Serotype Specific Sequences for Multi Test Line Nucleic Acid Lateral Flow Development

Sekuen Spesifik Serotipe Untuk Pengembangan Multi Test Line Nucleic Acid Lateral Flow

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Abstrak

Virus dengue (DENV) merupakan penyebab demam berdarah dan dengue shock *syndrome*. Virus ini memiliki 4 serotipe yang berbeda yaitu DENV1-4. Serotyping diperlukan untuk deteksi dan aktivitas surveillance penyebarb penyakit. Akhir-akhir ini dikembangkan metode *Nucleic Acid Lateral Flow* (NALF) untuk konfirmasi hasil amplifikasi yang mudah tanpa peralatan rumit. Metode ini menggunakan *capture probe* diimmobilisasi pada *test pad.* Studi yang telah dilakukan sebelumnya mendapatkan empat sekuen spesifik serotipe Virus Dengue yang akan dibuat *dipstick* NALF. Sekuen tersebut dianalisis *%GC content*, homologi target, panjang 100% homologi non target, suhu hibridisasi, dan struktur sekunder untuk memperkirakan kemampuan *capture probe* tersebut pada reaksi hibridisasi. Sekuen *capture probe* diaplikasikan pada NALF dan diuji menggunakan ssDNA sebagai sampel untuk melihat kemampuannya. Hasil ke-empat *capture probe* untuk DENV1,2,3,4 berturut-turut adalah CACCAGGGGAAGCTGTACCCTGGTGGT, GTGAGATGAAGCTGTAGTCTCACTGG, GCACTGAGGGAAGCTGTACCTCCTTGCA, AGCCAGGAGGAAGCTGTACTTCTGGTGG. Aplikasi pada NALF yang dibuat secara fabrikasi menunjukan tidak terjadi hibridisasi silang menggunakan larutan dapar *stringency* tinggi.

Kata kunci: *capture probe; hibridisasi; nucleic acid lateral flow;* penentuan serotipe; virus dengue

Abstract

Dengue virus that agent of dengue fever and dengue shock syndrome has 4 different serotypes. Serotyping is needed for diagnosing and surveillance activities of disease spreads. Recently, the Nucleic Acid Lateral Flow (NALF) technique has been extended to confirm the results of easy amplification without complicated equipment. The aim of this study was designing capture probe for serotyping dengue virus (DENV) using NALF method. This study have conducted an analytical study to obtain four specific sequences of Dengue Virus serotypes to develop serotype specific NALF. Several parameters were used to analyzed Dengue genome sequences i.e. % GC content, target homology, length of 100% homology continue of non-specific bases, hybridization temperature, and secondary structure to estimate the probe's capture capability in the hybridization reaction. The capture probes were applied to NALF and assayed using single strand DNA sample to check its performance. The result of four specific sequence capture probes, DENV1, 2, 3, 4 were CACCAGGGGAAGCTGTACCCTGGTGGT, GTGAGATGAAGCTGTAGTCTCACTGG, GCACTGAGGGAAGCTGTACCTCCTTGCA, AGCCAGGAGGAAGCTGTACTTCTGGTGG. Application to fabricated NALF gave no cross hybridization with high stringency buffer assay.

Keywords: capture probe; dengue virus; hybridization; nucleic acid lateral flow; serotyping

Introduction

Dengue virus has 4 different serotypes that can cause fatal disease. Surveillance activities to detect the cause of the disease uses serotyping by ELISA and RT-PCR method. Complicated equipment can be avoided by using Nucleic Acid Lateral Flow (NALF) to confirm the results of amplification reaction (Doyle and Uthicke, 2020; Antiochia, 2021). This method uses immobilized capture probe on the test pad (Yrad *et.al*., 2019). We have conducted an analytical study to obtain four specific sequences of Dengue Virus serotypes that NALF dipstick will be made. The sequence is analyzed according to its % GC content, homology target, 100% continue length of homology to non-target, hybridization temperature, and secondary structure to estimate the capture probe capability in the hybridization reaction.

Detection of DENV infection is generally based on clinics manifestation such as a sudden high fever and simple blood tests (platelets and hematocrit concentrations) (Muller *et.al* 2017; Triana *et.al*., 2020; Ngim *et.al*., 2021). ELISA examination based on the presence of antibodies can be done (IgM and IgG) (Lee *et.al*., 2019; Yow *et.al*., 2021; Prommool *et.al*., 2021). The virus isolation method takes at least 7 days to find out the results (Azhar *et. al.,* 2010; Kuczera *et. al*., 2016). ELISA examination based on antigen virus such as NS1 is performed at the beginning of infection before viremia ends but does not distinguish serotypes (Duong *et al*., 2011, Mardekian and Robert, 2015). Examination method based on nucleic acid is rarely performed due to the high-cost. Although it is good for detecting the presence of viruses at the beginning of infection and able to distinguish serotypes (Kao *et al*., 2005; Mardekian and Robert, 2015).

