NEUTRALIZATION CAPACITY OF PRE-MEMBRANE AND ENVELOPE ANTIBODY RESPONSE OF DENGUE SEROTYPE-1 DNA VACCINE AGAINST HOMOTYPIC AND HETEROTYPIC DENGUE VIRUSES

Angky Budianti[1], Dwi Hilda Putri[2], Beti Ernawati Dewi[1], Agus Sjahrurachman[1], Mulya Rahma Karyanti[3], T Mirawati Sudiro[1] and Fithriyah Sjatha[1]

[1]Microbiology Department, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital, Jakarta; [2]Department of Biology, Faculty of Mathematics and Sciences, Padang State University, Padang, West Sumatera;[3]Department of Child Health, Cipto Mangunkusumo Hospital, Jakarta, Indonesia

Abstract. Dengue virus (DENV) infection is still a burden in tropical countries such as Indonesia. Although approved dengue vaccine is now currently available, high viral dispersity and variety still remain challenges for vaccine efficacy. A DNA vaccine was developed based on pre-membrane and envelope (prME) gene of dengue serotype-1 (DENV-1) genotype-1 isolated from dengue patient during a dengue outbreak in Jakarta in 2009. PrME DENV-1 genes were inserted into pUMVC4, a mammalian expression vector, producing pUMVC RDS 59/09 recombinant DNA vaccine, followed by antigen expression by immunostaining. Individual mouse serum from immunized mice was tested for antibody response and pooled sera were analyzed using a focus reduction neutralization test (FRNT) to assess neutralizing antibody capacity against homotypic and heterotypic DENV-1 isolates. ELISA indicated induction of antibody levels and high $FRNT_{50}$ values of neutralizing antibodies were also obtained against homotypic DENV-1 genotype-1 and heterotypic genotype-4. The results show DENV DNA vaccine was promising approach, which merits further development as a dengue vaccine prototype.

Keywords: antibody, dengue, DNA vaccine, neutralization capacity

INTRODUCTION

Dengue virus (DENV) infection is still a major health challenge in tropical country such as Indonesia. Dengue hemorrhagic fever is the 8th among 10 top infectious diseases, with 0.91% case fatality in Indonesia (NIHRD, 2008; MOH, 2012). According to WHO, from 2004 to 2010 Indonesia ranks the world's number two country in terms of dengue cases, costing an estimated USD 300 million in care and treatment every year (Bhatt *et al*, 2013).

DENV, a member of Flavivirus family, has a 11 kbp genome size and sharing an envelope (E) protein with 70% amino acids homology, DENV is distinguished in having four different serotypes (Holmes and Twiddy, 2003). Among each serotype, DENV has diverged into several geno-

Correspondence: Fithriyah Sjatha, Microbiology Department, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital. Jl Pegangsaan Timur no. 16, Central Jakarta 10320, Indonesia.
E-mail: titi_sjatha@yahoo.com

types based on nucleotide sequence variations of E protein gene with different geographical distributions. DENV serotype-1 (DENV-1) comprises five genotypes (GI to V), now widely spread all over the world since the discovery in Japan in 1943 of Mochizuki strain GI (Villabona-Arenas and Zanotto, 2013). DENV-1 has spread to Southeast Asia, South America, Arabian Peninsula, Australia and Polynesian islands (Yamashita *et al*, 2016). In Indonesia, DENV1 is the predominant dengue serotype found in patients, indicating high virulence or pathogenicity compared to other three dengue serotypes (Prasetyo *et al*, 2011; Lestari *et al*, 2017).

DENV contains three structural and five non-structural proteins, with membrane (M) and integral envelope (E) proteins forming the viral particle (Perera and Kuhn, 2008). Being the outer part of the virion, E proteins are recognized by host immune system, and thereby several neutralizing epitopes of these proteins have been considered as suitable candidates for dengue subunit vaccines development (McArthur *et al*, 2013). A number of vaccine platforms are using pre(pr)M and E proteins, heterologously expressed as recombinant proteins in *Escherichia coli*, yeast and *Drosophilla* systems in preclinical studies (Yauch and Shresta, 2014). On the other hand, Konishi *et al* (2000) demonstrated the ability of a recombinant expression plasmid containing prME protein gene to induce antibody response in immunized animals, indicating a promising step towards a dengue DNA vaccine development.

