MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8587
Vol 6, No 1, March 2012, p 15-22

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.6.1.3

Cloning and Expression of Nonstructural Protein NS1 of Dengue Virus Serotype 2

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Early diagnosis of dengue virus (DENV) infection is affirmative for patient management and control of the disease. Detection of nonstructural-1 (NS1) antigen has been proven to provide early detection of DENV infection. Commercial NS1 antigen assays are available in Indonesia with variable sensitivity. In an attempt to develop an NS1-based diagnostic test, we successfully cloned NS1 gene of DENV2 to a glutathione S-transferase-based vector pGEX6P-1 in *Escherichia coli* system. The recombinant protein (pG2NS12) was expressed in *E. coli* BL21. After induction with isopropyl-β-D-thiogalactoside 0.1 mM for 4 h at 25 °C a recombinant protein GST-NS1 with molecular size of approximately 75 kDa was obtained. The fusion protein was insoluble and found in the pellet fraction of the cell lysate. Addition of lysozyme (10 mg mL^{-1}) and DNase-I (7.2 mg mL^{-1}) in the lysis buffer was necessary to collect proteins from the pellet fraction. The proteins in the cell pellet were fractionated through Sephadex-G100 column, and GST-NS1 was further purified with Glutathione-Sepharose 4B beads. To obtain pure recombinant NS1 protein to be used in the immunization of mice, the fusion protein was cut with PreScission Protease® by addition of 0.075% Triton-X 100 was necessary to cut the fusion protein. We found that antibodies that recognized the recombinant NS1 protein and DENV2 virus were produced in mice immunized with purified NS1 protein. Therefore, our recombinant NS1 could be used to produce antibody that is potentially useful for developing diagnostic assay to determine the presence of dengue virus NS1 antigen in patient sera.

Key words: cloning, dengue virus, expression, non structural protein NS1

Diagnosis dini infeksi virus dengue (DENV) amat penting dalam penatalaksanaan penderita dan pengendalian penyakit ini. Deteksi antigen *nonstructural 1* (NS1) telah terbukti mampu mendeteksi dini infeksi DENV. Uji antigen NS1 komersial telah tersedia di Indonesia dengan berbagai tingkat sensitivitas. Dalam usaha mengembangkan uji diagnosis berdasarkan NS1, kami telah berhasil mengklona gen NS1 DENV2 pada vector PGEX6P-1 yang berbasis glutathione S-transferase pada system *Escherichia coli*. Protein rekombinan pG2NS12 diekspresikan di *E. coli* BL21. Setelah induksi dengan isopropil-β-D-tiogalaktosida 0.1 mM selama 4 jam, pada suhu 25 °C didapatkan ekspresi fusi protein rekombinant GST-NS1 berukuran sekitar 75 kDa. Penambahan lisozim (10 mg mL^{-1}) dan DNase-I (7.2 mg mL^{-1}) dalam dapar pelisis diperlukan untuk mendapatkan protein rekombinan dari fraksi pelet sel. Protein rekombinan difraksinasi menggunakan kolom Sephadex-G100 dan dilanjutkan dengan purifikasi menggunakan Gluthatione-Sepharose 4B. Untuk mendapat protein NS1 murni yang digunakan dalam imunisasi mencit, protein fusi GST-NS1 dipotong dengan *PreScission protease*® dengan penambahan 0.075% Triton-X 100 pada reaksi. Imunisasi mencit dengan protein NS1 menginduksi antibodi yang dapat mengenali protein NS1 rekombinan dan virus DENV2. Hasil ini menunjukkan bahwa NS1 rekombinan dapat digunakan untuk memproduksi antibodi yang berpotensi dalam pengembangan suatu uji diagnostik untuk mendeteksi keberadaan antigen NS1 dalam serum penderita.

Kata kunci:, ekspresi, kloning, protein non struktural NS1, virus dengue

Dengue virus (DENV) infection is a major problem in Indonesia (Setiati *et al.* 2006). It can manifest as mild dengue fever (DF), severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), or even fatal. Early diagnosis of DENV infection is affirmative for patient management and control of the disease. Diagnosis of DENV infection is now mainly based on clinical observation and routine hematologic tests. Rapid serological test is available, but it gives good results only after the formation of IgG/IgM, i.e. five days after the onset of illness. Rapid early diagnostic test, such as RT-PCR test is also available, but they are expensive and need special equipment to conduct (Shu and Huang 2004). Thus, despite the availability of those tests, development of new rapid early diagnostic test to detect DENV infection remains a challenge.