Recently, amplicon product detection method has been developed using Lateral Flow Dipstick (LFD) based on Nucleic Acid Lateral Flow Immuno Assay (NALFIA) or Nucleic Acid Lateral Flow (NALF). The use of LFD fabrication NALFIA method takes less time to confirm the results of the amplification reaction based on whether there is an appearance of lines on the LFD test line or not (Nimitphak *et al*., 2010; Yrad *et.al*., 2019; Kabir *et. al*., 2021). Research on the use of LFD for NALFIA / NALF has been carried out for detecting pathogenic diseases in both animals and humans (Ding *et al*., 2010; Nimitphak *et al*., 2010; Kusumawati *et al*., 2015, Kusumawati and Fatimah, 2018; Ajie *et. al*., 2021). Detection of DENV infection and determination of DENV serotypes have been developed using NALFIA-based LFD with isothermal amplification such as RT-LAMP and NASBA (Yrad *et.al*., 2019; Griffioen *et. al*., 2020). Universal NALFIA format with the introduction through DNA sequences or specific probes which is labeled as introduction molecules (antigen-antibodies) so that the hybridization process is needed with amplification (amplicon) products (Antiochia, 2021).

To develop the NALF method based on the introduction of DNA hybridization reactions, capture probe is needed as target recognition which is immobilized in the test pad. This study is for analyzing the DENV genome to obtain the fourth specific serotypes sequence. In this analysis, the ability of the hybridization probes with specific serotypes was calculated.

Materials and Methods

Specific DENV Serotype Sequence Identification

Specific DENV serotype sequence identified by performing multiple alignment of DENV serotypes 1-4 obtained from gene bank. MEGA ver. 5.05 were employed to align all DENV sequences. After Specific DENV serotype sequence candidates were obtained, the prediction of its potential as hybridization probes candidates were analyzed based on several parameters, such as: % GC content, bases length, homology, secondary structure, hybridization temperature, secondary structure, and melting temperatures. To estimate the secondary structure, Clone Manager 9 bioinformatics software from Scientific & Educational Software (USA) was used. Melting temperature (Tm) is calculated using the formula from Sambrook and Russel (2001).

$$Tm = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0,41(\%G+C) - 675/n) - 1.0m - 0,72$$

Na^+: Na^+ concentration (M), n: sequence length, m: mismatch percentage, f: methanamide percentage.

Serotype Specific NALF Preparation

Four capture probe, DENV1, DENV2, DENV3, and DENV4, were supplied by Integrated DNA Technology (USA). A Capture probes (100μM) were immobilized on nitrocellulose membrane using Isoflow dispenser (Imagine Technology, USA) at dispense rate of 0,06 μL/mm. Gold nano particle which conjugated with streptavidin (GNP-SA) (15 nm, OD 20) was spread surface on nylon membrane at dispense rate of 2x0,25 μL/mm. Both capture probe and GNP-SA were dehydrated an hour using dehumidifiers. Capture probes were cross linked on membrane with expose to Ultra Violet lamp, 256 nm and 30 W, (G30T8 Phillip, USA) during 120 second. Nitrocellulose membrane, absorbent paper and nylon membrane that all of them were sticked on plastic backing sheet then sliced to 3,8 mm wide using cutting machine (Matrix 2360 Kinematic Automation, USA).

NALF Detection of Synthetic DNA Correspond to DENV Genome

To analyze the performance of NALF, synthetic DNA correspond to DENV 1 -4 genome sequence were employed. The synthetic DNA sequences were consisted of 73 (DENV2,3,4) and 98 (DEN1) bases of single stranded DNA labeled with biotin supplied by Integrated DNA Technology (USA).

