

Immunogenicity of Recombinant DNA Vaccine Encoding Non-Structural Protein-1 Dengue Virus Serotype-2 in Balb/c Mice

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Dengue Hemorrhagic Fever (DHF) is an infectious disease caused by the dengue virus (DENV) which spread widely in tropical and subtropical regions of the world. DENV is a single-positive strand RNA virus with a genome size of \pm 11kb which encodes three structural proteins, seven non-structural proteins, and two untranslated regions (UTR). The non-structural protein-1 (NS1) of DENV is known to have important role in dengue pathogenesis also promising to be developed as dengue vaccine. Lately, novel vaccine approach by DNA immunization have given new perspective for a safe, stable, and immunogenic vaccine platform. Previously, we had successfully constructed DNA vaccine encoding NS1 protein of DENV2 (pUNSI) which expressed recombinant NS1 protein in mammalian cells line. Thus, in this current study the ability of pUNSI to induce humoral immune response will be further analyzed by in mice immunization. Sixteen BALB/c mice aged of 4 weeks were immunized 3 times with 100 μ g of pUNSI or pUMVC4a on 2 weeks interval. Blood sampling was carried out just before immunization and termination was done 2 weeks after last immunization. Antibodies titer from individual mice sera against DENV-2 were measured with in-house ELISA. Anti-dengue NS1 IgG titer from mice group immunized with recombinant pUNSI Showed ELISA absorbances five times higher than pUMVC4a group. This result suggested the ability of pUNSI to induce humoral immune response against NS1 DENV-2 in vivo. Recombinant pUNSI can induce humoral immune response in mice.

Key words: dengue virus serotype-2 (DENV-2), humoral immune response, NS1, recombinant DNA

Demam Berdarah Dengue (DBD) adalah infeksi yang disebabkan oleh virus dengue (DENV) yang tersebar luas di wilayah tropis dan subtropis di dunia. DENV merupakan virus RNA rantai tunggal dengan ukuran genom \pm 11kb yang mengkode tiga protein struktural, tujuh protein non-struktural, dan dua daerah yang tidak ditranslasikan (UTR). Protein non-struktural (NS1) DENV diketahui memiliki peran yang sangat penting dalam patogenesis infeksi DENV dan sebagai pengembangan vaksin dengue yang menjanjikan. Saat ini, pengembangan vaksin baru dengan DNA yang diimunitasikan memberikan perspektif baru karena aman, stabil, dan imunogenik. Pada penelitian sebelumnya, kami telah berhasil mengonstruksi vaksin rekombinan DNA yang mengkode protein NS1 dari DENV-2 (pUNSI) dan diekspresikan pada sel mamalia. Oleh karena itu, pada penelitian ini dilakukan analisis lebih lanjut untuk melihat kemampuan pUNSI dalam menginduksi respon imun humoral dengan immunisasi pada mencit. Sebanyak 16 mencit BALB/c yang berumur 4 minggu diimunitasi sebanyak 3 kali dengan 100 μ g pUNSI atau pUMVC4a dalam interval waktu 2 minggu. Pengambilan sampel darah mencit dilakukan sebelum immunisasi dan dilakukan terminasi 2 minggu setelah immunisasi terakhir. Titer antibodi dari serum masing-masing mencit diukur dengan ELISA in-house. Titer IgG anti protein NS1 dari DENV2 dari kelompok mencit yang diimunitasi dengan rekombinan pUNSI menunjukkan nilai absorbansi yang tinggi, 5 kali lebih tinggi dari kelompok pUMVC4a. Hal ini membuktikan kemampuan pUNSI dalam menginduksi respon imun humoral terhadap NS1 DENV-2 secara in-vivo. Rekombinan pUNSI dapat menginduksi respon imun humoral mencit.

Kata kunci: dengue virus serotipe 2 (DENV-2), DNA rekombinan, NS1, respon imun humoral

Dengue Hemorrhagic Fever (DHF) is an infectious disease caused by the dengue virus (DENV). Dengue virus infection is a global health problem that occurs in tropical and subtropical regions of the world. Based on data from the WHO (World Health Organization) this disease has progressed to cause nearly 390 million people to be infected each year, including more than 960,000 cases of severe dengue (WHO 2016).

