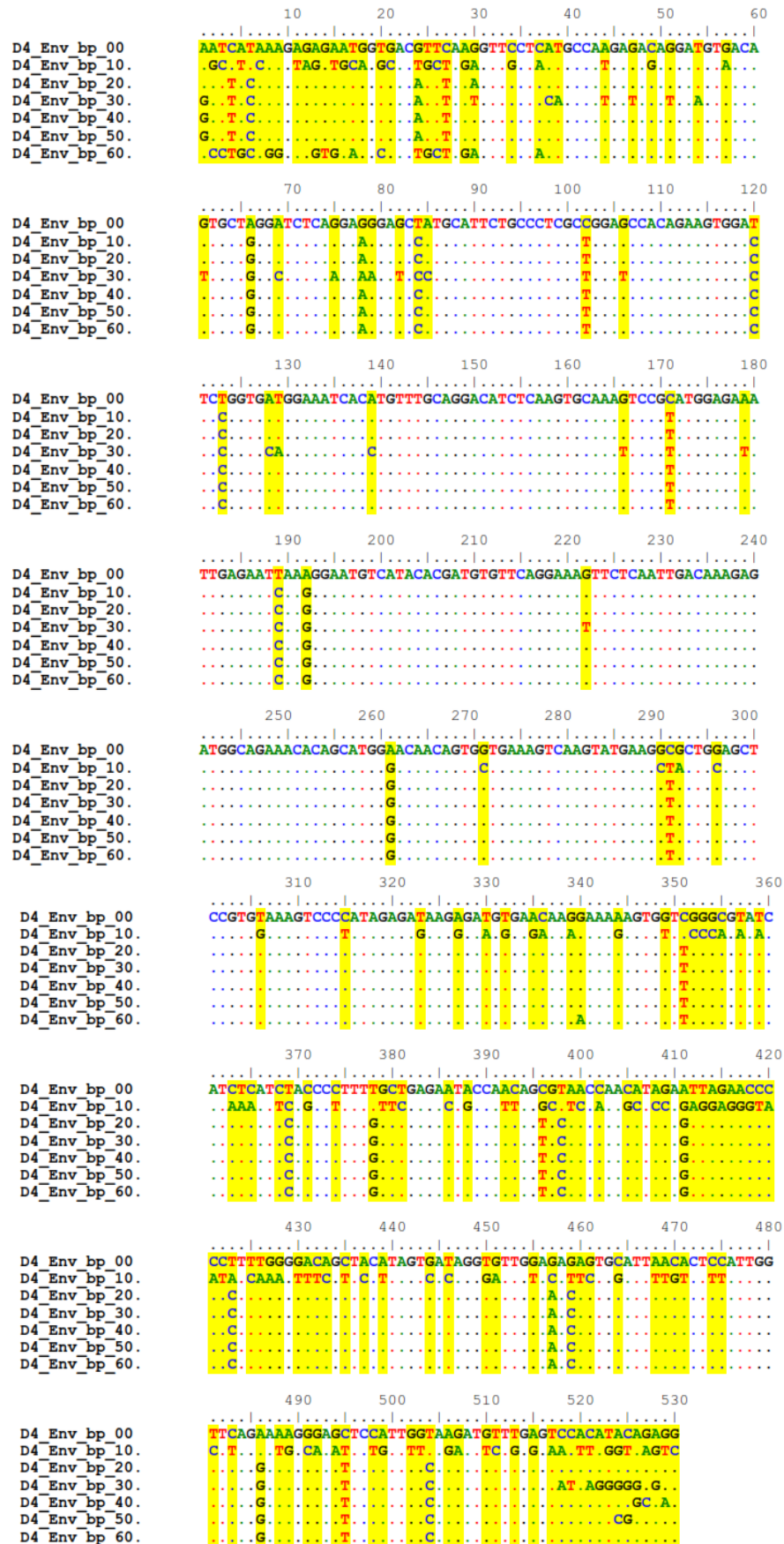


Figure 2 Analysis of the 0, 10, 20, 30, 40, 50, and 60th passage of Denv-4 nucleotide strands.

Variable sites from Bp 0 to Bp 10 there are 117 variable sites (22%), from Bp 0 to Bp 20 there are 28 variable sites (5.3%), from Bp 0 to Bp 30 there are 57 variable sites (10.8%), from Bp 0 to Bp 40

there are 31 variable sites (5.8%), from Bp 0 to Bp 50 there are 29 variable sites (5.4%), from Bp 0 to Bp 60 there are 43 variable sites (8.1%).