Detection of NS1 antigen has been proven to provide early detection of DENV infection (Alcon *et al*. 2002; Dussart *et al*. 2008). NS1 protein is a non-structural protein which is important in the replication of DENV (Perera and Kuhn 2008). It can be found

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intracellularly, or in association with micro particles but not with virions and extracellulary.

To develop an NS1-based diagnostic test, we attempted to clone NS1 protein of DENV2 in *E.coli* system. The glutathione S-transferase system is chosen because it provides integrated system for expression, purification and detection, and already widely used to produce various viral proteins (Matusan *et al.* 2001; Ma *et al*. 2008). In the process, we still faced some problems in the expression and purification of the recombinant protein. Here, we described the construction of the recombinant protein gene in a vector and several methods to purify the protein to find the best way to obtain pure NS1 protein, and also test of the antigenicity of the protein in mice.

MATERIALS AND METHODS

RNA Extraction. RNA DENV-2 was obtained from patient plasma with code number of DS31/06 in Jakarta in 2006. RNA was extracted from 140 µL of plasma using QIAamp® viral RNA kit (Qiagen, ICI Americas Inc.) according to the manufacturer's instruction. Complementary DNA (cDNA) strands were reverse-transcribed using Super Script II First Strand Synthesis System with random hexanucleotide primer according to the manufacturer`s instructions (Invitrogen, California, USA). The PCR amplification of the entire genome was performed using the cDNA products in a PTC-100 ™ Programmable Thermal cycles (MJ Research, Inc).

Recombinant Plasmid. pGD$_2$NS12 plasmid was constructed by inserting NS1 DV2 gene and its 72 bp upstream fragment into pGEX6P-1 plasmid (Amersham Pharmacia Biotech, 1997). First, cDNA was obtained as stated above. Sequencing of E and NS1 region of the virus was done preceeding the cloning process. Full length of NS1 gene was amplified by PCR with sense primer (d2 2329s*Bam*: 5'- CGCGA GGATCCTGGATAGGAATGAATTCACGC-3') and anti sense primer (d2 ns-1-350 c*Sal* : 5'- TCCGCT GTCGACTCAGGCTGTGACCAAGGAGTT-3'). The primers contained upstream *BamH*I and downstream *Sal*I restriction sites (underlined nucleotides). To improve protein expression, the amplification of NS1 region included 72 bp up stream of NS1 (Falgout *et al.* 1989).

For cloning, PCR product was purified with a Qiaquick® PCR purification kit (Qiagen, ICI Americas Inc) and digested with *BamH*I and *Sal*I enzymes. After purification with Gel extraction kit (Qiagen), the NS1 fragment was inserted into pGEX-6-1 using T4 DNA ligase (Promega) and the ligation mixtures were transformed into competent *E. coli* Top 10 (Invitrogen, California, USA) (Ausubel *et.al.* 1994). Selection of recombinant bacteria was done on LB agar containing ampicillin 60 µg mL^{-1}. The size and orientation of the recombinant plasmid pGEX-6-1-NS1 (Fig 1) was confirmed by *Bal*I restriction enzymes digestion and PCR with primer pair of NS1 sense and antisense primers recognizing the vector part. The recombinant pGEX-6-1-NS1 was sequenced to confirm the cloned NS1 gene nucleotide sequence. The nucleotide sequence of the cloned NS1 gene and its deduced amino acid sequence were aligned to the sequence of the original virus as well as to the published sequences for DEN-2 strain existed in GenBank database.

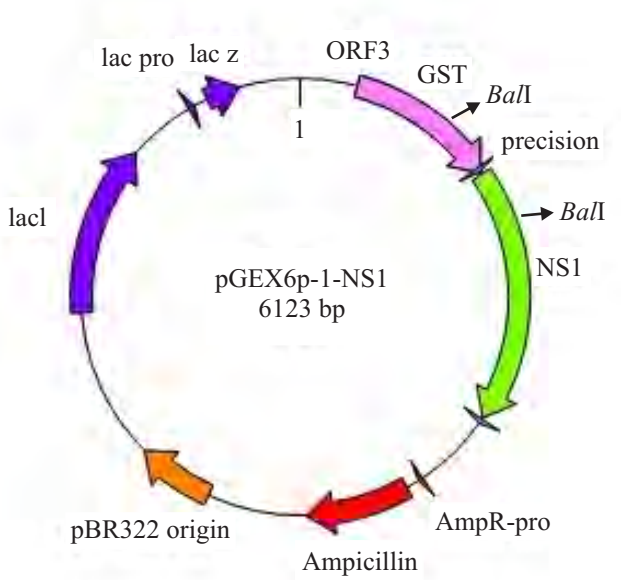

Fig 1 Construction of pGST-NS1 DV2. NS1 DNA fragment with 72 bp upstream sequence was inserted into *BamH*I and *Sal*I sites of vector pGEX-6P-1.