DENV belongs to the family Flaviviridae, of the genus *Flavivirus*. Dengue virion morphology is spherical with a diameter of \pm 50 nm. The outer part of the virion is covered by a sheath in the form of a membrane lipid with a sheath thickness of \pm 10 nm with Nucleocapsid inside about \pm 30 nm in diameter. DENV genetic material in the form of positive RNA single strand with a length of \pm 11 kb. The genome of this virus encodes a large polyprotein which is divided into three structural proteins (capsid (C); membrane precursor (prM); and envelope (E)), seven non-structural (NS)

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proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5), and two regions that are not translated or called the Untranslated Region (UTR) (Putnak *et al.* 2003; Lin *et al.* 2012). Based on genetically, dengue virus has four different serotypes are DENV-1, DENV-2, DENV-3, and DENV-4. Each DENV serotype has a protein E amino acid sequence homology of about 70% (Lin *et al.* 2012).

NS1 protein is a glycoprotein weighing 42-50 kDa from 353-354 amino acids encoded by 1056bp of genes (Clyde *et al.* 2006). This protein with other viral proteins can fulfill a structural role, helping to anchor the replication complex to the membrane and induce the formation of membrane components that facilitate viral RNA replication, assembly and release of viruses, immune avoidance, and various aspects of pathogenesis (Glasner *et al.* 2018). There are two forms of NS1 which expressed by infected host cell, on cell surface (mNS1 protein) and secreted into the extracellular environment (sNS1 protein). In the process of infection, mNS1 will be presented by Major Histocompatibility Complex (MHC) molecules and can induce the immune response. Meanwhile, sNS1 can directly bind to various components of the complement pathway which either trigger or inhibit the activity of complement also can be used as a marker because the presence of high levels of NS1 have shown an association between the severity of dengue virus infection (Henrique *et al.* 2014; Watterson *et al.* 2016).

The NS1 protein is able to activate endothelial cells directly and decrease the integrity of the endothelial cell barrier through dependent TLR4 pathway (Henrique *et al.* 2014). When DENV infects, the body can activate the cellular immune system and the humoral immune system in response to fight. As a result of the humoral immune system, dengue virus infection can lead to the formation of short-term protective antibodies in the form of immunoglobulin M (IgM) and long-term protective antibodies in the form of immunoglobulin G (IgG) against the virus (Shu P.Y. *et al.* 2000).

In contrast to viral structural proteins that induce neutralizing antibodies, NS1 protein can induce anti-NS1 antibodies. So, it's not inducing ADE (antibody dependent enhancement) activity which raised from M or E protein based vaccine (Hertz, *et al.* 2017). Recombinant DNA plasmid can be delivered to the host in several ways and methods so that it may mimic some aspects of the natural infection of the host cells. Compared to recombinant protein and recombinant virus vaccines, DNA vaccines are relatively

inexpensive, have low production costs, easy to manufacture and use, and safe because it is not pathogenic but still immunogenicity. This is based on the possibility of the DNA vaccine can induce CD8⁺ and CD4⁺ T cells so that stimulate the immune response through the MHC-I and MHC-II pathways (Leitner *et al.* 2007). In addition, a recombinant DNA plasmid is another potential strategy for the development of an NS1-based vaccine (Liu, Y. *et al.* 2016).

Several DENV vaccine approach based on NS1 protein are widely developed. In our previous study, we successfully constructed and proven that our recombinant pUNS1 (recombinant puMVC4.a encoding NS1 protein of DENV-2) able to express its protein in mammalian cell line (Sjatha *et al.* 2019). Furthermore, this current study will be analyzed the ability of our recombinant pUNS1 to induce humoral immune response in mice as initial evaluation of pUNS1 antigenicity as DNA vaccine.

MATERIALS AND METHODS

Ethics Statement and Mice. All protocols within this experiment have been ethically approved by Health Research Ethics Committee Faculty of Medicine Universitas Indonesia-RSCM number: KET-1350/UN2.F1/ETIK/PPM.00.02/2019. Animal experiments were carried out using 16 Balb/c mice in total, which were divided into two groups: (1) eight mice were immunized with pUMVC4.a control plasmid and (2) eight mice were immunized with pUNS1. Experiment in mice was carried out by experienced and certified animal welfare staff and under the supervision of attending veterinary. The animal experiment was carried out in the animal care facility of the Department of Microbiology, Faculty of Medicine Universitas Indonesia with a closed cage system and ad libitum feed-drink.

Viruses. The virus used as antigen for in-house ELISA was dengue virus serotype-2 (DENV-2) NCG (New Guinea C) strain harvested at 6 d.p.i titer of 5.02×10^7 FFU/ml.

