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Construction and expression of a synthetic gene encoding nonstructural glycoprotein NS1 of dengue 2 virus in *Pichia pastoris*

Fernita Puspasari[1], Riski Dwimalida Putri[1], Aisyah[1], Raden Roro Rika Damayanti[1], Anita Yuwita[2], Bachti Alisjahbana[3], Sukwan Handali[2], Ihsanawati[1], Dessy Natalia[1*]

[1]*Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institute of Technology 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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ABSTRACT

Objectives: To express and characterize NS1 of Indonesian-specific DENV2 virus in *Pichia pastoris* (*P. pastoris*).
Methods: A codon optimized synthetic gene derived from the DENV-2 NS1 amino acid sequences was synthesized commercially and inserted into the *P. pastoris* pPICZαA expression vector. The recombinant DENV-2 NS1 protein was purified by Ni-NTA affinity chromatography, and its antigenicity was tested.
Results: The recombinant DENV-2 NS1 protein was secreted as a protein with a molecular weight of ~45 kDa, and the optimal expression condition was achieved by induction with 2% (v/v) methanol for 72 h. The purified recombinant DENV-2 NS1 protein was able to interact with a monoclonal antibody of NS1 in a commercial rapid test.
Conclusions: The resulting recombinant DENV-2 NS1 protein produced in *P. pastoris* KM71 is a potential candidate for use in the development of a dengue diagnostic kit and vaccine.

1. Introduction

Dengue virus, carried by *Aedes aegypti* and *Aedes albopictus* mosquitoes, causing dengue disease, is one of the major health problems in more than 100 countries in tropical and subtropical regions. It is estimated that 390 million people in the world are infected by dengue virus every year [1]. Children and young adults are the major victims of the disease, with 5% mortality in young patients caused by dengue haemorrhagic fever [2]. To reduce the transmission, number of cases and fatalities, a rapid and affordable assay for early detection is urgently needed.

Dengue virus belongs to the Flaviviridae family and has a spherical shape with a diameter of 50 nm. It has a single-stranded, positive-sense RNA genome of approximately 11 kb encoding three structural proteins (capsid, envelope, and membrane) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [3]. The nonstructural proteins are essential for virus replication, virion assembly, and evasion of the host immune response [4].

The NS1 protein of dengue virus is generally composed of 352 amino acids with a variable size of 40–55 kDa, depending on its glycosylation degree. NS1 is produced early in viral infection, before the onset of antibody production in the infected host. Hence, dengue NS1 detection in the patient's blood is a suitable marker for detection in the early days of the fever [3]. Indeed, it has been reported that the use of NS1 antigen to diagnose dengue fever has high sensitivity and excellent specificity [5], and recombinant NS1 protein induces protective immunity in mice [2]. To meet the need for dengue diagnostic tools and vaccine development, a high level of NS1 protein must be produced in a heterologous expression system. The expression of dengue virus NS1 protein in *Escherichia coli* (*E. coli*) resulted in insoluble protein aggregates (inclusion bodies) [2,6]. In contrast, soluble NS1 protein was secreted in the yeasts *Pichia pastoris* (*P. pastoris*) [7] and *Kluyveromyces marxianus* [8]. This paper describes the expression of NS1 of Indonesian-specific DENV2 virus in methanol utilization slow (Mut^S) *P. pastoris* KM71. Furthermore, the ability of the recombinant DENV-2 NS1 to be recognized by a commercial

*Corresponding author: Dessy Natalia, Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung, 40132, Indonesia.
Tel: +62 22 2502103
Fax: +62 22 2504154
E-mail: dessy@chem.itb.ac.id (D. Natalia).

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dengue diagnostic kit and also IgG/IgM of dengue patient serum was demonstrated.

