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Production of Immunologically Active Untagged Recombinant DENV-2 NS1 in *Escherichia coli*

[1]Dessy Natalia, [1]Christian Heryakusuma, [1]Fernita Puspasari, [1]Linda Juniar, [1]Yovin Sugiyono, [2]Anita Yuwita, [3]Hofiya Djauhari, [3]Silvita F. Riswari, [3]Bachti Alisjahbana and [1]Ihsanawati

[1]Biochemistry Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia
[2]Pakar Biomedika Indonesia, Jl. Rancabentang 12B, Bandung 40142, Indonesia
[3]Faculty of Medicine, Universitas Padjadjaran, Jl. Raya Bandung Sumedang KM21, Jatinangor, Indonesia

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Corresponding Author:
Dessy Natalia
Biochemistry Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia
Tell: 62-22-2502103
Fax: 62-22-2504154
Email: dessy@chem.itb.ac.id

Abstract: Dengue is an infectious disease affecting 390 millions people in tropical and subtropical region annually. Dengue virus NS1 protein plays an important role in viral replication in the host cell and it is detected in high level in the infected patient serum. A synthetic gene of untagged DENV-2 NS1 with codons optimization for expression in *Escherichia coli* has been generated and then inserted into an expression vector pET16b. As a hydrophobic protein with aliphatic index of 71.21%, DENV-2 NS1 was produced as inclusion bodies in *E. coli* BL21(DE3). The DENV-2 NS1 aggregate was unfolded in 8 M urea and then refolded by reverse dilution method. The refolded DENV-2 NS1 has immunologically active structure as it is capable to interact with anti-NS1 antibody, hence making it as a potential vaccine candidate.

Keywords: Dengue Virus Serotype 2, Non Structural Protein 1, Vaccine Candidate, Refolding, *Escherichia coli*

Introduction

Disease caused by the Dengue Virus (DENV) varies from asymptomatic, non-specific fever, Dengue Fever (DF) to a deadly form of Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) (Guzman *et al*., 2013). A recent study estimated that 390 million people are infected with dengue virus each year, with 96 million patients exhibiting various disease symptoms (Lim *et al*., 2013). Therefore, WHO and many epidemic countries undertake strong actions to prevent and reduce the infection and mortality rate caused by dengue virus. One recommended method is through a rapid and accurate dengue diagnostic test in early stage of infection (WHO, 2012). It allows identification and confirmation of dengue infection thus enabling the best treatment before the disease develops into more severe and life-threatening conditions.

Dengue virus belongs to the flavivirus family (*Flaviviridae*), which includes three genera, namely *Flavivirus*, *Pestivirus* and *Hepacivirus* (Lim *et al*., 2013). DENV has a spherical shape with a diameter of 50 nm. The structures of mature and immature DENV particles have been solved by cryo-electron microscopy (Mukhopadhyay *et al*., 2005; Lim *et al*., 2013). The virus has a single-stranded positive-sense RNA genome approximately 11,000 nucleotides encoding for three structural proteins and seven non-structural proteins (Muller and Young, 2013). The three structural proteins are Capsid (C), Envelope (E) and membrane protein (prM). All structural proteins of DENV (C, E and prM) have been determined by X-ray crystallography or Nuclear Magnetic Resonance (Perera and Kuhn, 2008). The seven non-structural proteins of DENV are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Muller and Young, 2013). The nonstructural proteins are essential for virus replication, virion assembly, evasion of host immune response (Bollati *et al*., 2010).

The non-structural protein NS1, plays an important role in the human immune invasion and evasion mechanisms (Rastogi *et al*., 2016), RNA replication in the host cell (Sampath and Padmanabhan, 2009; Fan *et al.*, 2014), production of infectious viral particles (Scaturo *et al.*, 2015), autoimmune damage of the host tissue (Amorim *et al.*, 2014) and is a potential protein for vaccine candidate (Amorim *et al.*, 2014; Liu *et al.*,

2016). The NS1 protein is generally composed of 352 amino acids with highly variable size, i.e., 46-55 kDa depending on glycosylation degree (Muller and Young, 2013). NS1 is secreted into blood circulation in a few days after infection and can achieve high circulating level (≥600 ng/mL) within 72 h of illness (Libraty *et al*., 2002), making this protein a potential biomarker for early dengue infection detection. Among all four dengue serotypes (DENV-1, DENV-2, DENV-3 and DENV-4), DENV-2 was frequently associated with most severe cases and epidemics (Soo *et al*., 2016; Yung *et al*., 2015; Vicente *et al*., 2016)