Table 1. Synthetic DNA sequences to a analyze the performance of NALF

Serotype	Sequences
DENV1	Biotin-GGTCTCCTCTAACCTCTAGTCCTTACC ACCAGGGTACAGCTTCCCCTGGTGTTG
DENV2	Biotin-GGTCTCCTCTAACCTCTAGTCCTTTCA GTGAGACTACAGCTTCATCTCACCTTG GGCCCCCATTGTTGCTGCG
DENV3	Biotin-GGTCTCCTCTAACCTCTAGTCCTTTGC AAGGAGGTACAGCTTCCCTCAGTGCTC GGGCCCCGCTGCTGCGTTG
DENV4	Biotin-GGTCTCCTCTAACCTCTAGTCCTTCCA CCAGAAGTACAGCTTCCTCCTGGCTTC GGGCCCCCTTTTTTGCTGC

Assay was done using sample amplicon (100 pmol) and 100 μL buffer assay (0,125% Tween-20, 0,5% BSA, 15% methanamide, and 5% sucrose in 0.5x PBS). Glycerol was added to give 15% concentration into buffer assay (Hendarta *et al*., 2020). All reagents were dropped into nylon membrane. The successful hybridization reaction was showed with signaling of color which raise on test line of nitrocellulose membrane after 90 minutes dropping.

Results and Discussion

This study performed alignment analysis of complete DENV 1-4 genomes (around 10,000 bases). DENV isolates came from various regions in Indonesia and the surrounding regions or countries such as Singapore, Malaysia, Philippines and Thailand. The complete DENV 1-4 genome were taken from the Gen Bank database. The details are in Table 2.

Table 2. The DENV genome sequences used for alignment analysis

No	Origin	Serotype of DENV				Total
		1	2	3	4	
1	Indonesia	2	14	16	-	32
2	Malaysia	3	-	-	-	3
3	Singapore	3	3	-	3	9
4	Thailand	12	51	22	-	85
5	Philippines	1	-	-	1	2
	Amount	21	68	38	4	131

The alignment results (Figure 1) showed conserved bases sequence regions (> 70%) for all four serotypes (white background) and a conserved bases sequence (> 70%) which is specific to each of the serotypes (colored background). The bases sequence is spread throughout the regions in the DENV genome with a length of about 5-20 bases. The translation regions (400-10,000 position) were conserved regions (5-20 bases) for the four least serotypes and there are several regions of the sequence of conserved bases that are specific to each serotype. The 5 'end regions (0-400 position) and 3' end (10,000-10,500 position) which are untranslated region (UTR) areas have many conserved regions for the four serotypes

and there several sequences of conserved bases that are specific to each serotype. The region is suitable for amplification targets and serotype determination especially by hemi, nested and probing amplification. Researchers who use that region, are Lanciotti *et al.* (1992), Harris *et al.* (1998), Wu *et al.* (2001), Yong *et al.* (2007), Gurukumar *et al.* (2009), Sahni *et al.* (2013) and Anggraini (2016).
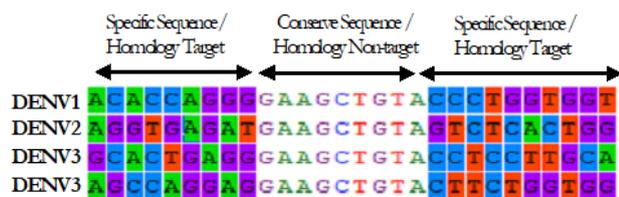

Figure 1. Specific and conserve sequence location on DENV genome

Lanciotti *et al.* (1992), Harris *et al.* (1998), Yong *et al.* (2007) used the 5'UTR region as conventional amplification target for RT-PCR

with serotyping based on the difference in target length of amplicon target produced. Wu *et al.* (2001) and Anggraini (2016) used 5'UTR region as amplification target of isothermal NASBA which aimed to perform serotyping based on probing. At 5' UTR region, there were specific sequences of DENV 1-4 serotype which flanked by bases conserve sequences (>90%) with 100-200 bases in length (Figure 1). Those regions had 2 long bases sequences which located near to 7-10 bases of specific conserve region (70-100% in homology) for each DENV1-4 serotype. DENV1 genome has specific regions bases sequence with 8 bases in length (CACCAGGG) with 100% homology and 10 bases (CCCTGGTGGT) with 100% homology. DENV 2 genome has a specific regions bases sequence with 8 bases in length (GG-T / C-GA-G / A-AT) with 75% homology and 10 bases (G / A-TCTC-A/G-CTG-G / A) with 70% homology. DENV 3 genome has a bases sequence with 9

Table 3. Oligonucleotide sequence of designed probe

Probe	Sequence	Genome position
DENV 1	**CACCAGGG**GAAGCTGTA**CCCTGGTGGT**	10.562–10.588[a]
DENV 2	**GGTGAGAT**GAAGCTGTA**GTCTCACTGG**	10.551–10.577[b]
DENV 3	**GCACTGAGG**GAAGCTGTA**CCTCCTTGCA**	10.535–10.562[c]
DENV 4	**AGCCAGGAG**GAAGCTGTA**CTTCTGGTGG**	10.481–10.508[d]

