MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.12, No.2, June 2018, p 53-60

Available online at
http://jurnal.permi.or.id/index.php/mioline
DOI: 10.5454/mi.12.2.4

Cloning and Expression of NS2B/NS3 Protein of DENV3 Indonesia Strain in *Saccharomyces Cerevisiae* Expression System for the Development of Dengue Vaccine

ASRI SULFIANTI*[1], SABAR PAMBUDI[1], ISMA NUR AZIZAH[2], DODDY IRAWAN SETYO UTOMO[1], AND ABINAWANTO[2]

[1]*Center of Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology (BPPT), Gedung 613, Puspiptek - Serpong, Tangerang Selatan, Indonesia;*
[2]*Departement of Biology, Faculty of Mathematics and Science, Universitas Indonesia, Depok, Jawa Barat, Indonesia*

NS3 protein is 618 amino acids (aa) in length containing serine protease and helicase domains required for DENV replication. Alignment of consensus amino acid sequences from all four DENV serotypes demonstrated that this protein is more conserved (78%) among the different dengue serotypes, which elicits a strong cellular immune response after viral infection in humans and animal models. Present study, a central hydrophilic region of NS3 cofactor, NS2B (NS2BH) with full length of NS3 genes DENV3 Indonesian strain were amplified from cDNA following PCR, and inserted to PYES2/CT shuttle vector. The recombinant plasmid was transformed and expressed in *Saccharomyces cerevisiae* expression system. As result, detection with Anti-His detector and dengue virus type 2 NS3 monoclonal antibody shown NS2BH/NS3 was expressed as 83 KDA protein band. We found that addition of NS2BH on NS3 full length construction plasmid increase the yield of protein expression in *S. cerevisiae*. In future study, our recombinant NS2B/NS3 protein can be used as recombinant protein in dengue vaccine development.

Key words: dengue, NS2B-NS3, *Saccharomyces cerevisiae*, vaccine

Protein NS3 adalah protein dengan panjang 618 asam amino yang teridiri atas daerah serine protease dan helicase yang dibutuhkan dalam replikasi virus dengue. Pensejajaran konsesus asam amino dari ke-empat serotipe virus dengue menunjukkan bahwa protein ini bersifat lestari (78%) pada semua jenis serotipe, yang mampu menginduksi respon imun kuat pada manusia dan hewan model. Pada penelitian ini, bagian tengah hidrofobik dari kofaktor NS3, yaitu NS2B (NS2BH) bersama dengan NS3 diamplifikasi dari cDNA melalui PCR, dan diinsersikan ke dalam plasmid PYES2/CT. Plasmid rekombinan yang dihasilkan ditransformasi dan diekspresikan dalam sistem ekspresi *Saccharomyces cerevisiae*. Sebagai hasil, deteksi dengan Anti-His dan Anti-NS3 menunjukkan bahwa protein rekombinan NS2BH/NS3 terekspresi sebagai protein dengan berat 83 KDA. Kami menemukan penambahan kofaktor NS2B pada konstruksi plasmid NS3 meningkatkan hasil ekspresi protein NS3 yang pada tingkat berikutnya, dapat digunakan dalam pengembangan vaksin dengue.

Kata kunci: dengue, NS2B-NS3, *Saccharomyces cerevisiae*, vaksin

Dengue is the most common mosquito-transmitted viral infectious disease in human. A 2016 study estimated nearly 60 million symptomatic dengue cases worldwide every year, resulting in about 10 000 deaths (Lancet and Diseases 2017). The dengue virus belongs to the family of Flaviviridae and are comprised of four antigenically related serotypes (DENV 1- 4). The genomes of the dengue viruses consist of a positive single-stranded RNA of approximately 10,700 bases in length, which contains a single open reading frame encoding a precursor polyprotein. Co- and post-translational processing of the precursor results in the formation of three structural (membrane (M), capsid (C), and envelope (E)), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The cleavage this polyprotein, which represents an essential step for viral replication, is performed by host enzymes and the NS3 viral protease (Laura *et al.* 2012; Shannon *et al.* 2016; Simmons, Sun and Putnak 2016).