Expression of DENV-2 NS1 from different origins has been reported in various host cells ranging from prokaryotic cells, such as *E. coli* BL21 (DE3) (Yohan *et al.*, 2017) and *E. coli* Rosetta (DE3) (Lemos *et al.*, 2013), to eukaryotic cells, such as *Pichia pastoris* X-33 (Zhou *et al.*, 2006), *P. pastoris* KM71 (Puspasari *et al.*, 2017) and insect cells (Rozen-Gagnon *et al.*, 2012). All of the recombinant proteins, however, had a different solubility depends on the host cells in which it was expressed. Those expressed in higher complex systems like eukaryotic cells and viruses were found as a soluble protein while those expressed in *E. coli* were found as an aggregate in a form of inclusion bodies. Nevertheless, the use of *E. coli* results in high expression level and it requires simple fermentation process.

Most attempts to produce recombinant NS1 used a tag or fusion system, such as His tag or thioredoxin, which requires removal of the tag or fusion partner, especially when the NS1 is designated for a vaccine candidate. In this studies, we reported the expression of untagged DENV-2 NS1 in *E. coli* BL21(DE3). The refolding process to obtain an immunological active DENV-2 NS1 recognized by NS1 antibody is described.

Materials and Methods

Microorganisms and Maintenance

E. coli strain TOP10F' (Invitrogen) was used for routine recombinant plasmid multiplication while *E. coli* strain BL21(DE3) (Novagen) was used for gene expression. Recombinant *E. coli* cells were subcultured in Luria-Bertani medium (LB) [0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl] at 37°C, supplemented with 100 µg/mL ampicillin.

Design of Indonesian DENV-2 NS1 Synthetic Gene

DENV-2 NS1 amino acid sequence was derived from NCBI database of Indonesian dengue virus (GenBank ADK37478). The nucleotide sequence was optimized according to expression codon in *E. coli* and synthesized comercially by GenScript (USA).

Construction of Recombinant Expression Vector

The DENV-2 NS1 synthetic gene was inserted into pET16b expression vector (Novagen) between *Nco*I and *Bam*HI restriction sites. The resulted recombinant plasmid was designated as pET16b-DENV-2 NS1.

Preparation of E. coli Competent Cell and Transformation

Single colony of *E. coli* BL21(DE3) was grown in 5 mL LB medium at 37°C with shaking of 150 rpm for 18 h. A total of 250 µL overnight culture was inoculated into 25 mL LB medium and incubated at 37°C with shaking of 150 rpm until OD_{600} of 0.2-0.4 was reached. The culture was transferred into a 50 mL centrifuge tube, incubated in ice for 10 min, then centrifuged at 2,700 × g for 10 min. Pellet was resuspended with 5 mL of 0.1 M iced-cold $CaCl_2$. After settling for 10 min on ice, the mixture was centrifuged at 2,700 × g for 10 min. Pellet was resuspended with 800 µL of 0.1 M iced-cold $CaCl_2$ and aliquoted into microtubes at 100 µL. Competent cells were ready to use after being stored at 4°C for 2-24 h.

E. coli was transformed by recombinant plasmid pET16b-NS1-DENV2 using modified heat-shock method (Sambrook *et al.*, 1989). Recombinant plasmid (1 ng) was added to a microtube containing 100 µL competent cells. The mixture was incubated for 30 min at 4°C, then heat-shocked at 42°C for 90 sec and cooled on ice for 2 min. After cooling the mixture, 900 µL LB medium was added and the mixture was incubated at 37°C for 1 h with shaking of 150 rpm. A total of 100-200 µL cell were spread on LB agar plate containing 100 µg/mL ampicillin and incubated at 37°C for 16-18 h.

Production of DENV-2 NS1 in E. coli BL21(DE3)

E. coli BL21(DE3) transformants were inoculated into 25 mL LB medium supplemented with 100 µg/mL ampicillin and then incubated for 18 h at 37°C with shaking of 150 rpm. A total of 2.5 mL overnight culture was inoculated into 250 mL LB medium and incubated at 37°C with shaking of 150 rpm until OD_{600} of 0.6-0.8 was reached. Cell was induced by adding 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 4 h. Culture was centrifuged at 9,800×*g*. Pellet was analyzed or stored at -80°C.

Isolation and Refolding of DENV-2 NS1 Protein

DENV-2 NS1 protein was isolated according to the method described previously by Athmaram *et al*. (2013) with some modifications. Cell pellet (2-3 g) was resuspended with 1:4 lysis buffer (50 mM Tris-Cl pH 8, 10 mM EDTA pH 8, 100 mM NaCl, 0.1 mM PMSF) and the suspension was sonicated with frequency of 10,000 Hz for 15 min in ice (30 s on and 30 s off). Pellet was collected by centrifugation at 17,400 × *g* for 30 min at 4°C.