GST-DV2NS1 Protein Expression. For expression purposes, the recombinant plasmid was sub-cloned into *E. coli* BL21. To optimize expression, several conditions were carried out, i.e.: 1) IPTG inducer concentrations (0.05, 0.1, 0.25, 0.5, 0.75, and 1 mM); 2) induction time (1, 2, 3, and 4 h); 3) induction temperature (room temperature (25 ºC) and 37 ºC). IPTG concentration and time of induction were considered in relation with the amount of protein product and the possibility of inclusion bodies formation which can interfere the purification of recombinant protein.

The recombinant *E. coli* BL21 carrying were

cultured in Luria Bertani (LB) agar supplemented with 60 µg mL^{-1} ampicillin at 37 °C for 18 h, followed by subculture in LB broth containing 60 µg mL^{-1} ampicillin at 37 °C shaked at 200 rpm. After 18 h, it was subcultured again with starting OD_{600} 0.01, and incubated in rotary shaker at 37 °C until it reached OD_{600} 0.5. Expression of the fusion protein was induced with isopropyl-1-thio-β-D-galactopyranosine (IPTG) 0.05 to 1 mM, 1 to 4 h, 37 °C.

Four hours after IPTG induction, the bacteria were resuspended in phosphate buffer saline (PBS) pH 7.3 containing lysozyme (10 mg mL^{-1}) and DNase-I (7.2 mg mL^{-1}) and incubated 5 min, 37°C. The bacterial cells were lysed by freeze-thaw method (30 cycles of dry ice and 60 °C bath) or by sonication (30 cycles of 30 output 30 s pulse on and 10 s pulse off). The lysate was centrifuged at 13 000 x g, 10 min. Then the supernatant was collected and the pellet was resuspended in PBS, and stored in -80 ºC until tested. The pellet and supernatant fractions were examined by SDS polyacrylamide gel electrophoresis 8% (SDS-PAGE).

Purification of GST-NS1 DENV2. To reduce the cellular proteins found in the cell pellet, the suspension of the cell pellet was fractionated through Sephadex-G100 colum, and the fractions were analysed by SDS-PAGE 8%. The fractions with high concentration of the expected protein were taken and GST-NS1 DENV-2 protein was further purified using Glutathione-Sepharose 4B beads (Bulk GST Purification Modules, GE Healthcare) by modified manufacturer's method. The fraction containing recombinant protein was added with 50% Gluthatione Sepharose 4B and was incubated at 4 ºC for 18 h. The mixture was passed through *Bulk* GST. Here, the protein bound to the Glutathion-Sepharose, and then eluted with elution buffer.

Excission of GST-NS1 with PreScission Protease. We used PreScission Protease ® (GE Healthcare) to cut the NS1 protein from GST-NS1 by modified manufacturer's protocol. Several conditions were carried out to optimized excition proceses, i.e.: 1) In column or in solution; 2) duration of reaction; 3) with or without addition of Triton-X100. In column, treatment was done by passing the protein into the 120 Glutathione-sepharose 4B (GE Healthcare) column, followed by adding the PreScission Protease and then mix them. The reaction was incubated at 5 °C for 6 to 20 h. The colum was then centrifuged at 500 g for 5 min at room temperature. In solution, treatment was done by incubating the fusion protein with the PreScission Protease at 5 °C for 6 to 20 h. After incubation, the reaction mixture was passed through the Glutathion-Sepharose column. The GST fragment of the fusion protein and the protease would bind to the glutathione, but the NS1 fragment would pass during elution.

Immunogenicity of Recombinant NS1 DENV2 Protein. Immunogenicity study was done in mice. Immunization of mice was carried out by method described previously (Ausubel 1994) with minor modifications. Six mice Balb/C aged 6 to 8 weeks were immunized. For the first immunization either GST-NS1 fusion protein or purified NS1 protein were injected intraperitoneally into four mice and two mice, respectively. A hundred µg protein in the suspension of complete Freund's adjuvant were injected to each mouse. Three and two weeks later, respectively, the animals were given the first booster using 25 µg of purified NS1 protein in suspension of incomplete Freund's adjuvant. This procedure was repeated as the second booster two weeks later. A week after the second booster samples of mice sera were taken from tail vein and tested for anti-NS1 antibody by ELISA. ELISA was done using purified NS1 protein and DENV2 virus as antigen.