At present, there is no antiviral therapy that is available for the prevention and treatment of acute dengue virus infections, and efforts to develop a vaccine against dengue have been ongoing for decades (Khumthong *et al.* 2002) . The CYD-TDV (marketed as Dengvaxia) is a live, attenuated tetravalent vaccine developed by Sanofi Pasteur. Dengvaxia was the first dengue vaccine licensed which used in regional mass vaccination program in Philippines and Brazil, with

*Corresponding author: Phone: +62-85885266350; Email: asri.sulfianti@bppt.go.id

targeting 1 million people (Savarino *et al.* 2018). However, reassessment data from the clinical trials in 2017, found Dengvaxia can increase the risk of severe dengue in particular circumstances (Schwartz *et al.* 2015; Lancet and Diseases 2017; Halstead 2018). As a consequence, the uses of these vaccine has been suspended, and other dengue vaccine platform still remains to be developed.

Vaccines against Flavivirus are generally based on the E protein, which contains most of the epitopes that elicit neutralizing antibodies. However, this protein may also induce non-neutralizing antibodies involved in the phenomenon of antibody-dependent enhancement (ADE) of DENV infection, which can be associated to the occurrence of increased numbers of DHF in secondary infections. Alternatively, some reports suggest the use of non-structural proteins for dengue vaccines to overcome such problem. The NS1 is also highly immunogenic and may generate antibodies with complement fixing activity, probably triggering the lyses of infected cells which present this protein on its surface. Nevertheless, antibodies against the NS1 may also cross-react with human proteins, which can be associated to some pathological effects of the dengue infection. In contrast, there are only few studies evaluating the use of the NS3 protein as a protective antigen against DENV (Costa *et al.* 2011; Laura *et al.* 2012).

The NS3 protein is 618 amino acids (aa) in length containing serine protease and helicase domains required for DENV replication, and at least 30 T-cell epitopes, 14 (47%) of which are clustered within a 124 aa-long stretch from aa 200 to aa 324. Alignment of consensus amino acid sequences from all four DENV serotypes demonstrated that this region is more conserved (78%) among the different dengue serotypes, which elicits a strong cellular immune response after viral infection in humans and animal models (Costa *et al.* 2011; Simmons, Sun and Putnak 2016). It's proven by studies which found the eliciting of IFN-γ production and neutralizing antibody titter in mouse serum after immunization with DNA vaccine based on NS3 protein and NS3-Helicase subunit recombinant from DENV2. In addition, immunization with those vaccine also increase mouse survival time (Costa *et al.* 2011).

Therefore, in the present work we develop the Dengue yeast recombinant vaccines based on NS2B/NS3 protein from DENV3 Indonesian Strain. A central 47aa hydrophilic region (NS2BH) spanning residues 49-95 intimately engages with full length of NS3, and expressed in *Saccharomyces cerevisiae* expression system. The yeast based protein dengue vaccine is a novel vaccine platform which never been developed before. *Saccharomyces cerevisiae* is an effective vector in therapeutic vaccines. Several studies demonstrated expressing foreign antigens in *Saccharomyces cerevisiae* can activate dendritic cells (DCs), elicit robust antigen-specific cytotoxic T lymphocyte (CTL) responses, and confer protective cell-mediated immunity against tumour challenge in mice. Additionally, yeast expression system also offering benefits such as cost-effectiveness and able to overcome problems which commonly found in *Escherichia coli* (Ardiani, Higgins and Hodge 2010; Lankenau 2016).

MATERIALS AND METHODS

Construction of Plasmids. The NS2BH/NS3 full length genes, respectively was amplified from CDNA following standard PCR condition using a forward 5'TAATAAGCTTaaaaatgcctGCAGACCTCACTGTA GAAAAAGCAGC-3', and reverse primer 5'-TAATctcgagCTTTCTACCAGCCGCAAAGTCCTT GAATTCC-3' containing *Hind*III and *Xho*I and restriction sites, respectively. These primers generated around 2.103 base pairs within the extension sequence around 31 base pairs in 5' and 3' for *Hind*III and *Xho*I sites. The PCR reactions were performed using *Pfu High fidelity DNA polymerase* (Thermo Fisher Scientific, USA) to minimize the mutation during the amplification process. The products were cloned to PYES2/CT shuttle-vector (Invitrogen, USA). The transformed bacteria with recombinant plasmid were selected on Luria Bertani agar medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% Sodium chloride) containing ampicillin (100 µg/ml). The positive clones were identified by digested the plasmid DNAs with *Hind*III and *Xho*I (Thermo Fisher Scientific, USA). The new recombinant vector was named as PYES2C/T + NS2BH/NS3 (Fig 1).