To date, neutralizing antibodies are still the main indicator of vaccine efficacy in protecting against DENV infection (WHO, 2007). Several studies showed neutralizing antibody capacity may differ against DENV of different serotypes and/or genotypes (Barban *et al*, 2012; Sjatha *et al*, 2013; Yamanaka *et al*, 2016). This phenomenon may appear in the form of a decline of neutralizing capacity and also, more worrisome, in appearance of antibody-dependent viral enhancement due to sub-neutralizing antibody titer, which leads to an increase of disease severity (Wahala and de Silva, 2011). Thus, a suitable dengue vaccine platform able to provide protection against DENV of a given serotype or genotype without inducing any enhancing activities is urgently needed.

Herein we report on the development and *in vivo* evaluation of a dengue DNA vaccine based on expression of prME protein from DENV-1 strains isolated from dengue patients in Jakarta, Indonesia. The results should constitute a step towards achieving a safe and effective dengue DNA vaccine for use worldwide.

MATERIALS AND METHODS

Cells and virus stock

Vero cells were maintained on minimal essential media (MEM) (Gibco, Dublin, Ireland) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich, Darmstadt, Germany) at 37°C under a humidified atmosphere 5% CO_2 and used for virus propagation, transfection and focus reduction neutralization test (FRNT). DENV-1 isolates used in the study (Table 1) were obtained from Microbiology Department, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital, Jakarta. DENV-1 Hawaii strain was obtained from the National Institute of Health Research and Development, Ministry of Health Republic of Indonesia. DENV serotype was confirmed by nested RT-PCR (Lanciotti *et al*, 1992) followed by

Table 1
$FRNT_{50}$ value of murine immune serum against DENV-1 isolates.

DENV-1	Genotype	$FRNT_{50}$
RDS 59/09	I	640
IDS 11/10	I	640
IDS 12/10	I	640
RDS 53/09	I	1,280
Hawaii	I	1,280
D1 073	IV	1,280
D1 915846	IV	1,280
DSA 11/10	ND	640
DSA 54/09	ND	1,280
IDS 80/10	ND	640
IDS 13/10	ND	640
IDS 41/10	ND	1,280
IDS 23/10	ND	1,280

$FRNT_{50}$, highest serum dilution producing 50% focus reduction compared to control pooled serum; ND, not determined.

genotype analysis through sequencing of E gene.

DNA vaccine construction

PrME protein gene of DENV-1 RDS 59/09 was generated through RT-PCR using specific primers and inserted into mammalian expression vector pUMVC4.a (Aldevron, Fargo, ND) as previously described (Hasnida, 2012). Correct construction of pUMVC RDS 59/09 was confirmed through PCR, restriction enzyme and sequencing analysis.

Antigen expression analysis

Vero cells were transfected with pUMVC RDS 59/09 using Lipofectamine (Thermo Fisher, Waltham, MA) according to manufacturer's protocol, incubated for 24 hours at 37°C, and treated with chilled acetone:methanol (1:1) followed by DENV-1-infected patient's immune serum (1:500 dilution), then rabbit peroxidase-conjugated anti-human IgG (Sigma-Aldrich) (1:1,000 dilution). Immunoreactive proteins were visualized by application of diaminobenzidine (Thermo Fisher); positive cells were stained red.

Mice immunization

Mice (n = 24), 4-5 weeks old (Bio-Farma, Bandung, Indonesia) were divided into two equal groups, one group immunized with 25 μg of pUMVC RDS 59/09 in phosphate-buffered saline (PBS) three times over a period of three weeks and the other group (control) with pUMVC4.a in a similar fashion. Blood (100-200 μl) from each immunized mouse was collected retro-orbitally just before immunization and three weeks after the last immunization for preparation of sera. Then mice were euthanized. Sera were prepared from control mice at the same time as the immunized mice.

Animal experiment protocols were approved by the Faculty of Medicine, Universitas Indonesia Ethics Committee (approval no. 120/H2.F1/ETIK/2014).