Note: Specific bases to serotype printed in bold; [a] based on DENV 1 *IND genomic RNA complete genome strain: 98901530 DF DV-1*; [b] based on DENV 2 *IND strain DENV-2/ID/1016DN/1975 complete genome*; [c] based on DENV 3 *IND genomic RNA complete genome strain: 98901403 DSS DV-3*; [d] based on DENV 4 SIN *isolate EHI310A129CY10 complete genome.*

Table 4. Capture probe analysis

Probe	% Homology to genome				Length of 100% homology continue of non-specific bases			
	DENV1	DENV2	DENV3	DENV4	DENV1	DENV2	DENV3	DENV4
DENV 1	>96,3	51,9	67,7	55,6	9	9	13	11
DENV 2	51,9	>88,9	37	59,3	9	9	9	9
DENV 3	64,3	35,7	100	53,6	13	9	9	11
DENV 4	57,1	60,7	53,6	>96,3	11	9	11	9

Table 5. Distribution analysis of specific bases, homology, and a series of non-specific bases for probe design

Probe	n	%G+C	T_m (°C)	Hybridization temperature (T_m-20°C)	Secondary structure ΔG. 28°C (Kcal)
DENV 1	28	61,7	48,53	28,6	-8,4
DENV 2	27	51,8	43,88	23,9	-3,3
DENV 3	28	57,1	47,07	27,1	-0,9
DENV 4	28	57,1	47,07	27,1	-

Notes: n: nucleotide length; %G+C: GC content percentage; Tm: melting temperature calculating using Sambrook and Russel (2001) with mismatch 1 base, Na+ 0,068 mM in PBS 0,5x, and methanamide 15%; Secondary structure was calculated using Clone Manager 9

bases in length (GCACTGAGG) with 100% homology and 10 bases (CCTCCTTGCA) with 100% homology. DENV 4 genome has a specific regions bases sequence with 9 bases in length (AGCCAGGAG) with 100% homology and 10 bases (CT-T / C-CTGGTGG) with 90% homology. Those two bases sequences are separated by conserved bases sequences (89%) with 9 bases in length (G / A-AAGCTGTA). These specific base sequences are used for capture probe on the DEN-NALF test line.

The probes position in Dengue Virus genome (Table 3) were chosen in the same or adjacent region (10481-10588). The adjacent position is very beneficial because it will require a short length of amplicon as target which short nucleic acid amplification provides high amplification efficiency. This position was the only region in the Dengue Virus genome where had serotype specific sequence in the adjacent position in four serotypes. The amplification method that is developed by Lanciotti *et al*. (1992), Harris *et al*. (1998), and Yong *et al*. (2007) uses the RT-PCR amplicon around 250-700 bp in length. Serotyping based on amplicon length uses visual appearance by using electrophoresis so that it requires a different length of amplicon to be able to distinguish amplicon positions based on different bases lengths. Whereas Wu *et al*. (2001) and Anggraini (2016) used a short amplicon (120 bp) since they used a specific capture probe of each serotype which adjacent bases position/region.

The shorter probe, the faster hybridization takes place. Shorter probes are better in discriminating towards the target genes (Farrell-Jr, 2010). The length of the probe bases is directly related to the hybridization temperature. As an oligonucleotide probe, hybridization takes place at 5-12 degrees Celsius below Tm (Sambrook and Russel, 2001). According to Rule *et al*. (1996), the maximum speed of DNA hybridization probes is generally 25°C below melting temperature (Tm). Working very close to the Tm temperature will result in a decreasing number of mismatched bases but also reduce the number (rate) of a perfect hybrid formation.

The capture probe worked at around 28°C of when working at this temperature, it will have the expected mismatch of 1 base. Low hybridization

temperatures will decrease stringency which can result in increasing mismatch bases or decreasing specificity even though the speed of hybridization increases (Sambrook and Russel, 2001). To increase hybridization stringency, methanamide can be used as it has been done by Rule *et al*. (1996). Methanamide can be added up to 50% in hybridization buffer to increase stringency. Methanamide concentrations of 20% and 25% in hybridization buffers or can eliminate mismatches as much as 10% and 20% bases on the optimum hybridization temperature (Tm - 25°C) which can be seen visually on nitrocellulose membrane paper (Rule *et al*., 1996). The decreasing of Tm by the addition of Methanamide can be calculated at 0.72°C for 1% of each Methanamide concentrations (Sambrook and Russel, 2001).