Transformation of Recombinant Plasmid to *Saccharomyces cerevisiae.* Transformation of recombinant plasmids (PYES2C/T + NS2BH/NS3) to INVSc1 strain (Invitrogen, USA) was done by heat shock. A mixture contains of 1 µg DNA plasmid, 100 µl of yeast competent, and 100 µg of denatured sheared salmon sperm were mixed with 1X LiAc/40% PEG-3350/1X TE, and incubated at 30°C for 30 minutes.

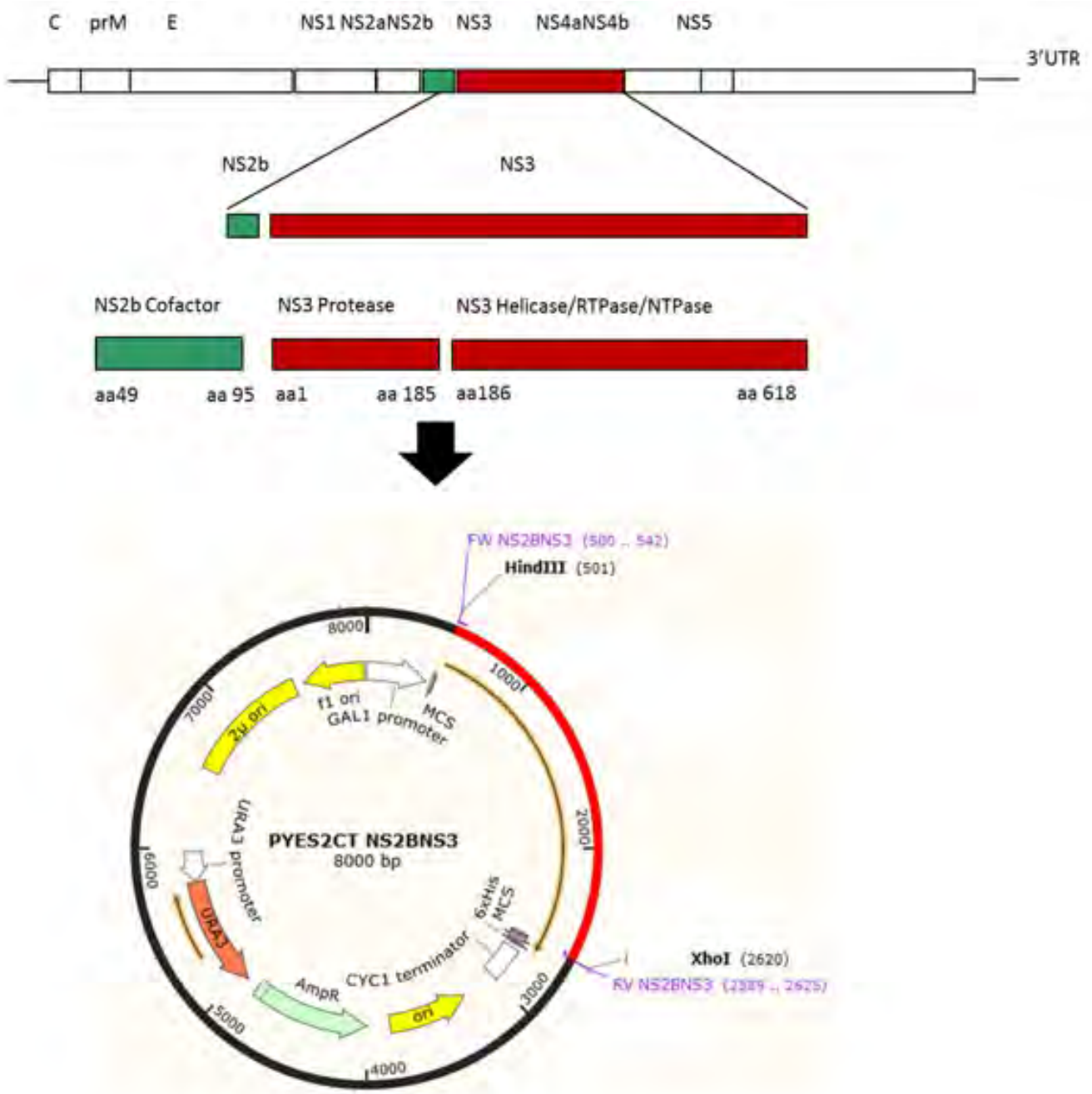

Fig 1 Construction of PYES2 C/T + NS2BH/ NS3. NS2BH (aa49-aa95) and NS3 full length (aa1-aa618) genes were inserted between HindIII and XhoI in the MCS (multiple cloning site) of PYES2 C/T vector (5963 bp) resulted recombinant plasmidtermed as PYES2C/T + NS2BH/NS3.

After 30 minutes, DMSO was added to the mixtures and shocked at 42°C for 7 minutes. The cells were pelleted by centrifugation and suspended with TE1 before it spread onto selective plate (Minimal Media agar). Identification of the recombinant yeast colonies were executed by PCR colony with PYES2 C/T primers (Forward Primer: 5'-AATATACCTCTATACTTTAACGTC-3'; Reverse Primer: 5'- GTCACGCTTACATTCACGC-3') which each bind to the GAL1 promotor and CYC1 terminator.

Expression of Recombinant Protein in *Saccharomyces cerevisiae.* The positive clones were inoculated into MM broth + 2% glucose, and grown overnight at 30 °C for 48 h at 250 rpm. As control, wild type *Saccharomyces cerevisiae* (INVSc1) also grown on YPD at the same condition. An OD600 = 0.4 of pellets were collected from overnight culture, and re-suspended with minimal media containing 2% galactose (induction media). A culture then grown at 30 °C for 48 h at 250 rpm. After 48 h, the cells were harvested by centrifugation at 1.500 g for 5 min at 4°C. The supernatants were removed, and dry pellets stored

at -80 °C until use.