Figure 3 Denv-4 amino acid analysis of 0, 10,20,30,40,50, and 60 passages

Amino acid variable sites from Bp 0 to Bp 10 there are 74 variable sites (41%), from Bp 0 to Bp 20 there are 4 variable sites (2,3%), from Bp 0 to Bp 30 there are 25 variable sites (14%) , from Bp 0 to Bp 40 there are 7 variable sites (3,9%), from Bp 0 to Bp 50 there are 6 variable sites (3,4%), from Bp 0 to Bp 60 there are 13 variable sites (7.3%). RNA viruses can undergo mutations on

average from 10<sup>-3</sup> to 10<sup>-5</sup> substitution per nucleotide copy or about 1% to 3%. This allows for rapid adaptation and evolution when viral RNA is subjected to selective pressure changes. These mutations are the result of high frequency in diverse mutant populations of the viral genome (Liu et al., 2008; Nor et al., 2010).

Table 2 BLAST Analysis of E Denv-4 Protein Coding Genes

<i>Description</i>	<i>Max score</i>	<i>Total score</i>	<i>Query cover</i>	<i>E value</i>	<i>Ident</i>	<i>Accession</i>
<u>D4 Env bp 10</u>	422	422	60%	2e-122	91%	41603
<u>D4 Env bp 20</u>	824	824	98%	0.0	95%	51077
<u>D4 Env bp 30</u>	695	695	96%	0.0	91%	2037
<u>D4 Env bp 40</u>	821	821	97%	0.0	95%	45581
<u>D4 Env bp 50</u>	819	819	98%	0.0	95%	46461
<u>D4 Env bp 60</u>	785	785	94%	0.0	95%	50537

Table 3 BLAST Analysis of Amino Acids for E Denv-4 Protein Coding Gene

<i>Description</i>	<i>Max score</i>	<i>Total score</i>	<i>Query cover</i>	<i>E value</i>	<i>Ident</i>	<i>Accession</i>
<u>D4 Env bp 10</u>	207	207	93%	1e-72	63%	42027
<u>D4 Env bp 20</u>	359	359	100%	2e-132	98%	36447
<u>D4 Env bp 30</u>	314	314	97%	1e-114	88%	5783
<u>D4 Env bp 40</u>	355	355	99%	7e-131	97%	52625
<u>D4 Env bp 50</u>	354	354	100%	3e-130	97%	43173
<u>D4 Env bp 60</u>	340	340	98%	1e-124	94%	14715

From Table 2, the closest similarity to BP 0 is BP 20, 40, 50, and 60, namely 95%, while BP 10 and 30 have similarity to BP 0 only 91%. Table 3 shows that BP 0 compared to BP 10 has only 63% similarity, BP 0 compared to BP 20 has 98% similarity, BP 0 compared to BP 30 has a similarity of 88%, BP 0 compared to Bp 40 has a similarity of 97%, BP 0 compared to Bp 50 has a similarity of 97%, BP 0 compared to BP 60 has a similarity of 94%. From existing data, BP 0 compared to BP 20 has the highest similarity, namely 98% and the lowest is seen in the comparison of BP 0 with BP 10.

According to Sasmono et al (2012) the occurrence of changes in the composition of

nucleotides can indicate the dynamics of the genetic characteristics of this virus because the virus is repeated in phase so that this virus adapts to cells by changing its genetic makeup. Chemical mutagenesis and analysis of Denv-4 sequencing results conducted by Blaney et al., 2001 have resulted in the identification of a large number of point mutations that result in amino acid substitution in all genes except Capsid and NS4A and point mutations in untranslated regions (UTRs). The mutations examined every few passages in vero cells, may be adaptive changes that give the Denv-4 increased efficiency of replication in vero cells.

## CONCLUSION

The nucleotide composition analysis had a high mutation rate of more than 3%, especially in the 10th passage compared to the 0th passage, which was 22% and the one with the lowest mutation rate was the 20th passage, namely 5.3% and the average mutation rate 57.4%. The analysis of amino acid composition has a high diversity of more than 3% because there are many site variables, especially in the 10th passage where the diversity reaches 41% while the lowest mutation rate is in the 20th passage of 2.3%, and the average -the mutation rate average was 71.9%.

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