2. Materials and methods

2.1. Microorganism and maintenance

The yeast strain used in this work was *P. pastoris* KM71 with the Mut^{s} phenotype (Invitrogen). The yeast was maintained on YPD agar plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar]. For long-term storage, cells were frozen at −80 °C in 20% (v/v) glycerol. *P. pastoris* KM71 cells were precultured in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] at 30 °C with 250 rpm shaking overnight (16–18 h). *E. coli* strain TOP10F' (Invitrogen) was used for routine recombinant plasmid multiplication. Recombinant *E. coli* cells were subcultured in low-salt Luria-Bertani medium (LSLB) [0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl] at 37 °C, supplemented with zeocin (25 μg/mL).

2.2. Design of Indonesian DENV-2 NS1 synthetic gene

The DENV-2 NS1 amino acid sequence was derived from the NCBI database entry of the Indonesian dengue virus (GenBank ADK37478). The nucleotide sequence was optimized for the expression codons in *P. pastoris* and synthesized commercially by GenScript (USA).

2.3. Construction of recombinant expression vector

The DENV-2 NS1 synthetic gene was inserted into the pPICZαA expression vector (Invitrogen) between the *Eco*RI and *Xba*I restriction sites, producing the recombinant plasmid pPICZαA-NS1. The gene was kept in-frame with the polyhistidine (6xHis) tag in the expression vector backbone.

2.4. Yeast transformation

The recombinant plasmid pPICZαA-NS1 was first linearized with the restriction enzyme *Sac*I, followed by the transformation of *P. pastoris* KM71 with the linearized recombinant plasmid using electroporation (Eppendorf) [9]. The transformed cells were spread on an YPD agar plate containing 100 μg/mL zeocin and then incubated at 30 °C for 2–3 days.

2.5. Selection of multicopy integrants

To select multicopy integrants, the yeast transformants were grown on YPD agar plates containing 2000 μg/mL zeocin. The presence of the *NS1* gene in the 2000 μg/mL zeocin-resistant yeast colonies was confirmed by PCR using AOX1 primers