Cells Destruction, SDS-PAGE, and Immunoblotting. Cells destruction was done by sonicate the yeast pellet on 8 Hz for 20 min with 20 sec pulse on, 20 sec pulse off in 4°C. The lysate contain proteins were boiled on 100°C for 15 min and analyzed by electrophoresis and western blot using Anti-His detector TM nickel-HRP (KPL) and dengue virus type 2 NS3 monoclonal antibody (Thermo Fisher GT2811 , USA). After electrophoresis, gel containing protein was transferred onto a PVDF membrane (Thermo Fisher Scientific, USA) with Bind™ Western Device (Thermo Fisher Scientific, USA). The membrane was blocked with skim milk Blotting Grade-Blocker 5% in PBST 0,05%, and incubated in 1:5000 anti-His (His detector TM nickel-HRP) / dengue virus type 2 NS3 monoclonal antibody for overnight. The membrane washed three times with PBST 0,05% washing buffer for each 5 min, and incubated in *Anti-Mouse HorseRadish Peroxidase* (HRP) (1:100.000) (Abcam, USA) for 1 h. After washing 3 times with PBST 0,05%, the membrane was incubated in Super Signal West Femto Membrane Sensitivity Substrate (Thermo Fisher Scientific, USA) for 5 min in dark room. For high sensitivity, the PVDF membrane can be transferred again into film paper [Kodak, Japan] for 1 min in dark room. The protein which printed into film paper then washed by developer buffer, water, fixer buffer and water, for each 1 min to get specific band target.

RESULTS

Identification of Plasmid Recombinant. The amplification of NS2BH/NS3 resulted a single expected DNA band at 2.134 base pairs, which consisted of 2.103 bp for NS2BH/NS3 gene, within extension sequence around 31 bp at 5' and 3' for *Hind*III and *Xho*I sites (Fig 2a). The recombinant plasmids identification by restriction enzyme released a 5.963 bp for PYES2C/T vector and 2134 bp for NS2BH//NS3 gene. Based on this identification, 4 clones shown positive contain NS2BH/NS3 gene (Fig 2b). Analysis on gene sequence revealed that the NS2BH/NS3 recombinant plasmid had a correct open reading frame and high similarity to DENV3 reference virus 141 Jakarta strain (93%). However, though there were found 3 amino acid sequences changed on NS2B/NS3 recombinant protein, the similarity of this protein were still high to its reference virus (Fig 3).