Hybridization was designed to be performed at room temperature (27-30°C). This was a low temperature so it needs high stringency condition to keep specificity to serotype. Low monovalent mineral concentration such as Na$^+$ was chosen to keep high stringency. The estimation resulted for the probes (Table 4.2) with Na$^+$ concentration was 0.068 M (PBS 0.5X) and hybridization temperature works at 20°C below Tm which still at room temperature. The calculation used Sambrook and Russel (2001) formula. There were also calculations to estimate whether there was a secondary structure to estimate the efficiency of hybridization (Table 5) and the analysis of target and non-target homologies to estimate the condition of hybridization (Table 4).

Our capture probes were designed to have non-target homology below 70%. These designs were able to prevent from cross hybridization signals. According to Kucho *et. al.* (2004), low homology towards the target (specific area) causes a high cross hybridization. Probe oligonucleotides should have a minimum homology of 30% with target (specific regions) or a maximum of 70% homology with a non-target (conserved regions) to ensure high specificity.

The percentage of capture probe's GC content is above 55% (DENV1 60.7%, DENV 3 and DENV4 57.1%) (Table 4). It could potentially cause the cross hybridization to

happen if stringency hybridization condition is low. Preferably % GC content is designed around 35-55% . The high % GC content will increase cross hybridization and reduce discriminant (Kucho *et. al.*, 2004; Whiteford *et al.*, 2005; Koehler and Peyret, 2005; Haslam *et al.*, 2008).

Preferably, the probe is designed to have no possibility of forming a secondary structure which has ΔG value more than -10 kcal / mol (Farrell-Jr, 2010) The existence of a secondary structure will reduce the efficiency of hybridization. Table 3 shows the capture probe and amplicon have a secondary structure, but still below the stable extreme (<-10 kcal / mol). However, the DENV1 capture probe has ΔG value -8.3 kcal / mol gives lower hybridization efficiency than other capture probe.

Kane *et al.* (2000) showed a continuous sequence of 100% non-homology for less than 14 nt with non-target homology conditions less than 75%, % GC content 51% and 50 mer of probe length, does not provide signal hybridization. While studies from Kucho *et.al.* (2004), the length of a continuous sequence of 18-23 nt with non-target homology conditions is 40-51%, 45 mers of probe length, with% GC content 35-55% does not indicate a cross hybridization signal. The percentage GC content above 55% gives a signal of cross hybridization. Table. 3 shows the capture probe and amplicon of our design have a non-target continuous homology sequences with 11 and 13 bases in length for DENV 1 capture probe towards DENV3 and DENV4 amplicons. Other than that, looking at % GC content for high capture probe (60.7%), there is a possibility of cross hybridization between DENV3 amplicon and DENV4 amplicon with DENV1 capture probe if the hybridization stringency conditions are not high.

Assay test using ssDNA DENV1-4 as targets worked well and showed no cross hybridization which the only color signal raised on target test line (Figure 2). Cross hybridization did not occur between DENV1 sample with DENV3 and DENV4 capture probe as based of calculating previously. Using buffer assay component with low Na^+ and methanamide in this study gave high stringency to more specific hybridization (Sambrook and Russel, 2001).

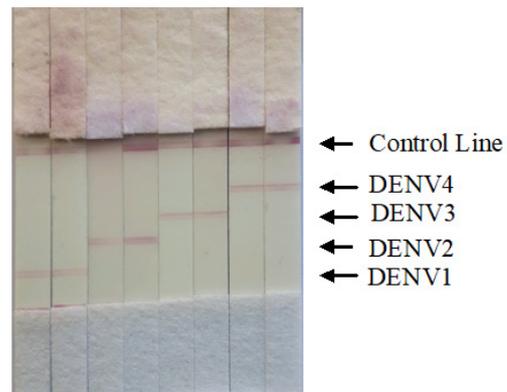Also, addition to glycerol showed no cross hybridization (Hendarta *et al.*, 2020)

Figure 2. Color signal on multi test line NALF

Conclusions

Through genome analysis, the capture probe design was obtained for NALF serotyping. Capture probe has 2 specific serotypes sequences that separated by a conserve sequence. Capture probe has a high % GC content (> 55%) and the length of % homology on a quite long non-target (11-13 nt) so that high stringency condition is needed to avoid cross hybridization. Application of designed capture probe to fabricated NALF gave no-cross hybridization on test line using high stringency buffer assay. It has prospect to future development. .

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