Pellet obtained, in a form of inclusion bodies, was washed by combining the method described previously by Athmaram *et al*. (2013) and Williamson *et al.* (1996) with some modifications. Pellet was resuspended with 1:7 wash buffer 1 [50 mM Tris-Cl pH 8, 10 mM EDTA pH 8, 100 mM NaCl, 0.5% (v/v) Triton X-100] and the suspension was stirred for 1 h at room temperature. Pellet was collected by centrifugation at 17,400 × *g* for 30 min at 12°C. The washing step was conducted three times where at the second time, the inclusion bodies were resuspended with 1:7 Milli-Q and at the third time, the inclusion bodies were resuspended with 1:7 wash buffer 3 (10 mM Tris-Cl pH 8, 5 mM EDTA pH 8, 200 mM NaCl, 1 M urea).

Inclusion bodies were solubilized by combining the method described previously by Athmaram *et al*. (2013) and Yohan *et al.* (2017) with some modifications. Washed inclusion bodies were added with 1:7 unfold buffer (50 mM Tris-Cl pH 8, 100 mM NaCl, 11 mM dithiothreitol, 8 M urea) and suspension was sonicated with frequency of 4,000 Hz for 5 min on (15 s on and 45 s off). Suspension was stirred for 20 min at room temperature and supernatant was collected by centrifugation at 17,400 × *g* for 30 min at 12°C. Supernatant (unfolded protein) was stored at room temperature.

The unfolded DENV-2 NS1 was refolded by reverse dilution according to the method described previously by Allonso *et al*. (2011) with some modifications. The unfolded protein was diluted with 1:20 refolding buffer (50 mM Tris-Cl pH 8, 100 mM NaCl, 0.2 M L-arginine, 1 mM reduced-glutathione, 0.1 mM oxidized-glutathione) with constant stirring at 4°C and the refolding buffer dropped-rate was set to 1 drop per 3 s. The mixture was then incubated with constant stirring for 30 min at 4°C. Protein aggregate during refolding step was separated by centrifugation at 17,400 × *g* for 30 min at 4°C.

The refolded DENV2-NS1 protein was then concentrated by 5-fold using centrifugal filters (Millipore) with 10 kDa molecular cut-off. The concentrated protein was centrifuged at 17,400 × *g* for 30 minutes at 4°C to remove protein aggregates and frozen at -20°C for 4 h prior to freeze-drying for 18 h.

Dengue NS1 Antigen Diagnostic Kit Test

Antigenic evaluation of the refolded DENV2-NS1 was performed by a commercial dengue diagnostic kit (SD Bioline). In addition, sera from patient with positive and negative dengue infection were also included.

ELISA

The refolded DENV2-NS1 protein was examined by commercial ELISA kit, Platelia Dengue NS1 Antigen (Bio-Rad). ELISA was performed using 25 µg of purified protein and 50 µL of ELISA positive and negative controls. The negative and positive controls were provided by the kit manufacturer. All sample and controls were analysed in triplicate. ELISA interpretation of results was based on the value of sample ratio and calculated according to the instruction of the kit manufacturer.

Results and Discussion

Construction of an Untagged DENV-2 NS1 Recombinant Plasmid

Indonesian originated sequence of DENV-2 NS1 (GenBank ADK37478) was chosen in order to obtain a recombinant DENV-2 NS1 protein with the highest similarity to the most dengue virus endemic in Indonesia. DENV-2 NS1 gene was synthesized with codon optimization for *E. coli* in order to achieve the best protein expression. Furthermore, codon usage bias adjustment which was represented by the high value of Codon Adaptation Index (CAI) and GC content in an ideal percentage range of 30-70% were considered as adequate requirements for successful heterologous gene expression (Sharp and Li, 1987). The synthetic gene used in this experiment had a high CAI value of 0.84 with GC content of 42.61%. The full length of the DENV-2 NS1 synthetic gene was 1,079 bp with *Nco*I and *Bam*HI restriction sites attached to 5'- and 3'-end of the gene for its insertion into pET16b expression vector. The expression of DENV-2 NS1 without additional taq sequence was regulated by *T7* promoter (Fig. 1).

Expression and Refolding of Untagged DENV-2 NS1

E. coli BL21(DE3), used in this study, expressed a high level of DENV-2 NS1 protein but, as expected, almost all of the protein aggregated and formed an inclusion bodies. The molecular weight of the expressed DENV-2 NS1 is about ~40 kDa based on SDS-PAGE analysis (Fig. 2).