ELISA

Antibody response from immunized mice were analyzed using ELISA. In brief, 96-well MaxiSorp plates (Invitrogen, San Diego, CA) were coated with $1x10^6$ pfu/ml DENV-1 RDS 59/09 in carbonate coating buffer and incubated at 4°C overnight, then 100 μl aliquot of blocking buffer (1% skim milk in PBS) was added to each well and plates were incubated for 1 hour at 37°C, followed by addition of individual mouse serum (1:100 dilution in PBS). Following 1hour incubation at 37°C, goat anti-mouse peroxidase-conjugated antibody (Sigma-Aldrich) (1:2,000 dilution in PBS) was applied to each well for 1 hour at 37°C, followed by addition of

tetramethylbenzidine substrate (Nacalai, Kyoto, Japan). After 10 minutes reaction was terminated by addition of phosphoric acid and $A_{450\,nm}$ measured using 800^{TM} TS Absorbance Reader (BioTek, Winooski, VE).

FRNT

FRNT was performed as previously described (Putri *et al*, 2015). In short, a mixture of preincubated serially diluted pooled mouse serum obtained after 3rd immunization and DENV-1 RDS 59/09 ($2.5x10^4$ pfu/ml) at 4°C overnight, was applied to wells of a 96-well plate containing 80% confluent Vero cells. After incubation at 37°C for one hour to allow virus infection, a semi-overlay media (1.5% methylcellulose) (Sigma-Aldrich) in maintained Vero cells media containing 2% FBS) was applied followed by incubation for two days at 37°C. Cells were washed with PBS and immunostaining using patients' pooled immune serum was performed as described above. Focus number was reported as highest serum dilution producing 50% focus reduction ($FRNT_{50}$) compared to control pooled serum.

RESULTS

ELISA antibody titer

Murine antibody titer against homotypic DENV-1 RDS 59/09 was determined by ELISA. Mean $A_{450\,nm}$ of individual mouse serum from pre-immunization, and from week 0 to week 3 immunization showed increase in antibody levels from immunized mice following each vaccination but significantly higher level than control was observed only after the third vaccination (p-value <0.05) (Fig 1).

$FRNT_{50}$ antibody response against DENV-1 isolates

Mice sera from three weeks post last

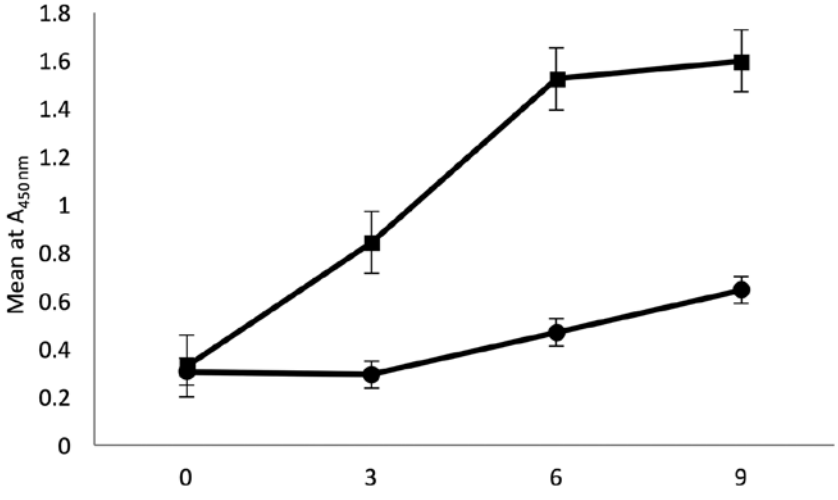

Fig 1-Mean ELISA value from immunized mice sera. Mice (n = 12) were immunized with 25 μg of pUMVC RDS 59/09 in phosphate-buffered saline three times over a period of three weeks and serum prepared from each mouse. Control group mice (n = 12) were immunized with pUMVC4.a (empty vector) in a similar fashion. ELISA was performed according to standard technique using goat anti-mouse peroxidase-conjugated antibody. (●) Immunized mice. (■) Control mice. Vertical line indicates SD. On X-axis, number indicates week.

immunization were pooled and neutralization capacity against a variety of DENV-1 isolates was evaluated using FRNT. $FRNT_{50}$ value against parental virus RDS 59/09 was obtained from homotypic isolates G-1 IDS 11/10 and IDS 12/10 but higher against RDS 53/09 and Hawaii, the latter value being obtained against heterotypic isolates G-IV D1073 and D1 915846 (Table 1). Against unidentified DENV- genotypes, $FRNT_{50}$ ranged as homotyic and heterotypic values.