Identification of *Saccharomyces cerevisiae* Recombinant and Expression of NS2BH/NS3 Protein in *Saccharomyces cerevisiae*. The heat shock of *Saccharomyces cerevisiae* with recombinant plasmids yielded 9 positive clones verified by PCR method (Fig 4). The DNA from non-recombinants did not show any amplification.

Out of those positive clones, only 2 clones (clone no. 3 and 6) shown the NS2BH/NS3 protein recombinant protein expression after 48 hours' induction with 2% galactose. It represented as 83 KDA protein band was detected by Anti-His detector and dengue virus type 2 NS3 monoclonal antibody (Fig 5).

DISCUSSION

The aim of our study was to develop new platform dengue vaccine based on yeast recombinant. We construct full length of NS3 with a central 47aa hydrophilic region (NS2BH) spanning residues 49-95. Studies shown that activity of NS3 protease (NS3pro) is depend on interaction with a cofactor, NS2B *(Yusof et al*. 2000). The NS2B protein is a 14-kDa integral membrane protein that contains three domains: two putative hydro- phobic trans membrane segments at both N and C termini and a central region (aa 49–96, 47 aa) that intimately bind to NS3pro, and essential cofactor of the NS3 protease (Li, Phoo and Luo 2014; Pan *et al*. 2017).

Interestingly, the recombinant of NS3pro with only central region of NS2B is sufficient to express NS3 protein. Its proven in our work which resulted a NS2BH/NS3 protein expressed in *Saccharomyces cerevisiae* expression system. As comparison, other plasmid construction with only NS3 did not show a high protein expression (Data not shown). It was concordant to other studies which found expression of NS3 protein alone did not lead to product an active protease. However, including a portion of NS2B with NS3 led to full proteolysis activity. Though, the mechanism by which NS2B contributes to high activation of NS3pro remains poorly understood (Wu *et al*. 2003; De Oliveira *et al*. 2014; Shannon *et al*. 2016).

Based on western blot with Anti-His and Anti-NS3, the NS2BH/NS3 recombinant protein expressed as 83 kDa in size. The recognizing of Anti-His to the protein represented that the gene inserted as one open reading frame to the poly-histidine (6xHis) tag in PYES2/CT vector (Invitrogen 2009)). In addition, the recognizing of monoclonal antibody Anti-NS3 to our protein shown there were an epitope in NS2BH/NS3 which reacted to