The inclusion bodies, however, were advantageous as it allowed a pre-purification of protein hence increasing the purity of DENV-2 NS1 protein for the later process. To change these inactive inclusion bodies into bioactive molecules, solubilization of the inclusion bodies followed by renaturation were required. During the unfolding of DENV-2 NS1 inclusion bodies, the recovery yield could reach just up to 60%. The low recovery yield might be due to the high content of hydrophobic amino acid which was indicated by the high value of aliphatic index (71.21%) of this protein, thus affecting the solubility of DENV-2 NS1 protein even in mild denaturing conditions.

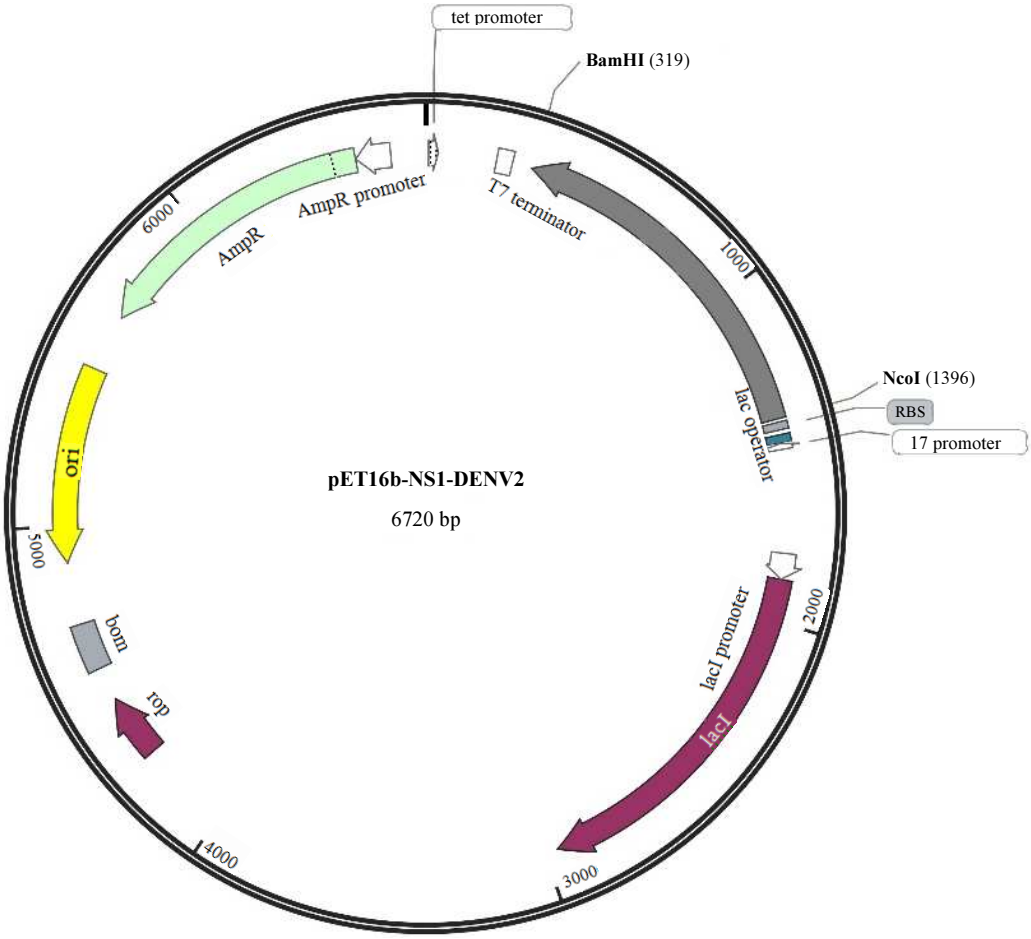

Fig. 1: Map of recombinant expression plasmid pET16b-NS1-DENV2. Illustrated by SnapGene viewer (from GSL Biotech; available at www.snapgene.com). Synthetic gene was inserted between *Nco*I and *Bam*HI restriction sites

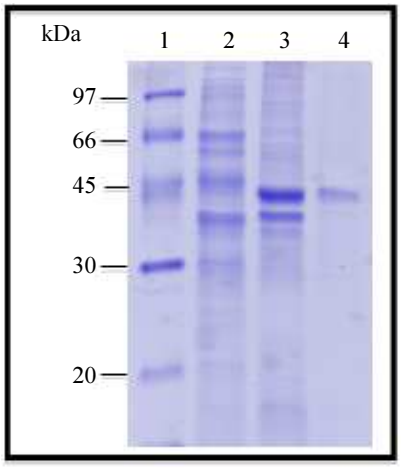

Fig. 2: SDS-PAGE of recombinant DENV-2 NS1 protein; Lane 1, protein marker; lane 2, supernatant; lane 3, inclusion bodies; lane 4, refolded DENV-2 NS1

In the next process, the urea concentration was lowered which allowed the refolding process to occur. Refolding process tried to return the structure of DENV2-NS1 back to its native state. This process will allow the sequence-specific structure that is crucial to the antigenic behaviour of the protein to be present on the surface of the protein and this structure could be identified using several antigenic test, such as dengue rapid diagnostic kit test and ELISA.