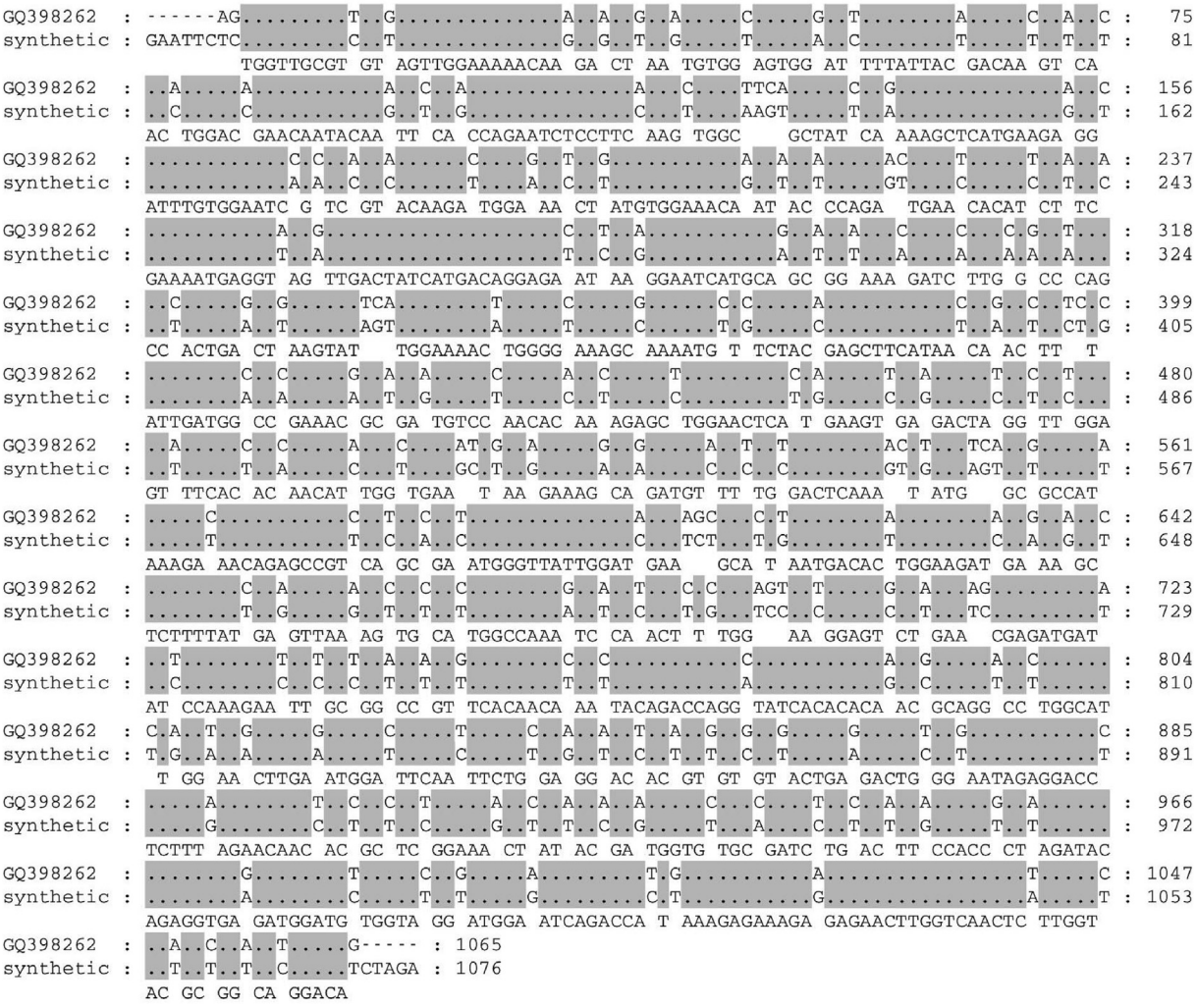

Figure 1. Alignment of reference DENV-2 NS1 (GenBank GQ398262) and the synthetic gene optimized for *P. pastoris*.

(Invitrogen) and specific DENV-2 NS1 primers (FP- GAATTCTC TGGTTGCGTCGTTAG and RP- CTCGAGT-TATTGTCCGTGACCAGC). The best-growing transformants in 2 000 μg/mL zeocin, verified with PCR, were then chosen for protein expression.

2.6. Expression of recombinant DENV-2 NS1 protein

Each selected colony was precultured in YPD broth at 30 °C with shaking at 250 rpm overnight. The preculture was then transferred into BMGY [1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin, 1% glycerol, 100 mM sodium phosphate, pH 6.0] at approximately 2% volume and grown until it reached an OD_{600} of 2–6. For induction, cells were first pelleted by centrifugation (6 000× *g*, 5 min, room temperature) and then resuspended in BMMY [1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin, methanol, 100 mM sodium phosphate, pH 6.0] using 10% volume of the original BMGY culture. Various concentrations of methanol [0.5%–3.0% (v/v)] were added every 24 h.

2.7. Purification of DENV-2 NS1

Cell-free culture supernatant was concentrated by ultrafiltration using a membrane with a molecular weight cut off of 5 kDa, and the medium was then exchanged with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole). The concentrated protein was mixed with Ni-NTA matrix (Thermo Scientific) and incubated for 2 h at 4 °C. The mixture was washed with Buffer A (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole), and the recombinant protein DENV-2 NS1 was then eluted using Buffer B (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole).

2.8. Antigenic evaluation of DENV-2 NS1

Antigenic evaluation of the recombinant protein DENV-2 NS1 was performed using a commercial dengue diagnostic kit (SD Bioline). In addition, sera from patients positive and negative for dengue infection were also included.

3. Results

3.1. Synthetic gene and construction of recombinant expression vector pPICZαA-NS1

Figure 1 shows the alignment of the reference DENV-2 NS1 gene (GenBank GQ398262) and the synthetic gene with codons optimized for *P. pastoris*. The synthetic gene had 79% identity to the reference gene. The full length of the synthetic gene DENV-2 NS1 was 1 064 bp, with *Eco*RI and *Xba*I restriction sites attached to the 5′- and 3′-end of the gene for insertion into the pPICZαA expression vector. The DENV-2 NS1 expression was regulated by the *AOX1* promoter (Figure 2), and DENV-2 NS1 was fused with an oligonucleotide encoding the pre-pro α factor signal sequence to allow its secretion into the culture medium.

