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Expression of Recombinant Non Structural 1 Protein of Dengue Virus Serotype-2 in Mammalian Cell Line

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Dengue infection is a global infectious disease with almost 100 million cases occurs annually in over more than 100 endemic countries. Dengue virus (DENV), the causative agent of dengue infection, is an 11 kbp RNA positive-strand virus which encode 3 structural and 7 nonstructural proteins within its genome. Non-structural 1 (NS1) protein of DENV is expressed in the earlier stage of infection and having a pathogenic role in disease severity. NS1 gene of DENV serotype-2 Indonesian strain was amplified through PCR method using specifically designated primers. NS1 amplicon was then cloned into pUMVC4.a and pcDNA3.1 mammalian expression vector which confirmed through colony PCR and sequencing method. Recombinant pUNS1 and pcNS1 plasmids were transfected into CHO-K1 mammalian cell line with lipid-based method. Recombinant NS1 protein expression was analyzed through immunostaining using dengue patient sera and rapid NS1 detection kit. Recombinant pUNS1 and pcNS1 plasmids were successfully constructed and recombinant NS1 protein was expressed in CHO-K1 mammalian cell line and shown to be reactive against dengue patient sera. Our recombinant NS1 protein also tend to be released outside the transfected CHO-K1 cells as detected in a rapid NS1 detection kit. Recombinant dengue NS1 protein was expressed in a mammalian cell lines in both intra and extracellularly and shown to be immunogenic.

Key words: CHO-K1 cell, dengue, Ns1

Infeksi dengue merupakan salah satu infeksi global dengan jumlah kasus mencapai 100 juta pert tahunnya di 100 negara endemis dengue. Virus dengue (DENV) selaku agen penyebab infeksi dengue merupakan virus RNA untai positif dengan genom berukuran 11 kb yang mengkode 3 protein struktural dan 7 protein non-struktural. Protein non-struktural 1 (NS1) virus dengue diekspresikan di fase awal infeksi dan memiliki peran penting dalam patogenisitas infeksi terkait dengan tingkat keparahan penyakit. Gen NS1 dari virus dengue serotipe 2 strain Indonesia diamplifikasi melalui teknik PCR menggunakan primer spesifik. Gen NS1 kemudian di klona kedalam plasmid ekspresi sel mamalia, pUMVC4.a dan pcDNA3.1. Konfimasi klona dilakukan melalui koloni PCR dan sekuensing. Rekombinan puNS1 dan pcNS1 kemudian ditransfeksikan kedalam sel CHO-K1 menggunakan penghantar lipid dan ekspresi protein NS1 rekombinan dianalisis melalui pewarnaan imunologis menggunakan serum pasien terinfeksi dengue dan kit komersial deteksi protein NS1 virus dengue. Plasmid pUNS1 dan pcNS1 rekombinan telah berhasil dikonstruksi dan protein rekombinan NS1 berhasil diekspresikan pada sistem sel mamalia CHO-K1. Protein rekombinan NS1 terbukti reaktif terhadap serum pasien dan dideteksi pada supernatan sel tertransfeksi yang mengindikasikan kemampuan protein rekombinan NS1 untuk dilepaskan keluar sel. Protein rekombinan NS1 dari virus dengue serotipe 2 telah berhasil diekspresikan pada sel mamalia baik intra maupun ekstrasel dan terbukti imunogenik.

Kata kunci: dengue, NS1, sel CHO-K1

Dengue fever and dengue hemorrhagic fever are infectious diseases caused by dengue virus (DENV) infection which globally spread in almost 100 endemic countries all over the world (WHO 2011). DENV is an arthropord borne virus transmitted by *Aedes aegypti* mosquito mainly in tropical and subtropical countries. Indonesia itself is one of an endemic countries with a long history of dengue infection. Since it was reported in 1968 in Jakarta and Surabaya, dengue virus is widely spread all over the Indonesia archipelago with four viral serotypes that were found to be circulated (Suwandono *et al*. 2006). Based on the Ministry of Health of Indonesia, dengue infection happened every year with five years outbreak periodically. During the 2007 outbreak, more than 127.687 and 1.296 deaths were reported, make it one of the largest outbreak ever happen in Indonesia. Until now, there is no antiviral treatment nor a globally approved vaccine available against DENV infection.