DISCUSSION

A major challenge in dengue vaccine development is the existence of four closely related DENV serotypes, each comprising of several genotypes (Holmes and Twiddy, 2003). Hence, sub-neutralizing antibody titer against DENV may lead an increase in virus numbers due to antibody dependent enhancement (ADE) (Flipse *et al*, 2013) and an effective dengue vaccine should be able to simultaneously induce neutralizing antibodies equally against all four DENV serotypes. Currently, a recombinant live attenuated dengue vaccine is available but with lower adequate neutralization capacity against DENV-2, presumably a result of discrepancy in the parental virus strain used in the vaccine preparation (Silveira *et al*, 2019).

We developed a dengue DNA vaccine based on prME gene of DENV-1 G-I that was able to induce in a mouse model significantly higher antibody response compared to control animal immunized with empty expression vector. The immunization regimen employed has been successfully reported (Konishi *et al*, 2006; Yunita, 2012).

FRNT revealed varying levels of neutralizing antibody against homotypic DENV-1 strains, but consistently low levels against known heterotypic DENV-1 strains. This might be due to differences in E protein sequences. To date, among all dengue vaccines only PUO359 strain originating from DENV-1 G-1 contains an E protein sequence more similar to that of RDS 59/09 used in our study than that of Western Pacific G-IV strain (Table 2) (Villabona-Arenas and Zanotto, 2013). It is worth noting $FRNT_{50}$ values differ only by two folds.

By only expressing prME protein, a virus-like particle (VLP) is able to form with a size similar to DENV particle (Urakami *et al*, 2017). VLP is a self-assembling particle consisting of viral structural protein, mimicking the conformation of native viral particle and is highly immu-

Table 2
Amino acid differencies of E protein from DENV-1 strains.

E protein region	RDS 59/09 *vs* PUO359*	RDS 59/09 *vs* Western Pacific*
Domain I	S8N, R14G, T171S; T180A	S8N, S155T, T171S, T180A
Domain II	S225T	A88T, K202E, E203K
Domain III	I320V, I324V, L356V	I320V, I324V, T329S
Stem-anchor	V436I, G449S	G449S, V461I

Amino acid alignment was performed using Genetyx Ver.10 software with nucleotide sequence of PUO359 (GenBank accession no. AF425630) and Western Pacific DENV-1 strain (GenBank accession no. U88535).

nogenic without any risk of becoming infectious or of reverting to a virulent virus as it lacks a genome. To date, VLP vaccine is an alternative feasible approach to live attenuated vaccines, which are currently used against HepBV and HPV (Choroboczek *et al*, 2014).

As only two representative DENV-1 strains of heterotypic genotype were used in our study, future studies will be required using additional virus strains with various genotypes. Another aspect that needs to be investigated is the presence of ADE activity in murine sera as a consequence of sub-neutralizing antibody titer.

In conclusion, this study lays a foundation for future research towards the development of safe and effective dengue DNA vaccine(s) suitable and affordable for developing countries including those in Southeast Asia.

ACKNOWLEDGEMENTS

The study was funded in part by JICA-JST SATREPS 2010-2014 collaboration between Kobe University and the Faculty of Medicine Universitas Indonesia. Publication was supported by PDUPT 2018 RISTEKDIKTI grant. Number 321/H2.R3.1/HKP.05.00.

CONFICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Barban V, Munoz-Jordan JL, Santiago GA, *et al*. Broad neutralization of wild-type dengue virus isolates following immunization in monkeys with a tetravalent dengue vaccine based on chimeric Yellow Fever 17D/dengue viruses. *Virology* 2012; 429: 91-8.

Bhatt S, Gething PW, Brady OJ, *et al*. The global distribution and burden of dengue. *Nature* 2013; 496: 504-7.

Choroboczek J, Szurgot I, Szolajska E. Virus-like particle as vaccine. Review. *Acta Biochimica Polonica* 2014; 61: 531-9.

Flipse J, Wilschut J, Smit JM. Molecular mechanisms involved in antibody-dependent enhancement of dengue virus infection in humans. Review. *Traffic* 2013; 14: 25-35.