The Refolded Untagged DENV-2 NS1 Interacts with Anti NS1 Antibody

The antigenicity of the refolded DENV-2 NS1 protein was evaluated using commercial dengue rapid diagnostic kit and ELISA. Interaction between the refolded DENV-2 NS1 with anti-NS1 monoclonal antibody on a commercial diagnostic kit was indicated by appearance of a red color on a test line (Fig. 3). Similar result was observed when blood serum sample of patient infected by dengue virus, verified by PCR, was spotted. However, the same red line was absence from a negative control containing proteins expressed by *E. coli* BL21(DE3) without DENV-2 NS1 gene.

Fig. 3: Dengue NS1 antigen diagnostic kit test. (A) Negative control (crude supernatant of pET-16b transformant), (B) positive control (blood serum sample of patient infected by dengue virus), (C) refolded DENV-2 NS1 protein

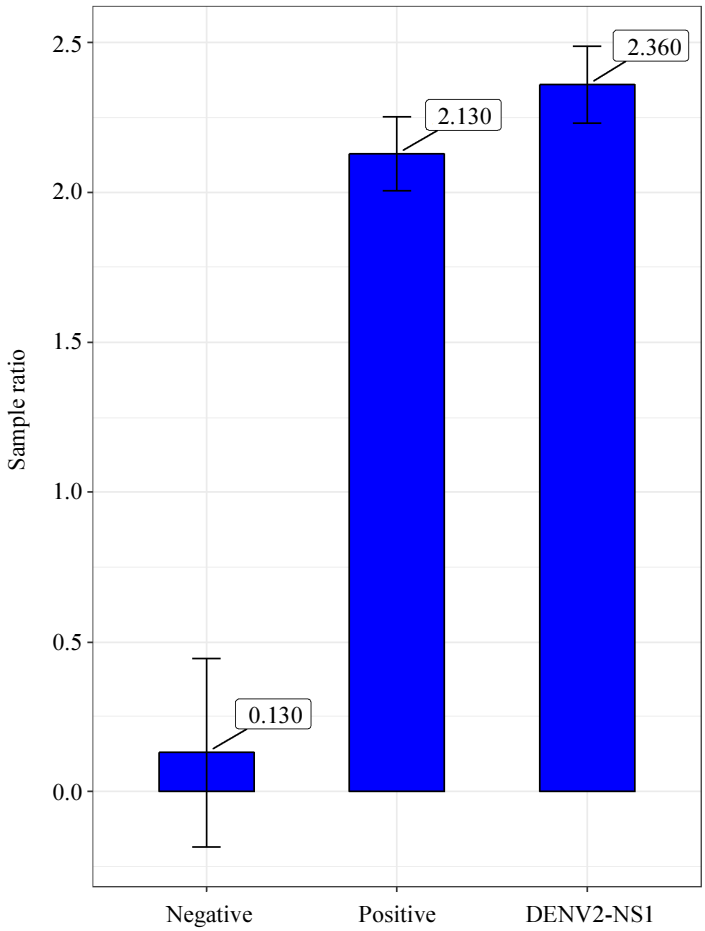

Fig. 4: ELISA graphic of purified DENV2-NS1 protein. All ELISA value bar represent an average value of three repeated assays. ELISA Sample Ratio (SR) was interpreted as followed: Reactive (SR ≥ 1.0), equivocal (0.50 ≤ SR < 1.0), non-reactive (SR ≤ 0.50)