DENV is an arthropod-borne virus which transmitted through *Aedes aegypti* and *Aedes*

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albopictus mosquito as a viral vector. Dengue virus is a member of Flavivirus family with 11 kb genome size, under one open reading frame which encode three structural (C, prM, and E) proteins and seven nonstructural (NS1, 2a, 2b, 3, 4a, 4b, 5) proteins. Based on the E (envelope) gene variation, dengue virus is divided into four distinct serotypes which antigenically different: DENV1-4 (Guzman *et al*. 2010; Back TA 2013) Three structural proteins (C, prM and E) were proteins that composed viral particle and having major antigenic site in the immune response. Among seven non-structural proteins of DENV, non-structural 1 (NS1) protein is a conserved glycoprotein with 46-55 kDa in size. The immature form of this protein was found as a monomer in reticulum endoplasmic, meanwhile the mature protein, with 352 amino acids residue, can be found extracellularly soluble (sNS1) or membrane bound (mNS1) in infected cells (Gutsche *et al.* 2011). NS1 protein can be detected in blood from day-1 of onset until the day-9 with concentration 0.01-50 μg mL^{-1} in the range which make this protein a suitable marker in early diagnosis of dengue infection (Alcon *et al.* 2002). Moreover, NS1 protein has a role in viral replication and allegedly related to dengue infection pathogenesis. The NS1 protein is known can bind with heparan sulfate of epithelial cells, fibroblast, hepatocytes and endothelial cells which lead to plasma leakage through complement activation. On the other hand, an antibody against NS1 was found to be cross-reactive with thrombocytes and endothelial cells. In summary, all of these phenomena can lead the increase in disease severity (Amorim *et al*. 2014).

The recombinant NS1 protein is widely developed for several purposes especially in developing diagnostic tools for dengue infection. Recombinant NS1 protein expressed in *E.coli* or yeast expression system were found to be antigenic and immunogenic in triggering both humoral and cellular immune response in the animal model (Dewi *et al*. 2012; Tripathi and Shrivastava 2018). In this study, we develop a recombinant plasmid expressing NS1 protein of DENV2 originated from Indonesia, pUNS1 and pcNS1 which proved to express the recombinant NS1 protein in both intracellular and extracellularly. Our recombinant NS1 protein is also recognized by dengue patient sera indicating its promising immunogenicity to be further used in developing dengue diagnostic kits.

MATERIALS AND METHODS

NS1 Gene, Plasmids, Bacterial Strain, Mammalian Cell, and Patient Sera. NS1 gene of DENV2 was originated from pGEXNS9.1 recombinant plasmid in a previous study (Dewi, 2012). Original pcDNA3.1 (Themo) and pUMVC4.a (Aldevron) mammalian expression plasmid vector were propagated in *E.coli* DH5α (Thermo) which cultured in LB medium containing 100 μg mL^{-1} of ampicillin for pcDNA3.1 or 30 μg mL^{-1} of kanamycin for pUMVC4.a. CHO-K1 mammalian cell from Kobe University, Japan used for plasmid transfection, was cultured in MEM media containing 10% FBS, 1% NEAA and 1% antibiotic-antimycotic at 37 °C and 5% CO_2 respectively. Dengue patient sera used for immunostaining was derived from Dengue Community Study with ethical approval from Research Ethical Committee Faculty of Medicine, Universitas Indonesia No. 71/PT02.FK/ETIK/2009.

PCR and Cloning. NS1 gene was amplified through PCR method using designated primers for cloned to pcDNA3.1; F2329s*Bam* (5'-CGCGAGGATCCTGGATAGGAATGAATTCACG C-3') and R ns-1-350c*Sal* (5'- TCCGCTGTCGA CTCAGGCTGTGACCAAGGAGTT-3'). Meanwhile for puMVC4.a amplification, *Bam*H1 restriction site in forward primer was changed into *Pst*1 restriction site respectively. Both primer pairs were used to amplify NS1 gene from pGEXNS1.9 template using PrimeSTAR master mix (Takara) reagent. Amplified NS1 gene was then ligated into pcDNA3.1 or pUMVC4.a plasmid and transformed into DH5α *E.coli* using heat shock method. Schematic insertion of NS1 gene into pcDNA3.1 and pUMVC4.a were depicted in figure 2. Recombinant pUNS1 and pcNS1 plasmid were confirmed for its NS1 insertion PCR method using insert primer pair in GoTaq Master Mix (Promega) reagent. Insert sequence and orientation were then confirmed through the sequencing method by Eijkman Institute.