3.2. Yeast transformation and selection of multicopy integrant

The integration of the recombinant pPICZαA-NS1 into *P. pastoris* KM71 genome produced zeocin-resistant transformants.

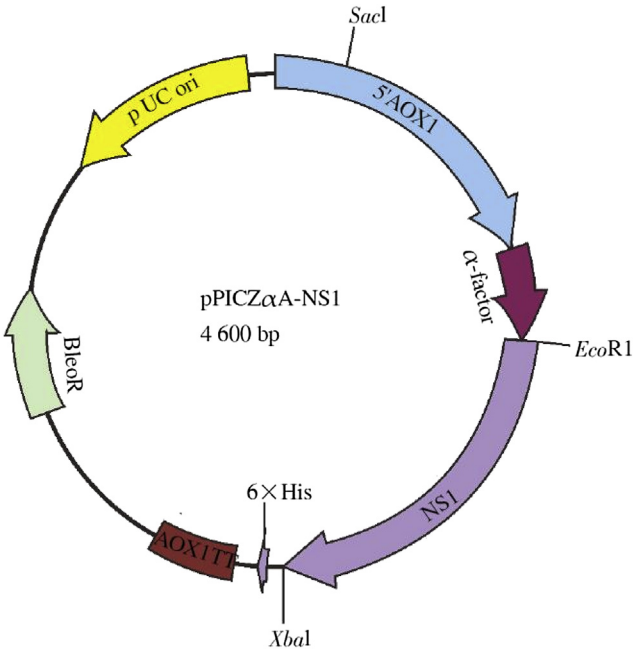

Figure 2. Map of recombinant plasmid pPICZαA-NS1.

P. pastoris KM71 transformants harbouring DENV-2 NS1 were subjected to multicopy integrant selection on growth media containing zeocin in the concentration range of 100 to 2 000 μg/mL. Several transformants were able to grow on YPD agar plates containing 2 000 μg/mL zeocin, which indicated the presence of multicopy integrants. A DNA fragment of approximately 1.5 kb was obtained when the multicopy integrants were amplified using AOX1 primers, while a 1.0 kb band was observed using specific DENV-2 NS1 primers (Figure 3). Therefore, the PCR results further confirmed the successful integration of pPICZαA-NS1.

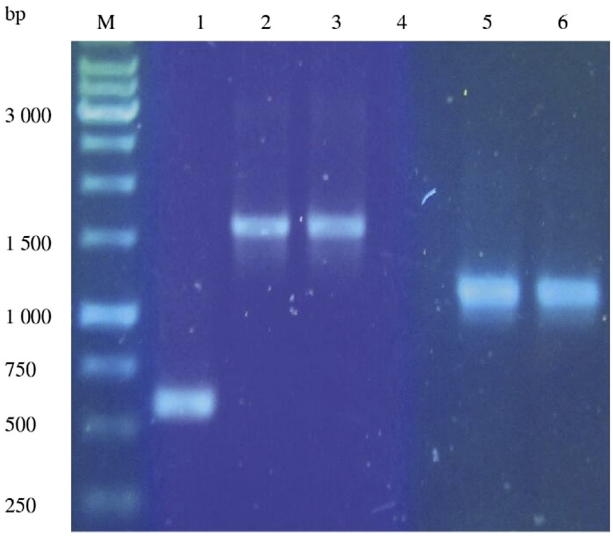

Figure 3. DENV-2 NS1 gene amplification of transformed *P. pastoris* by PCR.
M. DNA marker: 1. *P. pastoris* KM71 with integrated pPICZαA using AOX1 primers; 2. *P. pastoris* KM71 with integrated pPICZαA-NS1 using AOX1 primers; 3. Positive control (isolated PICZαA-NS1) using AOX1 primers; 4. *P. pastoris* KM71 with integrated pPICZαA using DENV-2 NS1 primers; 5. *P. pastoris* KM71 with integrated pPICZαA-NS1 using DENV-2 NS1 primers; 6. Positive control (isolated PICZαA-NS1) using DENV-2 NS1 primers.