SDS-PAGE, Western blotting, and ELISA. SDS-PAGE and Western Blotting were used to determine whether rNS1 protein was expressed. The *E. coli* pellets were added with 100 ng mL^{-1} lysozyme in Tris EDTA and incubated for 5 min at 37 ºC. The mixture was dissolved in loading buffer (1% SDS, 1% of 2–mercaptoethanol, and 200 mM DTT) and then boiled for 5 min, chilled on ice for 5 min and separated by 8% SDS-PAGE in vertical electrophoresis unit (Biorad, California, USA). Two gels were run at a time. One of the gels was stained with coomasie blue staining solution and the molecular size was determined using the molecular weight standard (Sigma-Aldrich Co). The other gel which was unstained was transferred onto nitrocellulose membrane (Amersham Pharmacia) by a transblot TM cell (Biorad, California, USA) filled with methanol-Tris glycine buffer. To confirm protein transformation, membrane was stained with Ponceau S solution for 5 minutes at RT and destained with *washing buffer* (500 mL 1xPBS and 100 µL Tween 0.1%). The membrane was blocked with 5% skim milk in PBS for 18 h at 4 ºC. NS1 protein expression was determined by incubating the membrane with dengue positive sera and monoclonal antibody anti-Gst-HRP conjugate. NS1 protein was visualized by addition of DAB substrate with H_2O_2 for 5 min at RT.

Indirect ELISA was done according to the method described by Igarashi A (2000) in Technical manual of arbovirus study with special emphasis on Japanese

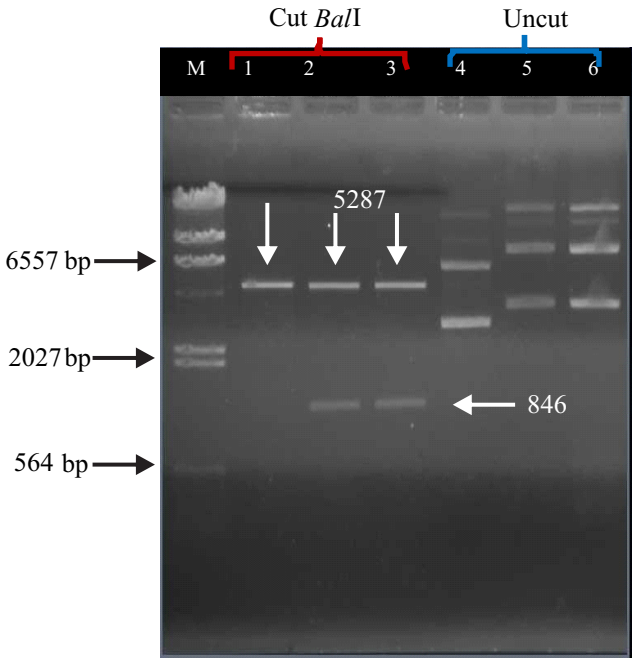

Fig 2 Analysis of recombinant clones. M: λ *Hind*III ; lane1 and 4. pGEX-6P-1 WT; lane 2 and 5. pG2NS9; lane 3 and 6: pGNS12. Lane 1 to 3: the plasmids were cut with *Bal*I; lane 4 to 6: the uncut plasmids.

enchepalitis and dengue viruses. ELISA plates were coated with 100 µL NS1 (2.3µg μL^{-1}) in 1:25 coating buffer and incubated overnight at 4 °C. After adding 300 µL blocking buffer containing 5% low fat milk (Tropicana Slim, PT Nutrifood Indonesia, Jakarta) in PBS pH 7.3 for 1 h at room temperature, they were rinsed with 300 µL washing buffer (PBS/Tween 20) three times. After that, the test sera diluted 1:50 were added and incubated for one hour at 37 °C, followed by rinsing with washing buffer. The secondary antibody (goat antimouse IgG HRP or goat antihuman IgG HRP) (Sigma Aldrich, Missouri) diluted 1:5000 in skim milk 1% was added and incubated for one hour at 37 °C, before the third rinsing. For detection, 100 µL substrate H_2O_2 + TMB (3,3′,5,5′-tetramethylbenzidine) (Kirkegaard & Perry Laboratories, Maryland) 1:1 was incubated for 10 min at room temperature in the dark. The reaction was stopped by addition of 100 µL H_2SO_4 3N, and the OD_{450} was read by ELISA reader (Bio-Rad Model 550, California).