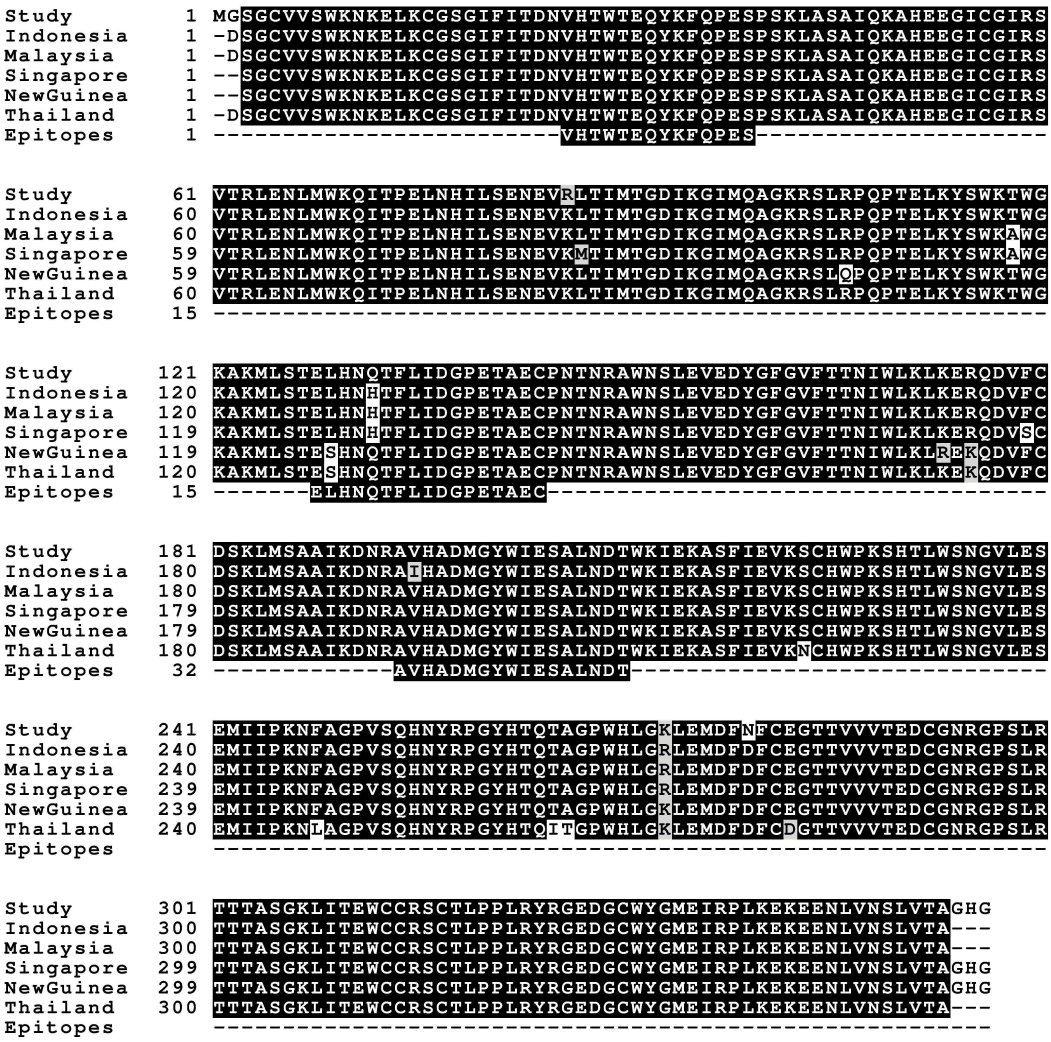

Fig. 5: Sequence alignment of recombinant DENV2-NS1 proteins from different countries with its conformational epitopes. The accession number of each amino acid sequences used in the alignment are as followed: this study (ADK37478), Indonesia (AHK09923), Malaysia (CAZ72152), Singapore (ABW82013), New Guinea (AAC59275) and Thailand (P29990). The conformational epitopes are based on DENV2-NS1 epitope study conducted by Rocha *et al*. (2017)

Further analysis of antigenicity of refolded DENV2-NS1 protein was conducted using ELISA. The DENV-2 NS1 protein gave a high value of A_{450} which was interpreted as a reactive protein to anti-NS1 monoclonal antibody (Fig. 4). The commercial rapid diagnostic kit and ELISA result suggested that the interaction between epitope presence in the surface of the DENV-2 NS1 protein with paratope of anti-NS1 monoclonal antibody on a commercial diagnostic kit and the complex antibody-antigen-antibody interaction on ELISA were occurred. The presence of these interactions proved that the refolded DENV-2 NS1 protein has immunologically active structure.

The result of these antigenic tests corroborated that sequence-specific structures or the conformational epitopes which contributed to the antigenicity of DENV-2 NS1 protein were present on the surface of the protein. These conformational epitopes are ^{25}VHTWTEQYKFQPES^{38}, ^{127}ELHNQTFLIDGPETAEC^{143}, ^{193}AVHADMGYWIESALNDT^{209} and located in three different regions of protein 3D structure (PDB ID: 4O6B) (Rocha *et al.*, 2017). The first epitope is located in an external loop, the second one is located in beta-sheets in an external region and the third one is located in beta-sheets in an internal region (Rocha *et al.*, 2017). Sequence alignment between recombinant DENV2-NS1 protein in this study and several other recombinant DENV2-NS1 proteins from different countries with the conformational epitopes showed 99% identity score and revealed the conservation of NS1 sequence and its epitopes within the proteins (Fig. 5). Though some differences were observed on the second and third epitopes (127-143, 193-209), the recombinant proteins were all immunogenic (Yohan *et al.*,

2017; Lemos *et al.*, 2013; Rozen-Gagnon *et al.*, 2012; Zhou *et al.*, 2006). Out of the 3 conformational epitopes, the second epitope, ^{127}ELHNQTFLIDGPETAEC^{143}, was reported as an immunodominant B cell epitopes in DENV2-NS1 protein (Jiang *et al.*, 2010).