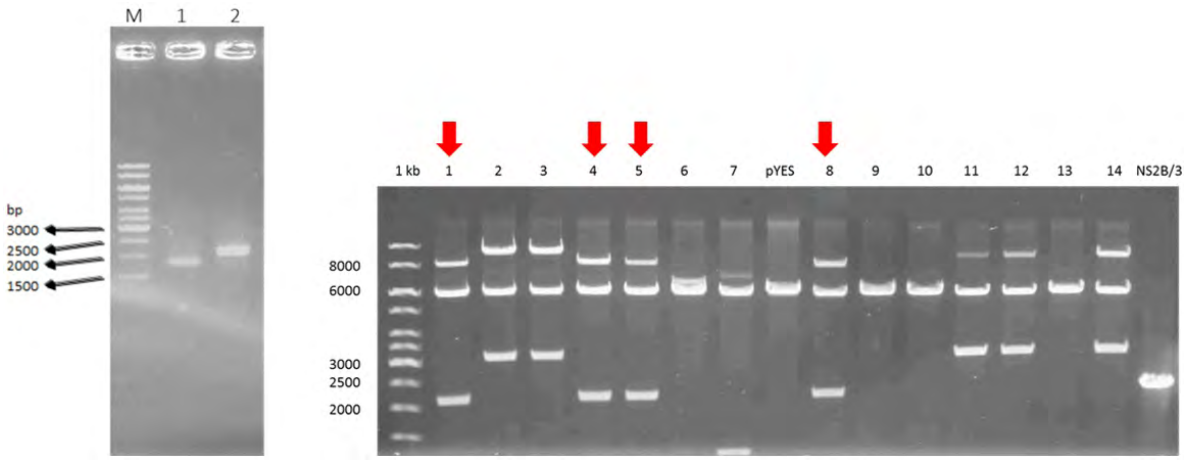

Fig 2 Visualization of $NS2B_H$/NS3 DNA fragment and NS2BH/NS3 plasmid recombinant. (a) The $NS2B_H$/NS3 PCR product in agarose 1%. Lane M: DNA 1 KB marker (Thermo Fisher Scientific); Lane 1: NS3 PCR product (1,9 kb). Lane 2: PCR $NS2B_H$/NS3 PCR product (2.1 kb). (b) Identification of recombinant plasmids with *Hind*III and *Xho*I restriction enzymes resulted 4 clones contain $NS2B_H$/NS3 as inserted (red arrows). Lane 1kb: DNA 1KB marker; Lane 1-14: DNA recombinant plasmids from clone number 1-14; Lane PYES: PYES2C/T wild type vector as control; Lane $NS2B_H$/NS3: PCR product of $NS2B_H$/NS3 gene.