RESULTS

Construction of pGST-NS1 DV2. The amplified and purified NS-1 gene fragment was inserted into expression vector pGEX-6P-1 to generate a recombinant plasmid pGEX-NS1. The purified and digested NS-1 gene was cloned in the correct frame with the GST at C-terminus of pGEX-6P-1 for high level of protein expression in *E. coli*. Transformation of ligation mixture into *E. coli* Top10 resulted in about 120 colonies. Random 16 colonies were chosen for further analysis. Recombinant clones (pGD_2NS9 and pGD_2NS12) with correct size were selected for protein expression (Fig 2). Sequencing of the inserts showed that no mutations found in four known B-cell epitopes of NS1 of the selected recombinant plasmid (Fig 3).

Expression of GST-NS1 Dv2. The expected 75kD protein of GST-NS1 DV2 can be seen after induction of *E. coli* BL21 containing pGD_2NS12 (Fig 4 and 5). The best result was obtained when induction was done with 0.1mM IPTG for 4 h at room temperature (data not shown), but the protein was mainly insoluble, and located in the pellet fraction of the cell lysate. The recombinant protein might be expressed as inclusion bodies. To increase the solubility of the protein in supernatant phase we tried other lysis buffers such as NTT buffer (1.5% N-Lauroysarcosine, 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 8.0), or lysis buffer containing 10 mM Tris pH.8,0; 0.1% Triton-X; 0.5 mM PMSF ; 0,1% lysozyme, and 5 mM imidazole). But these buffers seemed to interfere with the affinity of the fusion protein to the Glutathione-sepharose beads during purification step. So we did not use additional reagents for further processess, and instead, we focused on optimizing the purification of the protein from the pellet fraction. Addition of lysozyme and DNase into PBS in lysis buffer significantly improved cell lysis as could be seen by translucent appearance of the final results and after centrifugation the pellet could be easily resuspended in PBS.

Purification of GST-NS1 DV2 fusion protein. The GST-NS1 protein was in Gluthatione-sepharose 4B slurry and the protein was cut with PreScission Protease® with the protocol provided by the manufacturer. Cleavage was performed in both solution and in column, however the protease failed to cut the protein. Addition of a new enzyme did not improve the cleavage. So we tried to add Triton X-100 into the reaction, and the results showed that an addition of 0.075% triton X-100 cut the fusion protein successfully. Both cleavage in solution and in glutathione-sepharose column gave similar results. However, cleavage in glutathione-sepharose column had the advantage of reducing the amount of enzyme used. After cleavage, GST fragment and the protease remained bound to the glutathione and the NS1 protein passed through the column.