Recombinant pUNS1 and pcNS1 Transfection. Recombinant pUNS1 and pcNS1 plasmids were isolated by Midi Plasmid Purification Kit (Thermo). One microgram of recombinant pUNS1 and pcNS1 plasmids were transfected into CHO-K1 cells using Lipofectamine Plus transfection reagent (Thermo) according to manufacture instruction. Native pcDNA3.1 and pUMVC4.a were also transfected as a negative control. Briefly, 500 ng of each plasmid were transfected into 80% confluent of CHO-K1 cells at M6 well plates in the presence of Lipofectamine reagents for 4 hours at RT. The transfection mixture was then replaced by cell media followed by incubation at 37 °C

and 5% CO_2 until further analysis.

Immunostaining. Twenty four hours after transfection, the supernatant of CHO-K1 culture were discarded and cells were washed three times with excess PBS and air dried. Cells were then fixated with chilled absolute ethanol for thirty minutes at 4 °C. After fixation, 1:1000 diluted dengue patient sera in PBS were applied to transfected cells and incubated for 1 h at RT. Followed by 1:5000 secondary rabbit anti human IgG labelled with HRP (Sigma) was then added followed by another incubation for 1 h a RT. Metal DAB peroxidase substrate (Thermo) was then applied into the cells and positively stained cell will appear in brown color under microscope observation.

NS1 Detection using Commercial Kit. Seventy two hours supernatants of transfected CHO-K1 cells were directly dropped into NS1 detection kit (BioLine) followed by incubation at RT for 15 minutes. Red band color will appear in the sample line as a positive result.

RESULTS

Recombinant pUNS1 and pcNS1 Construction. Recombinant pUNS1 and pcNS1 were successfully achieved with the ligation-transformation rate was 80% respectively. As shown in figure 1, all single colonies of suspected recombinant pUNS1 and pcNS1 were having NS1 gene insertion at 1000 bp size based on colony PCR analysis, thus named as pUNS1.1, pUNS1.2 and pUNS1.3 for puMVC4.a backbone and pcNS1.20, pcNS1.21 and pcNS1.22 for pcDNA3.1 backbone.

Three positive plasmids from each construct confirmed on colony PCR were further analyzed through sequencing for its nucleotide and orientation analysis as predicted in figure 2. From the sequencing result, there is no mutation in the nucleotide sequence from the NS1 gene (data not shown).

Immunostaining. Immunostaining was performed to confirm the ability of the recombinant pUNS1 and pcNS1 to express the recombinant NS1 protein 24 h after transfection. In immunostaining, dengue patients sera were used as primary antibody in the presence of anti-NS1 IgG which confirmed by commercial dengue detection kit. As depicted in figure 3, all recombinant pcNS1 and pUNS1 constructs (pcNS1.20-22 and pUNS1.1-3) give red colored stained cells indicating the expression of the recombinant NS1 protein. In this experiment, pcDNA3.1 and puMVC4.a backbone plasmids were also transfected into CHOK1 cells and show no red colored cell indicating the absence of recombinant NS1 protein and there is no non-specific binding occurs in the use of dengue patients sera. For positive transfection and staining control, a recombinant pUMD2 (pUMVC4.a plasmid encoding prM and E gene of DENV2, (Putri *et al.* 2015)) was used and shown similar red-colored cell indicating the transfection and staining process iscorrectly done.

Extracellular NS1 Detection. Extracellular recombinant NS1 was detected using a commercial NS1 detection kit which commonly used in dengue diagnosis (SD Bioline Dengue Duo). Briefly, supernatant from 72 hours post transfected CHO-K1 cells were dropped into the detection kit and leave it at RT for 15 minutes. As depicted in figure 4, all recombinant pUNS1 constructs (puNS1.1 – 3) and pcNS1.21 gives positive red band indicating the presence of recombinant NS1 protein in the supernatant.