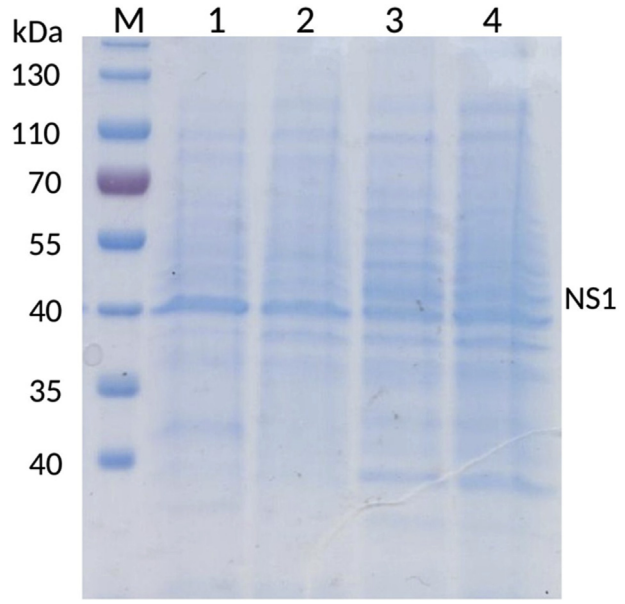

Figure 4. SDS-PAGE of recombinant DENV-2 NS1 protein by induction with 2% (v/v) methanol.
Proteins were analysed after 0, 24, 48 and 72 h of induction (1–4). M. protein marker.

3.3. Expression and purification of recombinant DENV-2 NS1 protein

Small-scale expression of recombinant DENV-2 NS1 protein was performed to determine the optimal inducer methanol concentration and induction period. Testing methanol concentrations of 1%, 2%, and 3% (v/v) suggested that the highest DENV-2 NS1 expression was achieved in 2% methanol (data not shown). Furthermore, it was found that the optimum condition for DENV-2 NS1 expression in 2% methanol was achieved after 72 h of induction (Figure 4).

The recombinant DENV-2 NS1 protein was purified by metal affinity chromatography Ni-NTA (Figure 5). SDS-PAGE analysis of cell-free culture supernatants showed that the recombinant DENV-2 NS1 protein had a molecular weight of ~45 kDa, which is in agreement with the deduced amino acid residues of DENV-2 NS1. In addition to the expected protein band of recombinant DENV-2 NS1 at ~45 kDa, two additional protein bands were observed at higher molecular weight. These protein bands could be the glycosylated and dimer forms of the recombinant DENV-2 NS1 protein.

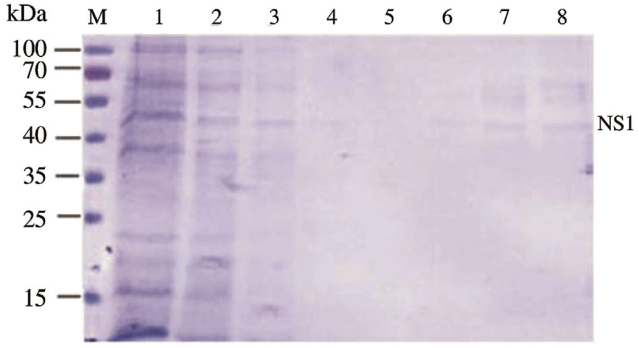

Figure 5. SDS-PAGE of recombinant DENV-2 NS1 protein.
M. protein marker: 1. crude supernatant; 2. unbound protein; 3–4. Washed protein; 5–8. Purified recombinant NS1-DEN2 protein.