Immunogenicity of NS1 Protein. Mice were

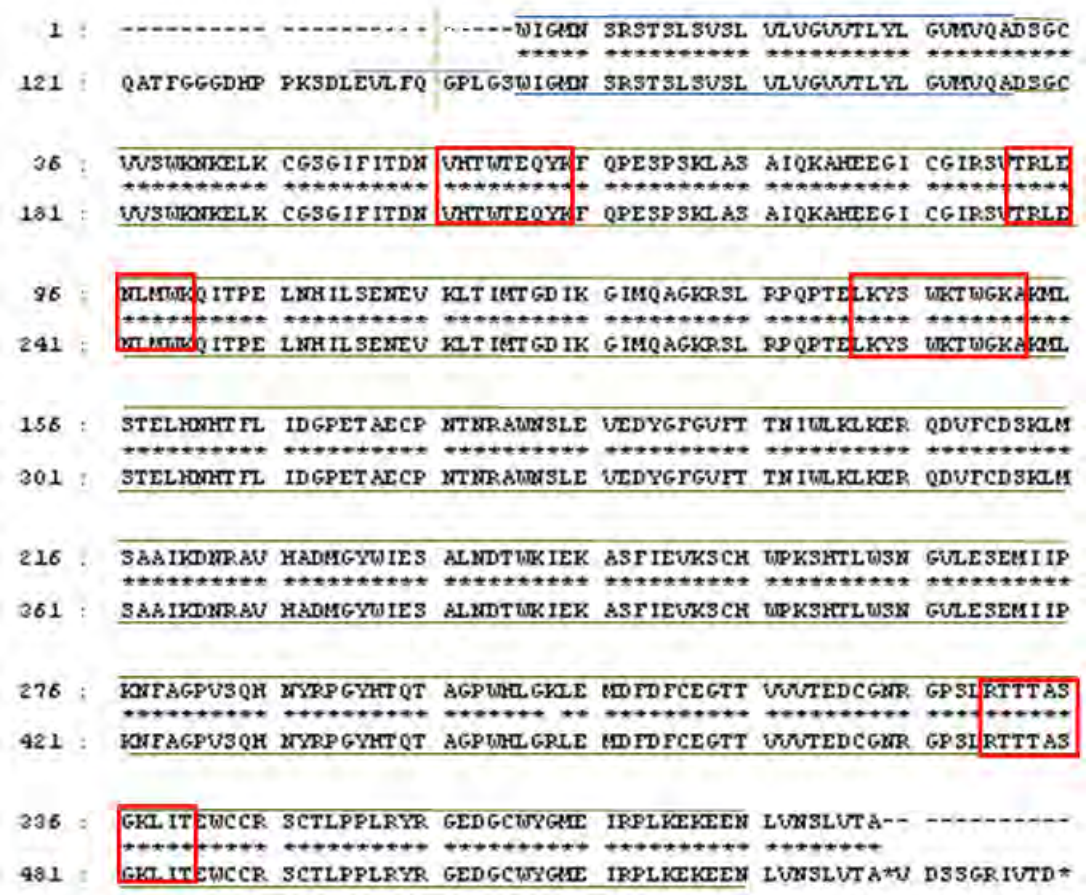

Fig 3 Deduced amino acid of NS1 insert in pGST-NS1 DV2 in comparison to NS1 sequence of the original DS31/06 sequence. Squares showed the positions of B-cell epitopes in NS1 protein.

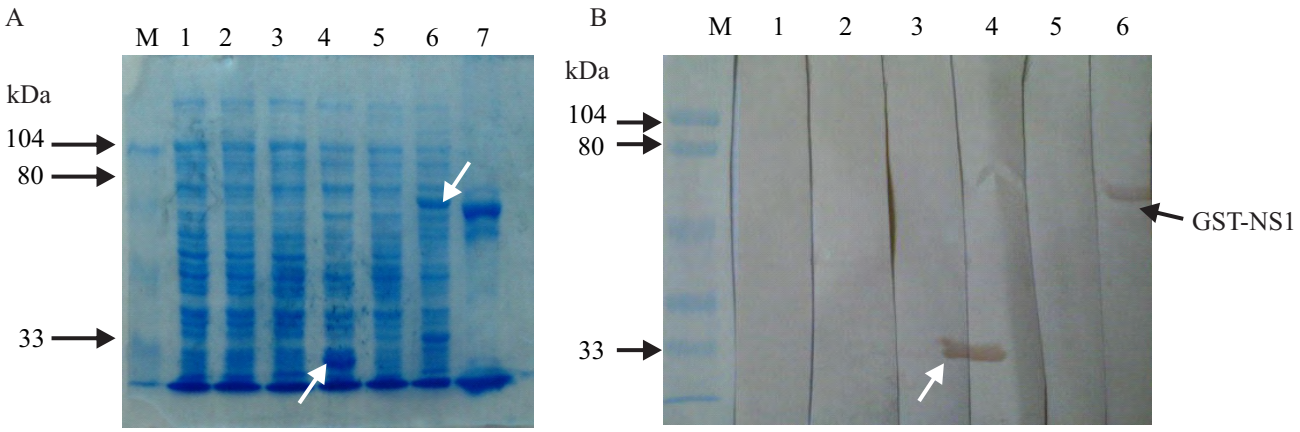

Fig 4 Analysis of the expressed fusion protein GST-NS1 DENV2 after induction with IPTG (A) on SDS-PAGE 8%; (B) WesternBlot using anti GST antibody. Arrows show the expected product GST-NS1 (75 kDa) and GST (26 kDa). Lane M: Protein marker; lane 1: *E. coli* BL21 without induction; lane 2: *E. coli* BL21 with induction; lane 3: *E. coli* BL21 containing pGEX-6P-1 without induction; lane 4: *E. coli* BL21 containing pGEX-6P-1 with induction; lane 5: *E. coli* BL21 containing pGD_2NS12 without induction; lane 6: *E. coli* BL21 containing pGD_2NS12 with induction; lane 7: DENV2.

immunized intraperitoneally with NS1 protein. One week after the second booster, samples of mice sera were tested by ELISA using purified NS1 and DENV2 as antigen. The immunized mice sera recognized both the recombinant antigen and the dengue virus (Fig 6). These results suggested that the NS1 protein expressed in *E.coli* could be served as a good antigen to induce antibody anti-NS1 in mice. This antigen can be used further in the production of antibody, and also can be used as antigen in the detection of antibody.