Propagation of *Escherichia coli* Harboring Plasmid. DH5a competent cells *Escherichia coli* that has carried the recombinant pUNS1 and pUMV4.a plasmid were propagated in liquid Luria Bertani (LB) medium. To produce starter cultures, bacterial clones containing recombinant plasmids were streaked on solid LB medium containing 50 µg/µl kanamycin and incubated at 37°C for 24 hours, then one loop of bacterial colony was inoculated in 5 ml liquid LB

medium containing 50 µg/µl kanamycin and incubated at 37°C, 200 rpm. After 18-20 hours, 2 ml of liquid culture was moved in to 200 ml of fresh liquid LB medium containing 50 µg/µl kanamycin. Then, re-incubated at 37°C, 200 rpm for 18-20 hours. When the cell density reach OD equal to 3-4 x 10⁹ cells/ml, the cells were harvested. The bacterial culture was centrifuged at 4000 rpm for 10 minutes at 17°C to get bacterial cell pellet.

Plasmid Isolation. Plasmid isolation from bacterial pellet was carried out following the HiSpeed® Plasmid Midi Kit (Qiagen, catalog no: 12643) protocol. Pure DNA plasmids were confirmed by gel electrophoresis and stored at -30°C. Plasmid concentrations were measured using spectrophotometry method.

Confirmation of pUNS1 by PCR. PCR reaction was performed using (Invitrogen, catalog no: 10342-053). Composition of PCR reaction as much: 2.5 µl of PCR master mix; 15.25 µl of DW; 2.5 µl of dNTP 2mM; 1 µl of MgCl₂ 50 mM; 0.8 µl of Reverse primer 350 *XbaI* (10 mM); 0.8 µl of Primary Forward 2329 *sBam* (10 mM); 0.15 µl of *Taq DNA polymerase enzyme* (Invitrogen, catalog no: 10342-053).; and 2 µl of plasmid template. The PCR cycles used were: 95°C for 5 minutes; followed by 40 cycles of 95°C for 30 seconds; 55°C for 30 seconds; 72°C for 90 seconds; and ended was 72°C for 5 minutes. Marker used was λ DNA *HindIII* (Tiangen, catalog no: Md202).

Mice Immunization. Sixteen Balb/c mice were immunized with 100 µg pUNS1 and pUMVC4.a plasmid using NFI (needle free injector, ShimaJet, Japan) apparatus. Immunizations were held within 3 doses in 2 weeks interval and retro-orbital blood were taken prior to immunization under the sedation of ketamine-xylazine treatment. Two weeks after third immunization, mice were terminated through cervical dislocation and blood were drawn by cardiac puncture. Mice blood were then centrifuged at 3000 rpm for 5 minutes and collected sera were stored at -70°C until further examination.

Anti-NS1 IgG of DENV2 analysis with in-house ELISA. Termination individual mice sera from both groups were tested for IgG recognizing NS1 of DENV2 with in-house ELISA. Briefly, flat bottom 96-well ELISA plate (NUNC, catalog no: 442404) were coated with 10⁴ FFU/ml DENV2 in bicarbonate buffer pH 9.6 overnight at 4°C. Plate were washed third with PBS containing 0.05% tween 20. Negative control antigen was PBS 1x, negative control sera were pre-immunized sera, and positive control were mice sera

immunized with DENV-2. ELISA plate wells were coated with DENV-2 antigen with the same titer in each well. The coating process is carried out overnight at 4°C. The plate washed 3 times with PBS containing 0.05% Tween 20, followed by 5% skim milk blocking at 37°C for 1-hour incubation. Then, ELISA plate was washed followed by addition of 100 µl of diluted (1:200) serum samples and incubation at 37°C for 1 hour. After incubation, plate was washed and diluted horse anti mouse-IgG HRP labeled (1:5000; Vector Laboratories, Catalog No: PI-2000) were added followed by incubation at 37°C for 1 hour. After incubation, plate was washed and 80 µl of TMB substrate (1-Step, Catalog No: 34028) was added followed by incubation in dark with gentle agitation for 20 minutes at RT. Finally, 30 µl of stop solution was added and absorbances were measured at 450 nm.

Statistical Analysis. Numerical data obtained from ELISA were analyzed using SPSS program, tested for its normality by Shapiro Wilk test, followed by t-test. For significances at p value <0.05 and confidence level 95%.

RESULTS

Recombinant Plasmid pUNS1 Confirmation. PCR was performed to confirm the NS1 gene in pUNS1. The pUMVC4.a plasmid was used as negative control. As seen in Figure 1, the presence of NS1 could be detected in the recombinant plasmid pUNS1. This is indicated by the presence of a band in the pUNS1 lane. Recombinant pUNS1 had NS1 gene insertion of 1056 bp size based on colony PCR analysis.