Conclusion

This study demonstrated that recombinant DENV-2 NS1 without additional tag sequence simplifies the procedure to produce DENV-2 NS1 as a vaccine candidate. Although the NS1 protein presented in this study is only one of the four dengue serotypes, the result shows that there is a great chance to further develop untagged NS1 recombinant proteins from other serotypes.

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Author's Contributions

Dessy Natalia: Designed the whole experiment, analysed the results and wrote the manuscript.

Christian Heryakusuma: Performed refolding and purification experiments and wrote the manuscript.

Fernita Puspasari: Designed synthetic gene, constructed the recombinant vector and wrote the manuscript.

Linda Juniar: Performed the refolding experiment.

Yovin Sugiyono: Performed cell production and protein denaturation experiments.

Anita Yuwita and Hofiya Djauhari: Performed immunological assay.

Silvita F. Riswari: Analysed immunological assay results.

Bachti Alisjahbana: Designed and analysed immunological assay results.

Ihsanawati: Designed the unfolding and refolding experiments.

Ethics

The authors declare their responsibility for any ethical issues that may arise after the publication of this manuscript.

References

Allonso, D., M.S. Rosa, D.R. Coelho, S.M. Costa and R.M.R. Nogueira *et al*., 2011. Polyclonal antibodies against properly folded Dengue virus NS1 protein expressed in *E. coli* enable sensitive and early dengue diagnosis. J. Virol. Meth., 175: 109-116. DOI: 10.1016/j.jviromet.2011.04.029

Amorim, J.H., R.P.S. Alves, S.B. Boscardin and L.C.S. Ferreira. 2014. The dengue virus non-structural 1 protein: Risks and benefits. Virus Res., 181: 53-60. DOI: 10.1016/j.virusres.2014.01.001

Athmaram, T.N., S. Saraswat, P. Misra, S. Shrivastava and A.K. Singh *et al*., 2013. Optimization of Dengue-3 recombinat NS1 protein expression in *E. coli* and in vitro refolding for diagnostic applications. Virus Genes, 46: 219-230. DOI: 10.1007/s11262-012-0851-5

Bollati, M., K. Alvarez, R. Assenberg, C. Baronti and B. Canard *et al*., 2010. Structure and functionality in flavivirus NS-proteins: Perspectives for drug design. Antiviral Res., 87: 125-48. DOI: 10.1016/j.antiviral.2009.11.009

Fan, J., Y. Liu and Z. Yuan, 2014. Critical role of dengue virus NS1 protein in viral replication. Virol. Sinica, 29: 162-169. DOI: 10.1007/s12250-014-3459-1

Guzman, M.G., M. Alvarez and S.B. Halstead, 2013. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: An historical perspective and role of antibody-dependent enhancement of infection. Archive Virol., 158: 1445-59. DOI: 10.1007/s00705-013-1645-3

Jiang, L., J.M. Zhou, Y. Yin, D.Y. Fang and Y.X. Tang *et al*., 2010. Selection and identification of B-cell epitope on NS1 protein of dengue virus type 2. Virus Res., 150: 49-55. DOI: 10.1016/j.virusres.2010.02.012

Lemos, G., I. Guillen, J.R. Fernandez, T. Diaz and A.B. Colarte *et al*., 2013. Expression and purification of a full-length recombinant NS1 protein from dengue 2 serotypes viral isolate. Biotecnol. Aplicada, 30: 187-193.