Hasnida A, Sudiro TM, Putri DH, *et al*. Immunogenicity of DNA vaccine candidate expressing PRM and E proteins of dengue-1 virus Indonesian Strain in mice. Singapore: Singapore International Conference on Dengue and Emerging Infections, 2012. (Oral presentation).

Holmes EC, Twiddy SS. The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol* 2003; 3: 19-28.

Konishi E, Kosugi S, Imoto J. Dengue tetravalent DNA vaccines inducing neutralizing antibody and anamnestic responses to four serotypes in mice. *Vaccine* 2006; 12: 2200-7.

Konishi E, Yamaoka M, Kurane I, *et al*. A DNA vaccine expressing dengue type 2 virus pre-membrane and envelope genes induces neutralizing antibody and memory B cells in mice. *Vaccine* 2000;18:1133-9.

Lanciotti RS, Calisher CH, Gubler DJ, *et al*. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992; 3: 545-51.

Lestari CSW, Yohan B, Yunita A, *et al*. Phylogenetic and evolutionary analyses of dengue viruses isolated in Jakarta, Indonesia. *Virus Genes* 2017; 017: 1474-7.

McArthur MA, Sztein MB, Edelman R. Dengue vaccines: recent developments, ongoing challenges and current candidates. *Expert Rev Vaccines* 2013; 12: 933-53.

Ministry of Health (MOH) Republic of Indonesia. Indonesia health data profile 2011. Jakarta: Ministry of Health Republic of Indonesia, 2012 (in Bahasa Indonesia).

National Institute of Health Research and Development (NIHRD). Basic medical research 2007. Jakarta: Ministry of Health, 2008 (in Bahasa Indonesia).

Perera R, Kuhn RJ. Structural proteomics of dengue virus. *Curr Opin Microbiol* 2008; 11: 369-77.

Prasetyo DS, Budianti A, Dewi BE, *et al*. [Association between dengue virus serotypes and type of dengue viral infection in department of child health, Cipto Mangunkusumo Hospital, Jakarta, Indonesia]. *Den Bul* 2011; 35: 205-13.

Putri DH, Sudiro TM, Yunita R, *et al*. Immunogenicity of a candidate DNA vaccine based on the prM/E genes of a dengue type 2 virus cosmopolitan genotype strain. *Jpn J Infect Dis* 2015;68:357-63.

Silveira LTC, Tura B, Santos M. Systematic review of dengue vaccine efficacy. *BMC Infect Dis* 2019; 19: 750.

Sjatha F, Takizawa Y, Kotaki T, *et al*. Comparison of infection-neutralizing and –enhancing antibody balance induced by two distinct genotype strains of dengue virus type 1 or 3 DNA vaccine in mice. *Microbes Infect* 2013;15:828-36.

Urakami A, Tun MMN, Moi ML, *et al*. Envelope-modified tetravalent dengue virus-like particle vaccine: implication for flavivirus vaccine design. *J Virol* 2017; 91: 01181-7.

Villabona-Arenas CJ, Zanotto PMA. Worldwide spread of dengue virus type 1. *PLOS One* 2013; 8: e62649.

Wahala MPBW, de Silva AM. The human antibody response to dengue virus infection. *Viruses* 2011;3:2374-95.

World Health Organization (WHO). Guidelines for plaque reduction neutralization testing of human antibodies to dengue virus. Geneva: WHO, 2007.

Yamanaka A, Moi ML, Takasaki T, *et al*. Neutralizing and enhancing antibody responses to five genotypes of dengue virus type 1 (DENV-1) in DENV-1 patients. *J Gen Virol* 2016;98: 166-72.

Yamashita A, Sakamoto T, Sekizuka T, Kato K, Takasaki T, Kuroda M. DGV: dengue genographic viewer. *Front Microbiol* 2016; 7: 875.

Yauch LE, Shresta S. Dengue virus vaccine development. *Adv Virus Res* 2014; 88: 315-72.

Yunita R. Immunogenity of recombinant DNA vaccine candidate using premembrane and envelope genes of dengue virus type 2 in mice. Jakarta: Universitas Indonesia, 2012. 115pp. Thesis.