DISCUSSION

Non-structural 1 (NS1) protein is a conserved glycoprotein in DENV which expressed soon after viral particle infecting a cell. This glycoprotein can be expressed in soluble (sNS1) and membrane-bound (mNS1) form which both of these forms can induce humoral and cellular immune response through the different pathways (Akey *et al.* 2014; Rivino *et al.* 2013; Avirutnan *et al.* 2007). Since NS1 protein can be detected in acute phase of DENV infection within various concentration level, NS1 protein is widely used as dengue diagnostic approach in detecting DENV infection by using many platform. Rapid test or ELISA based methods are the two common methods which have been developed for this detection system (Alcon *et al.* 2002). To develop such diagnostic method, the NS1 antigen is needed and *E.coli* and yeast expression system are the most common platform which has been used to obtain recombinant NS1 protein for further use in developing dengue diagnostic (Huang *et al.* 2001; Bragança *et al.* 2015; Zhou *et al.* 2006). Moreover, since NS1 protein is a non-structural protein which doesn't compose viral particle, NS1 protein is now also used as one approach to develop novel dengue vaccine. NS1 protein not posses the *Antibody-dependent enhancement* (ADE) activity as structural protein has as one of the advantages for using this protein which considers being safer than using structural protein (Amorim *et al.* 2014).

For our construction, we use the NS1 gene derived from DENV2 of Cosmopolitan genotype originated

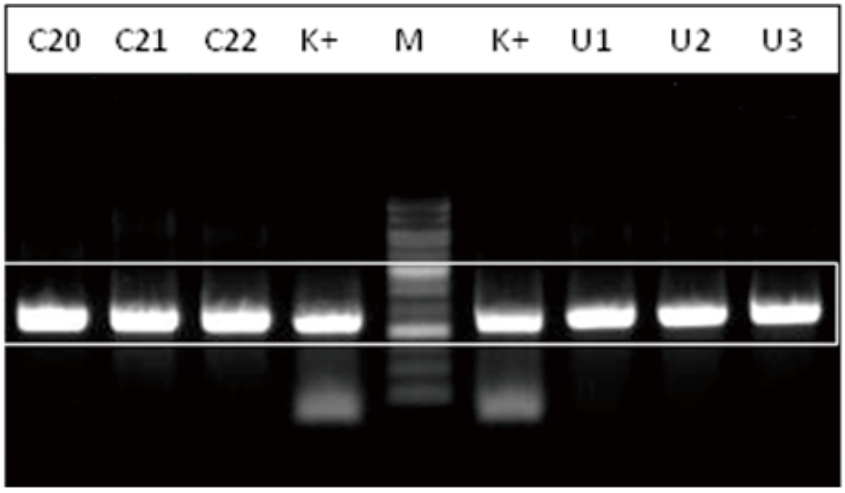

Fig 1 Colony PCR screening. C20-C22 : recombinant pcNS1, K+ : positive control for NS1 insert cloned to pcDNA3.1, M : 1 Kb DNA ladder, U1-3 : recombinant puNS1, K+ : positive control for NS1 insert cloned to pUMVC4.a. The white box showed positive NS1 target band at approximately 1 kb size.