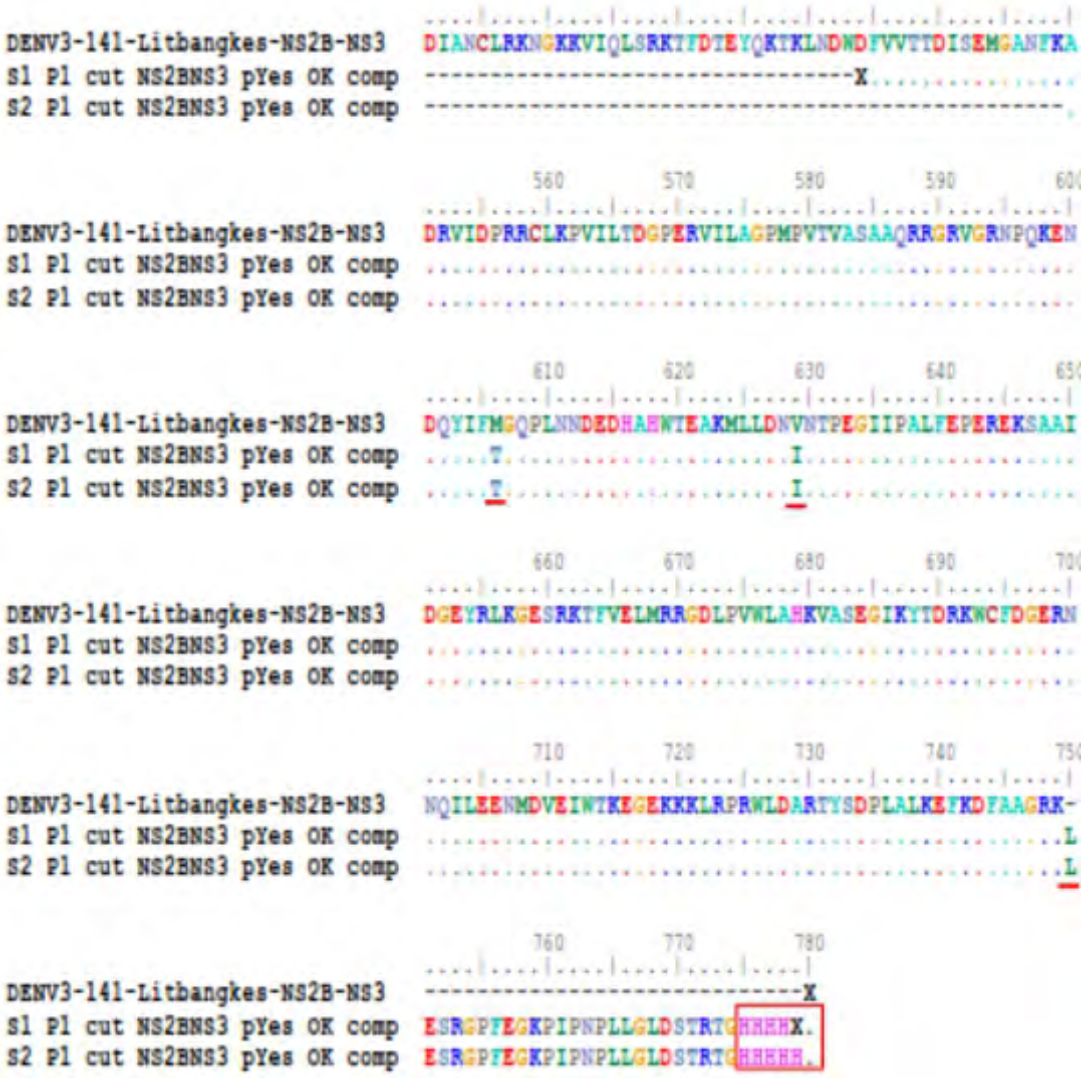

Fig 3 Amino acid sequence alignment between NS2B/NS3 recombinant and DENV3 reference virus 141 Jakarta strain. The highlighted alphabet shown the difference amino acid between recombinant and its reference. The boxes shown the poly-histidine sequence on the terminal of both proteins.

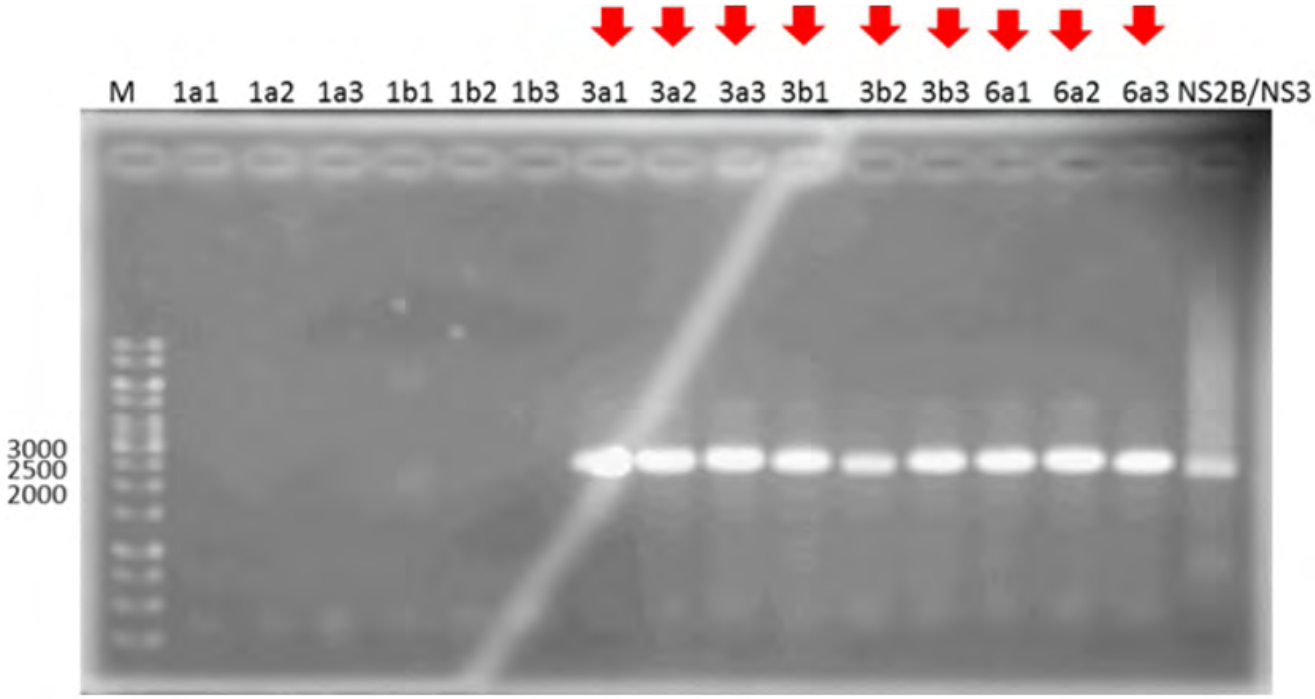

Fig 4 Visualization of PCR product on genomic DNA *Saccharomyces cerevisiae* recombinant. Transformation resulted 9 positive clones contain NS2B/$_{H}$NS3 gene (Lane 3a1, 3a2, 3a3, 3b1, 3b2, 3b3, 6a1, 6a2, 6a3: red arrows); Lane NS2B$_{H}$/NS3: PCR product of NS2B-cofactor/NS3 gene; Lane M: DNA 1 KB marker.