DISCUSSION

Rapid and early diagnostic of dengue virus

infection can lead to early therapeutic intervention and significantly related to the recovery of the patients. In several viral infections, virus load is greatest during the early symptomatic phase and immediately following the onset of symptome. In dengue virus infection, the peak of dengue viral load was before onset the fever (Vaughn *et al*. 2000). Dengue NS1 antigen detection is suggested as a helpful tool for the early diagnosis of dengue infection after the onset of fever in primary and secondary infection. It has been reported that NS1 antigen was found circulating from the first day after the onset of fever up to day 9, once the clinical phase of the disease is over (Shu *et al*. 2002). The NS1 protein could be detected even when viral RNA was negative in reverse transcriptase-PCR or in the presence of immunoglobulin M antibodies (Alcon *et al.* 2002). The circulating NS1 in acute phase serum sample is within the range of 10 ng mL^{-1} to 50 µg mL^{-1}, which does not differ significantly in primary or secondary infection (Alcon *et al.* 2002).

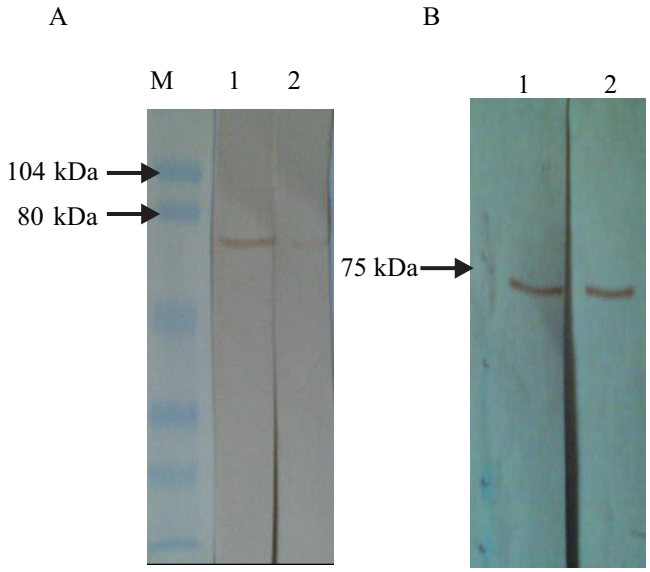

Fig 5 Western blot analysis of expressed GST-NS1 after purification of pellet and supernatant phase with Glutathione-sepharose 4B slurry. M: protein marker; lane 1: pellet; lane 2: supernatant. (A) Western blot done using anti-GST monoclonal antibody. (B) Western blot done using serum of patient with DENV2 infection.

Recently, commercial diagnostic NS1 kits are available in Indonesia with various specificity and sensitivity values. The sensitivity of some NS1 antigen assays ranged from 29 to 88%, and the specificity ranged from 89 to 100% (Guzman *et al*. 2010; Wang and Sekaran 2010). The reasoning behind the different sensitivities for different kits, different serotypes and different geographical sites requires further study. The difference may reflect different levels of avidity of the test mAbs for the relevant epitope(s) in NS1 from different serotypes, and potentially, different lineages from the same serotype, as well as the different virus burden caused by different serotypes (Guzman *et al.* 2010). The other limitation of of NS1 diagnostic kit is unability to distinguish within dengue serotypes. Qiu *et al* developed NS1 antigen assay using monoclonal antibody which successfully differentiated DENV-2 from other serotypes by 83.3% sensitivity and 100% specificity (Qiu *et al*. 2009). Overall, results of these studies suggest that the currently available NS1 antigen detection kits still need to be improved, mainly in sensitivity. In this study we cloned NS1 from DENV-2 virus isolated in Jakarta. We expected that using locally circulating strain as source may improve test sensitivity