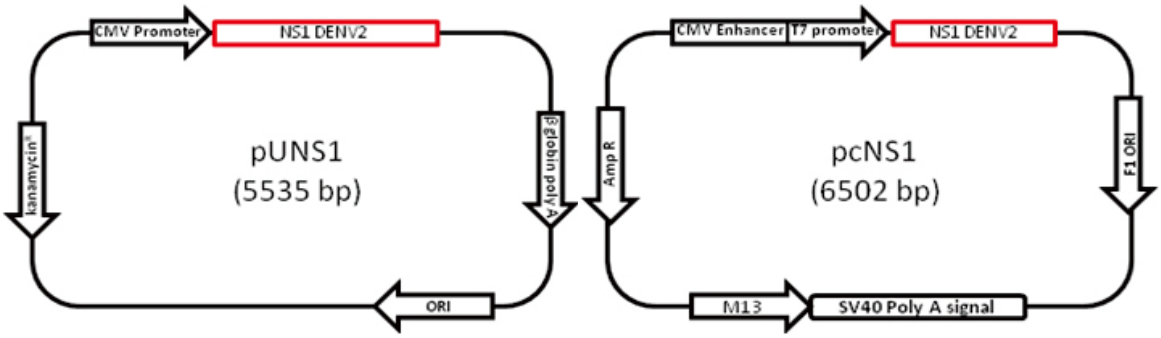

Fig 2 Schematic figure of recombinant pUNS1 and pcNS1. NS1 gene of DENV2 has inserted in vectors MCS regio between *Pst*-I and *Sal*-I restriction sites for puMVC4.a vector; and *Bam*-HI and *Sal*-I restriction sites for pcDNA3.1.

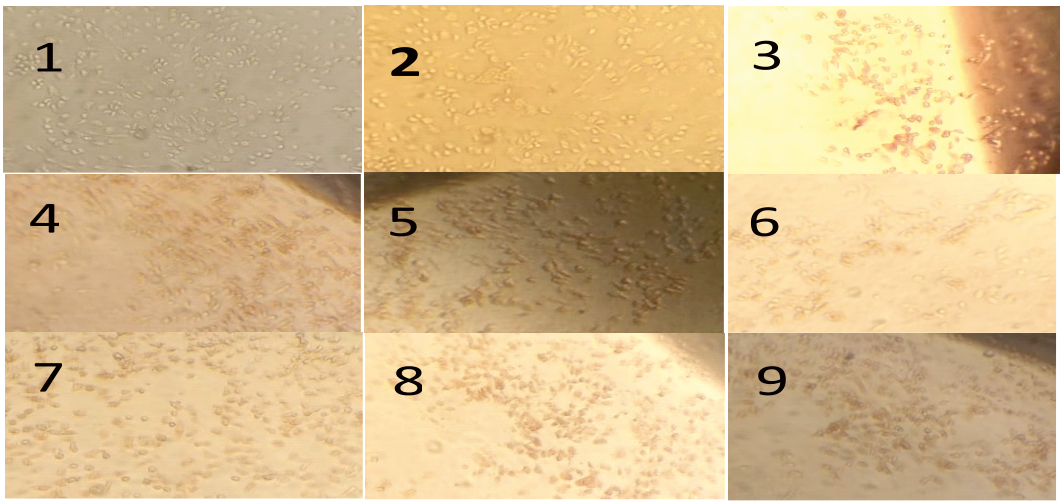

Fig 3 Immunostaining of transfected CHO-K1 cells. Cells were transfected with: 1. pcDNA3.1; 2. pUMVC4.a; 3. pUMD2; 4 – 6. puNS1.1 – 3; 7 –9. pcNS1.1 – 3.

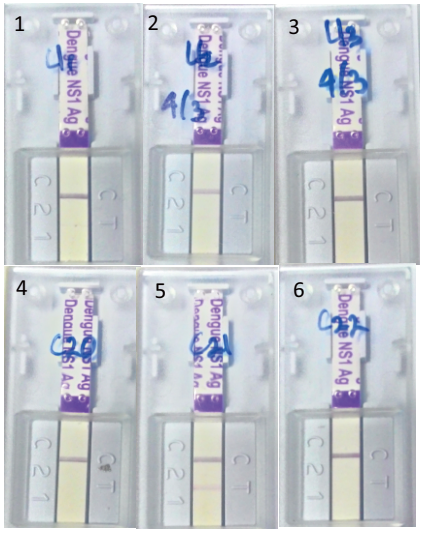

Fig 4 Extracellular recombinant NS1 detection. 1. pUNS1.1; 2. pUNS1.2; 3. pUNS1.3; 4. pcNS1.20; 5. pcNS1.21; 6. pcNS1.22. The positive result is shown as a red line on T.

from dengue patient in Indonesia. The cosmopolitan the genotype of DENV2 is the common circulated genotype of DENV2 in Indonesia and the South East Asia region (Putri *et al.* 2015). Although NS1 protein relatively a conserve glycoprotein, several antigenic properties which unique related to certain DENV genotype is found so it is more suitable to use any gene or viral sources derived from circulating viral in a certain area for a better result (Amorim *et al.* 2014; Chen *et al.* 2018).