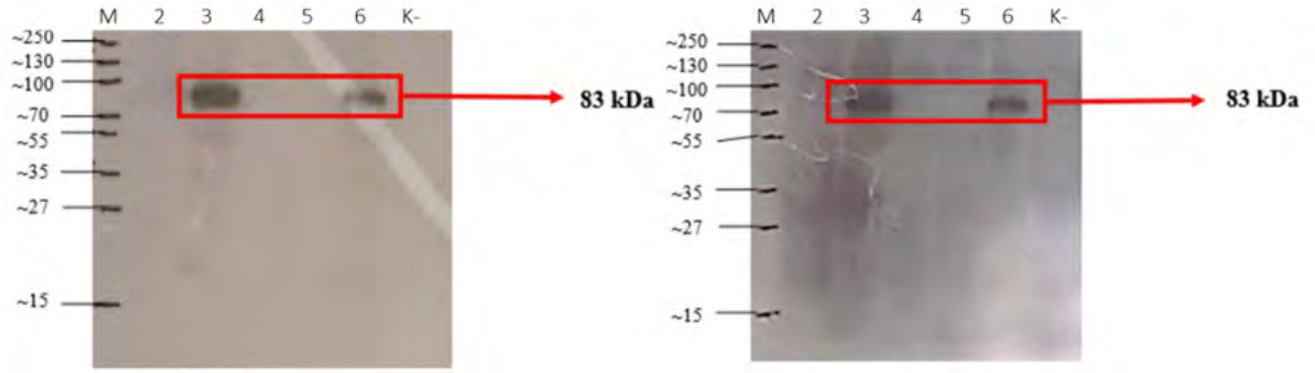

Fig 5 Western blot with two different antibodies resulted same size around 83 kDA of NS2B$_{H}$/NS3 band protein in film paper (Left: protein detection by Anti-His. Right: protein detection by Anti-NS3). Lane M: Prestained Protein Plus Ladder Marker [Thermo Fisher Scientific]; Lane 2-6: clone sample number; Lane K-: wild type *Saccharomyces cerevisiae* (INVSc1).

the Anti-NS3. In present study, we construct NS2BH region with full length of NS3. The NS3 protein comprised 2 domains. The serine protease domain within the first 180 amino acids at the N-terminal portion (residues 1–180), while the final two-thirds of NS3 protein in C terminal region (residues 180–618) contain three enzymatic domains such as, RNA stimulated nucleoside triphosphate (NTPase), RNA helicase and RNA 59-triphosphatase (RTPase) (Perera and Kuhn 2008; De Oliveira *et al*. 2014). Based on Simmon *et al*. (2016), it found at least 30 T-cell epitopes, 14 (47%) of which are clustered within a 124 aa-long stretch from aa 200 to aa 324 of NS3 Helicase which considered as a main target for CD4+ and CD8+ T cell response(Simmons, Sun and Putnak 2016).

Expression of NS2BH/NS3 dengue protein virus in *Saccharomyces cerevisiae* gave some benefits. *Saccharomyces cerevisiae* is an attractive heterologous expression platform that offers several advantages such as posttranslational modification, secretion, and ease of genetic manipulation and culturing; it is also regarded as a generally recognized as safe (GRAS) organism (Nguyen *et al*. 2013). The study of vaccine based heat-inactivated yeast recombinant

demonstrated that this vector can deliver multiple antigens into the *MHC class* I and II antigen presentation pathways to stimulate potent CD4+ and CD8+ T cell responses. In addition, it not readily neutralized in vivo and is therefore amenable to repeated administration, enabling the application of long-term immunological pressure, ideal for the elimination of chronic intracellular infections. Recent work has also shown that the this platform triggers a reduction in the number and immunosuppressive activity of regulatory T cells, likely due to the natural ability of yeast to elicit IL-1b production and Th17 T cell differentiation at the expense of regulatory T cells (King *et al*. 2014).

As conclusion, in this study we report a novel finding that the addition of NS2BH on NS3 full length construction plasmid successfully expressed in *Saccharomyces cerevisiae*. Moreover, this protein expressed also has an epitope which reacted with Anti-His and dengue virus type 2 NS3 monoclonal antibody. In future study, our recombinant NS2B/NS3 protein can be used as recombinant protein in Dengue vaccine.

ACKNOWLEDGEMENT

We are grateful to the Center of Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology for financial support.

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