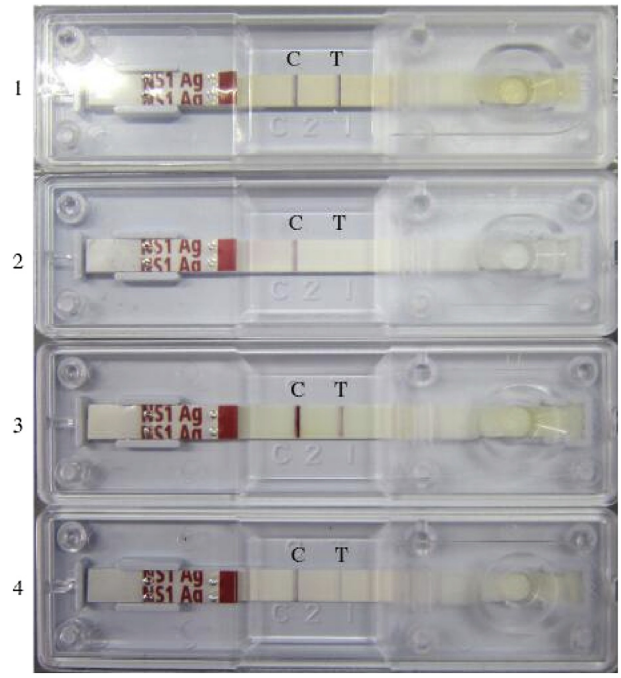

Figure 6. Interaction between recombinant DENV-2 NS1 protein and anti-NS1 monoclonal antibody in a commercial dengue diagnostic kit.
1. Blood serum sample of patient infected by dengue virus; 2. Crude supernatant of pPICZαA transformant; 3. Crude supernatant of pPICZαA-NS1 transformant; 4. Purified recombinant DENV-2 NS1 protein.

3.4. Antigenicity of recombinant DENV-2 NS1 protein

The antigenicity of the recombinant DENV-2 NS1 was evaluated using a commercial diagnostic kit. The interaction of recombinant DENV-2 NS1 with anti-NS1 monoclonal antibody in the commercial diagnostic kit was indicated by the appearance of a red line on the test line (Figure 6). A similar result was observed when a blood serum sample from a patient infected by dengue virus, as proven by PCR, was spotted, while the red line was absent from a negative control containing proteins secreted by *P. pastoris* KM71 without the DENV-2 NS1 gene.

4. Discussion

Dengue NS1 has two N-linked glycosylation points at Asn 130 and Asn 207 [10]. Due to its ability to perform post-translational modification, including glycosylation [11], *P. pastoris* is a suitable host for the expression of DENV-2 NS1. The Indonesian originated sequence of DENV-2 NS1 was chosen to obtain recombinant DENV-2 NS1 with high similarity to the most dengue patients in Indonesia. The DENV-2 NS1 gene was synthesized with codon optimization for *P. pastoris*. The synthetic gene and reference DENV-2 NS1 gene shared 79% identity. For codon usage bias adjustment, the synthetic gene had a codon adaptation index (CAI) of 0.84, which is regarded as a good indication for successful heterologous gene expression [12]. The GC content of the synthetic gene was 42.61%, which was considered to be in the ideal percentage range of the GC content (30%–70%).

Under its optimum expression conditions, the recombinant DENV-2 NS1 protein was secreted with a molecular weight of approximately 45 kDa. Two additional bands from the purification of recombinant DENV-2 NS1 protein suggested the

presence of dimer and hyperglycosylated forms. Other studies have reported that DENV-2 NS1 formed a dimer in *E. coli* [13] and *P. pastoris* [7]. The recombinant DENV-2 NS1 protein gave positive interaction with monoclonal NS1 antibody in a commercial diagnostic kit. Although we have only tested this antigen against the Rapid NS1 detection kit, it is supposed to provide 100% specificity, so it can be assumed that the structure and antigenic properties are correct.

In conclusion, this study demonstrated that Mut^{s} phenotype *P. pastoris* KM71 is a good host for the expression of DENV-2 NS1, and the resulting recombinant DENV-2 NS1 protein is a potential candidate for use in a diagnostic kit and vaccine development. Although this NS1 is still only one of the 4 serotypes affecting Indonesia, this result shows that there is an excellent chance to further develop recombinant proteins for other dengue serotype NS1s.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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