Evaluation of anti DENV2 IgG Titer. Pre and post-immunization serum of individual mice sera from both pUNS1 and pUMVC4.a groups were used to measure IgG antibody titers induced by recombinant pUNS1 against DENV2. As seen in Figure 2, pre-immunization serum from both pUNS1 (Mean ± SD: 0.137 ± 0.008) and pUMVC4.a (Mean ± SD: 0.138 ± 0.009) group showed similar absorbances value. However, post-immunization serum mice's with pUNS1 (Mean ± SD: 1.508 ± 0.039) had anti DENV2 IgG value higher than mice immunized with pUMVC4a (Mean ± SD: 0.294 ± 0.034). This difference is said to be significant with a confidence level of 95% (p < 0.05). These results indicate that the pUNS1 recombinant was able to induce humoral immune response in mice, with 5 times fold higher than pUMVC4.a control group in recognizing DENV-2.

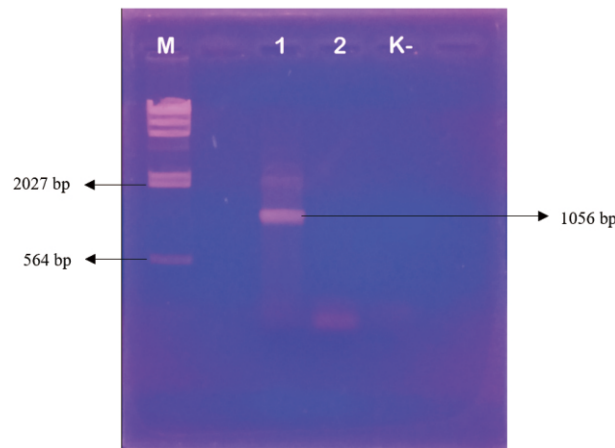


Fig 1 NS1 insert confirmation by PCR. M: Marker, 1: recombinant plasmid pUNS1, 2: plasmid pUMVC4.a, K-: negative control PCR.

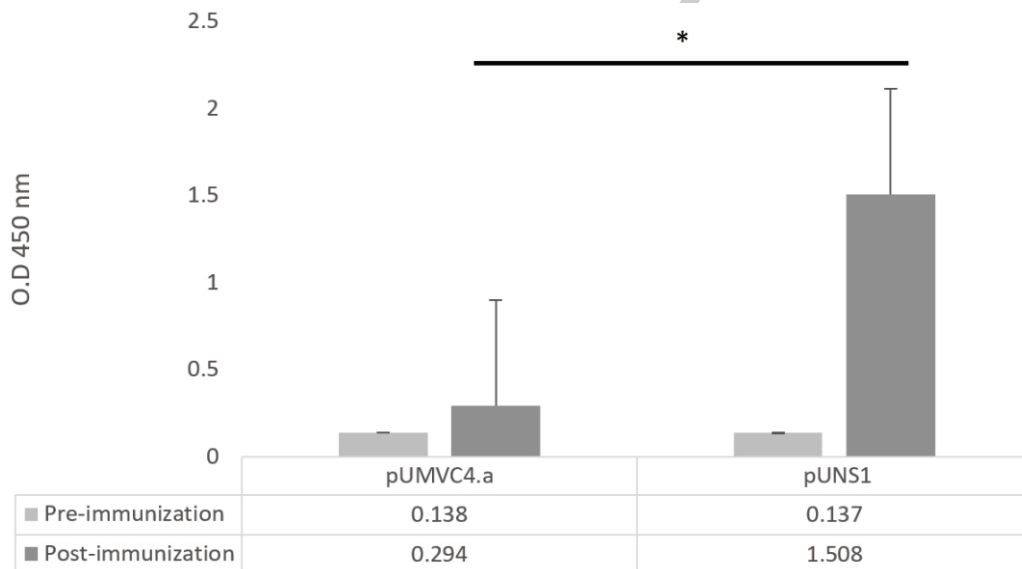


Fig 2 Antibody titer from mice group immunized with pUNS1 and pUMVC4a using in-house ELISA (* $p < 0.05$).