Libraty, D.H., P.R. Young, D. Pickering, T.P. Endy and S. Kalayanarooj *et al*., 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J. Infect. Dis., 186: 1165-1168. DOI: 10.1086/343813

Lim, S.P., Q.Y. Wang, C.G. Noble, Y.L. Chen and H. Dong *et al*., 2013. Ten years of dengue drug discovery: Progress and prospects. Antiviral Res., 100: 500-519. DOI: 10.1016/j.antiviral.2013.09.013

Liu, Y., J. Liu and G. Cheng, 2016. Vaccines and immunization strategies for dengue prevention. Emerg. Microbes Infect., 5: e77-e77. DOI: 10.1038/emi.2016.74

Mukhopadhyay, S., R.J. Kuhn and M.G. Rossmann, 2005. A structural perspective of the flavivirus life cycle. Nature Rev. Microbiol., 3: 13-22. DOI: 10.1038/nrmicro1067

Muller, D.A. and P.R. Young, 2013. The flavivirus NS1 protein: Molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antiviral Res., 98: 192-208. DOI: 10.1016/j.antiviral.2013.03.008

Perera, R. and R.J. Kuhn, 2008. Structural proteomics of dengue virus. Curr. Opin. Microbiol., 11: 369-377. DOI: 10.1016/j.mib.2008.06.004

Puspasari, F., R.D. Putri, R.R.R. Aisyah, A. Damayanti and B. Yuwita *et al*., 2017. Construction and expression of a synthetic gene encoding nonstructural glycoprotein NS1 of dengue 2 virus in *Pichia pastoris*. Asian Pac. J. Trop. Biomed., 7: 689-693. DOI: 10.1016/j.apjtb.2017.07.017

Rastogi, M., N. Sharma and S.K. Singh, 2016. Flavivirus NS1: A multifaceted enigmatic viral protein. Virol. J., 13: 131-131. DOI: 10.1186/s12985-016-0590-7

Rocha, L.B., R.P.S. Alves, B.A. Caetano, L.R. Pereira and T. Mitsunari *et al*., 2017. Epitope sequences in dengue virus NS1 protein identified by monoclonal antibodies. Antibodies, 6: 14-14. DOI: 10.3390/antib6040014

Rozen-Gagnon, K., N.J. Moreland, C. Ruedl and S.G. Vasudevan, 2012. Expression and immunoaffinity purification of recombinant dengue virus 2 NS1 protein as a cleavable SUMOstar fusion. Protein Expression Purificat., 82: 20-25. DOI: 10.1016/j.pep.2011.11.003

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Cold Spring Harbor Laboratory Press, ISBN-10: 0-87969-309-6.

Sampath, A. and R. Padmanabhan, 2009. Molecular targets for flavivirus drug discovery. Antiviral Res., 81: 6-15. DOI: 10.1016/j.antiviral.2008.08.004

Scaturo, P., M. Cortese, L. Chatel-Chaix, W. Fischl and R. Bartenschlager, 2015. Dengue virus non-structural protein 1 modulates infectious particle production via interaction with the structural proteins. PLoS Pathogens, 11: e1005277-e1005277. DOI: 10.1371/journal.ppat.1005277

Sharp, P.M. and W. Li, 1987. The codon adaptation index: A measure of directional synonymous codon usage bias and its potential applications. Nucleic Acids Res., 15: 1281-1295.

Soo, K.M., B. Khalid, S.M. Ching and H.Y. Chee, 2016. Meta-analysis of dengue severity during infection by different dengue virus serotypes in primary and secondary infections. PLoS One, 11: e0154760-e0154760. DOI: 10.1371/journal.pone.0154760

Vicente, C.R., K.H. Herbinger, G. Froschl, C. Malta Romano and A. de Souza Areias Cabidelle *et al*., 2016. Serotype influences on dengue severity: A cross-sectional study on 485 confirmed dengue cases in Vitoria, Brazil. BMC Infect. Dis., 16: 320-320. DOI: 10.1186/s12879-016-1668-y

Williamson, R.A., D. Natalia, C.K. Gee, G. Murphy and M.D. Carr *et al*., 1996. Chemically and conformationally authentic active domain of human tissue inhibitor of metalloproteinases-2 refolded from bacterial inclusion bodies. Eur. J. Biochem., 241: 476-483. DOI: 10.1111/j.1432-1033.1996.00476.x

WHO, 2012. Global strategy for dengue prevention and control 2012-2020. World Health Organization.

Yohan, B., P. Wardhani, A. Aryati, H. Trimarsanto and R.T. Sasmono, 2017. Production of recombinant dengue Non-Structural 1 (NS1) proteins from clinical virus isolates. Protein Expression Purificat., 129: 53-59. DOI: 10.1016/j.pep.2016.09.007

Yung, C.F., K.S. Lee, T.L. Thein, L.K. Tan and V.C. Gan *et al*., 2015. Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, singapore. Am. J. Tropical Med. Hygiene, 92: 999-1005. DOI: 10.4269/ajtmh.14-0628

Zhou, J.M., Y.X. Tang, D.Y. Fang, J.J. Zhou and Y. Liang *et al*., 2006. Secreted expression and purification of dengue 2 virus full-length nonstructural glycoprotein NS1 in *Pichia pastoris*. Virus Genes, 33: 27-32. DOI: 10.1007/s11262-005-0036-6