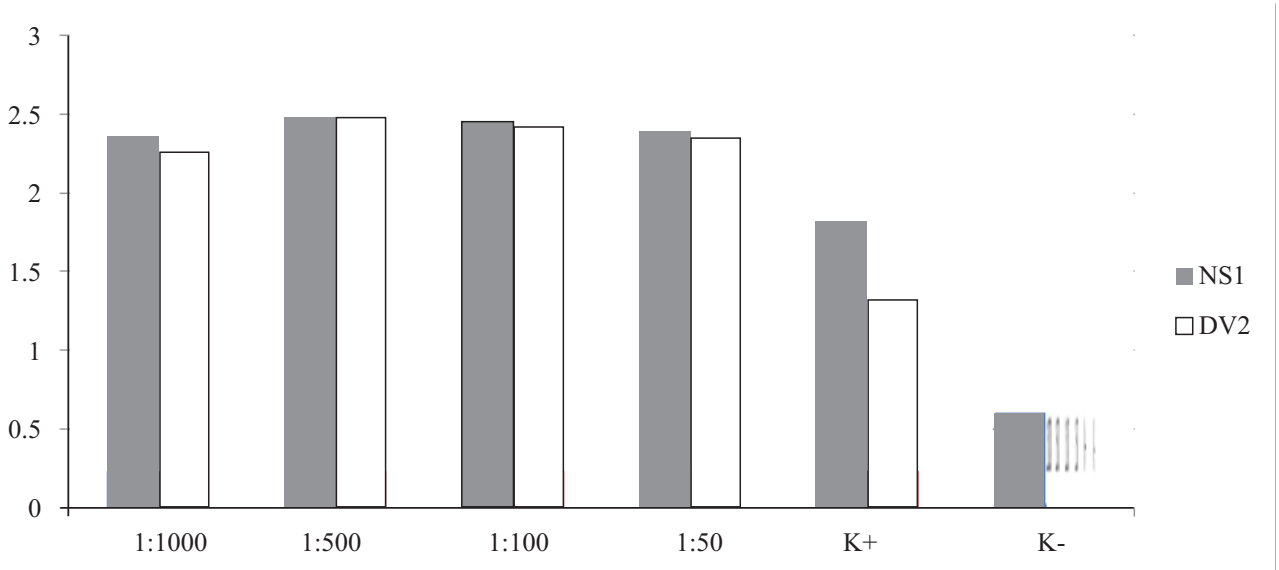

Fig 6 ELISA results to test sera from mice immunized with recombinant NS1 protein. OD_{450} value was the average of two tests. Recombinant NS1 and DENV-2 whole virus were used as antigen. K+ is positive control; K- is negative control.

if the test is to be used in Indonesia. However, this must be further investigated.

In this study, we used 72 bp upstram of NS1 region to express NS1 protein. Expression of NS1 dengue virus gene products involves specific proteolytic cleavages of a precursor polyprotein. Falgout *et al.* showed that the 24-residue hydrophobic sequence preceding NS1 was necessary and sufficient for the production of glycosylated NS1 and that this sequence was cleaved from NS1 in the absence of most dengue virus proteins. This hydrophobic sequence serves as an N-terminal signal sequence that is cleaved by signal peptidase .

Recombinant NS1 DENV2 in this study was expressed in a GST system in *E. coli*. Many eukaryotic genes can not be expressed efficiently in *E. coli* host due to the difference in codon preference as well as toxicity of foreign protein or mRNA. It is also known that heterologously expressed eukaryote protein are not post-translationally modified when it is expressed in *E. coli*. It is also difficult to express soluble protein or facilitate the secretion of expressed protein into culture media. Furthermore, proteins expression in large amounts tend to precipitate, forming inclusion bodies (Das *et al*. 2009) and present a difficulty in the purification. On the other hand, fusion proteins produced in this system have several advantages: they are produced at high level, are relatively stable, and can be easily be purified (Nasoff *e al*. 1991). In infected cells, monomeric NS1 is hydrophilic, but upon dimerization NS1 becomes more hydrophobic and membrane-associated (Winkler *et al*. 1989). In this study, GST-NS1 protein was also insoluble and remained in the pellet phase of the cell lysate. We failed to improve the solubility of the fusion protein into the supernatant fraction of the cell lysate. By adding lysozyme and DNAse in the lysis buffer and gluthatione-sepharose purification system, the GST-NS1 could be isolated from the pellet fraction. Despite this success of isolation, improvement of our method to increase the pure protein yield is still necessary. For diagnostic purpose, actually GST-NS1 can be used without cleavage (Nasoff *et al.* 1991). However, to improve the specificity of antibody anti-NS1 we produced, we think it is necessary to purify NS1 protein. For cleavage of GST-NS1, an addition of triton X-100 was necessary. This result suggested that the secondary structure of the fusion protein may cover the cleavage site of the protease. Immunization of mice with our recombinant NS1 also showed that the protein retained its ability to induce antibody that recognize both recombinant NS1 and DENV2 virus.

ACKNOWLEDGEMENTS

This research was in part funded by RISBIN IPTEKDOK 2008, Universitas Indonesia Reseach Grant (RUUI) 2009 (407M/DRPM-UI/A/N1.4/2009), and Competitive Grant DGHE Republic of Indonesia (Hibah Riset Berdasarkan Prioritas Nasional) Batch II 2009 (744A/DRPM-UI/A/N1.4/2009).

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