In this study, we develop a recombinant mammalian expression system plasmid encode NS1 gene of DENV2 to express recombinant NS1 protein. We use pcDNA3.1 and pUMVC4.a mammalian expression system with the consideration that pcDNA3.1 is a high copy number the of plasmid which can amplify the number of genes. Moreover, with the additional CMV enhancer in the upstream of T7 promoter tend to increase the expression level of recombinant antigen. It can be seen by a high level of recombinant NS1 expression which detected by commercial Ns1 agent kit. It shown as positive band of NS1 antigen which appear from the pcNS1.21 construct compare to the other pUMVC4.a based construct indicating its high expression level although the initial and termination condition of transfection were similar for all recombinant constructs. Another expression system that we use in this study is the pUMVC4.a vector which also a mammalian cell line expression system with kanamycin resistance gene as a selection marker. A kanamycin is an antibiotic which less usable for human compare to ampicillin antibiotic which is safer to be applied in human research. From our result, recombinant pUNS1.1-3 were able to express the recombinant NS1 antigen but we assume the amount of the recombinant protein are less than pcNS1.21 construct since there is only weak positive red band appear when testing using commercial NS1 detection kit. The negative band was obtained from pcNS1.20 and pcNS.22 constructs which we assume that less NS1 antigen was released outside transfected cells by these two constructs although the presence of NS1 antigen was positive at 24 h post transfection as proven by immunostaining analysis. Intracellular NS1 antigen for pcNS1.20 and pcNS1.22 at 72 h post transfection need to be further analyzed.

Originally, NS1 protein can be found in two form of soluble nor membrane bound protein which in this study both form of proteins were detected as immunostaining indicate the expression of intracellular NS1 antigen (membrane-bound located will need further analysis) and NS1 in the supernatan of transfection cell. Our positive immunostaining result show that our recombinant NS1 protein is recognized by dengue patients sera indicating the immunogenicity and antigenicity of our recombinant protein. Among dengue non structural protein, NS1 protein is the most antigenic and immunogenic compare to the other NS protein as several studies shows that there are several B-cell epitope and T-cell epitope were located in NS1 protein which relatively conserved compare to the epitopes which located in the envelope (E) or membrane (M) protein of dengue. Our NS1 recombinant protein is proven to have to conserve B-cell nor T-cell epitopes when amino acid sequences were analyzed (Dewi *et al.* 2012). Immunogenicity and

antigenicity of NS1 protein also one reason to use NS1 protein in developing novel dengue vaccine against DENV infection, highlighting it needs to modify in order to reduce the risk of cross-reactivity of anti NS1 antibody against endothelial cell that can lead to plasma leakage.

In this study, we choose mammalian cell expression system to obtain correct folding, correct protein structure or protein modification of our recombinant NS1. However, the mass production of mammalian cell line is less desirable for it highly cost production. We believe by using this expression system the antigenicity and immunogenicity of the NS1 protein can be maintained to achieve better antigenicity and immunogenicity properties. In this study, we use CHO-K1 cell line derived from Chinese Hamster Ovary wich commonly used in the recombinant protein expression studies and well known for its stability and high yield of protein production. CHO-K1 cell is easy to nurture and also widely used for mass production in recombinant protein technology (Dahodwala and Sharfstein 2017; Genzel 2015; Chen TH *et al.* 2018). From this study result, our system proved that the NS1 recombinant antigen is transported outside the transfected cells as the antigen is detected with commercial rapid NS1 antigen kit in the supernatant of transfected cells. This extracellular transport is one advantage as our recombinant NS1 protein will be much easier to be isolated and purified for further used compare with procaryotic expression systems such as *E.coli* which tend to make inclusion bodies to compensate the high expression pressure. The presence of inclusion bodies in procaryotic expression system is widely known as one of the obstacles in protein isolation and purification process which commonly resulting high protein loss and decreasing protein yields (Wingfield *et al.* 2014; Hoffmann *et al.* 2017).

In conclusion, in this study we successfully obtain recombinant pUNS1 and pcNS1 plasmid construct which able to express recombinant NS1 protein in CHO-K1 mammalian cell line. And our recombinant NS1 protein is reactive against dengue patients sera, indicating it is immunogenic and antigenic to be further used in developing dengue diagnostic kit or dengue vaccines.

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