DISCUSSION

Secreted and membrane-associated of DENV NS1 are highly immunogenic because it can induce immune system correspond to the production of antibody against NS1 which also detected in infected patients (Chuang, Y-C, *et al.* 2013). It has been found that DENV NS1 protein with high levels is associated with severity of infection and levels >600 ng/ml in the first 72 hours of disease occurrence associated with the development of DHF (Libraty *et al.* 2000). Thus, NS1 is also developed as DENV vaccine approach since its advantages for not inducing ADE (antibody dependent enhancement) activity which raised from M or E protein based vaccine (Hertz, *et al.* 2017).

Although NS1 can induce non-neutralizing

antibodies, there have been reported problems. Anti-NS1 antibodies can cross-react with coagulation-related cells or molecules, such as human plasminogen, thrombin, platelets, and endothelial cells (Chuang, Y-C, *et al.* 2013). These anti-NS1 autoantibodies can cause thrombocytopenia and apoptosis mediated by nitric oxidation in endothelial cells in vitro (Lin, C-F, *et al.* 2002). Because of the homology of the sequences between the NS1 DENV protein and proteins in platelets and endothelial cells, it is possible that these autoantibodies are induced by NS1 through molecular mimicry (Lin, Y-S, *et al.* 2011). Although in vitro has shown the potential of cross-reaction between anti-NS1 antibodies and endothelial cells and platelets, this finding has not been supported or proven in vivo (Sun, D-S, *et al.* 2007).

An alternative vaccine delivery strategy can be performed to get better vaccine outcome. In this study, we use NFI (needle free injector) as apparatus to introduce DNA vaccine in Balb/c mice which proven to be more effective in delivering DNA in to target cell compare to syringe-based immunization. In the present needle-free immunization strategy, the vaccine plasmid could directly enter a relatively large number of muscle cells in the thigh of host animals (Imoto, J-I, *et al.* 2005).

After immunization, DNA vaccine can be uptake by the muscle cells and neighboring antigen presenting cells. The injected DNA vaccine will express the recombinant protein as endogenous antigen and presented by MHC class I. On the other hand, antigen can be released through extra cellular and further taken up by circulated APCs which lately presenting recombinant protein by MHC class II. The antigen-expressing APCs then migrate to lymph nodes where they activate the T and B lymphocytes to induce cellular and humoral immune responses (Leitner *et al.* 2007).

Although antigen presentation by both MHC class I and II, DNA vaccine have other advantages. DNA vaccine are safer, more stable for storage and shipping, easy to handle, induce cellular immune responses, licensed veterinary vaccine, relatively generic construction and production, and potent prime in animal studies (Khan 2013; Liu 2010). One of disadvantages of DNA vaccine is inducing antibody production against DNA (Khan 2013). In our result, IgG response of control pUMVC4.a group is also detected in low level of absorbances. This result may occur because of factors influencing immune responses against the vector such as route of administration, dose of vector, host-related factors, and promoters (Bessis *et al.* 2004). Antivector immunity was found in mice injected with the plasmid intraperitoneally, intravenously, or subcutaneously. Besides producing antibodies against the inserted gene, specific cytotoxic T cells were also found in the plasmids used. However, in mice injected with plasmids intramuscularly, the humoral immune response was obtained but with a lower cytotoxic T cell response (Brockstedt *et al.*, 1999). A study of plasmid-induced endothelial cells demonstrated the productivity of the cytokines TNF- α , IL-1 β , and IL-4 which increased promoter activity (Ritter *et al.* 2000). Sjatha *et al.* 2019, showed that levels of the cytokine TNF- α in CHO-K1 mammalian cell line which transfected by pcNS1 was higher compared control.

Also in our result showed IgG recognizing DENV-2 obtaining from pUNSI group has shown 5 times higher absorbances level compare to control pUMVC4.a group, indicating high production of recombinant NS1 expression and its immunogenicity in inducing humoral IgG response. Kim *et al.* 2010, showed DNA vaccines may have a relatively poor immunogenicity. This can be overcome by increasing the potential mechanisms of DNA vaccine such as plasmid alterations, increasing the stability of the DNA by formulations and encapsulation, delivery, and augmentation of immunity (Liu 2010).

To evaluate our recombinant pUNSI as DNA vaccine candidate, induced antibody may further analyzed for its ability to overcome DENV infection in-vitro or in-vivo. And also to evaluate the possibility of cross-reaction with other DENV serotype or other *Flavivirus*, cross-recognizing of antibody against NS1 protein to endothelial cell which lead to plasma leakage and also its ability to trigger complement cascade as vaccine safety concern.

In conclusion, we successfully prove the ability of our pUNSI to induce humoral immune response in mice. Further analysis for its antibody protection against DENV can be performed as one parameter in vaccine development.

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