รายงานวิจัยฉบับสมบูรณ์

โครงการ
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โดย ชุติธร เกตุลอย และคณะ

ธันวาคม 2556

สัญญาเลขที่ MRG5480150

รายงานวิจัยฉบับสมบูรณ์

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“ Immunogenicity in mice of a chimeric dengue DNA vaccine based on DENV-4 E domain III replaced in DENV-2 prM/E backbone”

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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งานวิจัยและพัฒนาดีเอ็นเอวัคซีนเพื่อป้องกันโรคไข้เลือดทั้ง 4 สายพันธุ์ จากหลายแห่ง รวมทั้งวัคซีนต้นแบบที่เราได้พัฒนาขึ้นมานั้น ต่างประสบกับปัญหาในการกระตุ้นให้เกิดภูมิคุ้มกันต่อเชื้อไวรัสเด็งกี่ซีโรไทป์ 4 ได้ต่ำที่สุดเมื่อเปรียบเทียบกับซีโรไทป์อื่น งานวิจัยนี้จึงพัฒนาขึ้นเพื่อเพิ่มระดับภูมิคุ้มกันต่อเชื้อไวรัสซีโรไทป์ 4 ซึ่งประกอบไปด้วย 2 กลวิธี 1) การปรับโครงสร้างของแอนติเจนด้วยการออกแบบให้ดีเอ็นเอวัคซีนเป็นแบบลูกผสมที่ใช้ส่วนที่สามของโปรตีนเปลือกหุ้มของเชื้อไวรัสเด็งกี่ซีโรไทป์ 4 แทนที่ใน prM/E ของไวรัสเด็งกี่ซีโรไทป์ 2 ที่เป็นยีนแกนหลัก หรือเรียกว่า "chimeric D4EDIII-D2prME" 2) การเปลี่ยนสายพันธุ์ไวรัสอ้างอิงที่ใช้ในการทดสอบ plaque reduction neutralization test (PRNT): strain C0036 (เชื้อที่แยกได้ในปี 2006) กับ strain 1036 (เชื้อที่แยกได้ในปี 1976)

การทดสอบการแสดงออกของโปรตีนใน Vero cells พบว่าดีเอ็นเอลูกผสม (chimeric D4EDIII-D2prME) ที่ได้พัฒนาขึ้น สามารถสร้างโปรตีนได้ในระดับที่ใกล้เคียงกันเมื่อเปรียบเทียบกับดีเอ็นเอต้นแบบของเชื้อไวรัสเด็งกี่ซีโรไทป์ 2 (D2prME) และ ไวรัสเด็งกี่ซีโรไทป์ 4 (D4prME) การทดสอบประสิทธิภาพในการกระตุ้นภูมิคุ้มกันในหนูทดลอง โดยการฉีดดีเอ็นเอลูกผสม และดีเอ็นเอต้นแบบของเชื้อไวรัสเด็งกี่ซีโรไทป์ 4 ปริมาณ 25 μg จำนวน 3 ครั้ง ด้วยวิธี *in vivo* electroporation พบว่าเมื่อใช้เชื้อเด็งกี่ซีโรไทป์ 4 strain 1036 ในการทดสอบ จะไม่สามารถตรวจพบ NtAb ของหนูที่ได้รับวัคซีนทั้งสองชนิดได้เลย แต่เมื่อเปลี่ยนมาใช้เชื้อเด็งกี่ซีโรไทป์ 4 strain C0036 ในการทดสอบ พบว่าระดับของ NtAb เพิ่มสูงขึ้นอย่างมีนัยสำคัญทาง โดยมีค่า median PRNT50 เท่ากับ 80 และ 160 เมื่อได้รับดีเอ็นเอลูกผสมและดีเอ็นเอต้นแบบของเชื้อไวรัสเด็งกี่ซีโรไทป์ 4 ตามลำดับ ดังนั้นเชื้อเด็งกี่ซีโรไทป์ 4 strain 1036 จึงไม่เหมาะสมในการนำมาใช้เป็นไวรัสอ้างอิงที่ใช้ในการทดสอบ PRNT ในปัจจุบัน ผลจากการศึกษาครั้งนี้แสดงให้เห็นถึงความจำเป็นในการตรวจสอบการวิวัฒนาการของเชื้อไวรัสเด็งกี่อย่างต่อเนื่อง ซึ่งอาจมีผลกระทบต่อการออกแบบเอนติเจนสำหรับดีเอ็นเอวัคซีน และการเลือกใช้ไวรัสสายพันธุ์อ้างอิงในการทดสอบ PRNT ต่อไปในอนาคต

คำหลัก : chimeric DNA vaccine, domain III, DENV-4, DENV-2

ABSTRACT

Project Code : MRG 5480150

Project Title : "Immunogenicity in mice of a chimeric dengue DNA vaccine based on DENV-4 E domain III replaced in DENV-2 prM/E backbone"

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Several studies, including our dengue tetravalent DNA vaccine have been suffered from the lowest immunogenicity against dengue serotype-4 (DENV-4). This issue remains unexplained. In this study, two strategies were investigated to improve the DENV-4 immunogenicity: 1) restructuring the antigenic antigen by designing a new chimeric DENV-4 envelop glycoprotein domain III (EDIII) replaced in DENV-2 prM/E (D2prME) backbone (designated as "chimeric D4EDIII-D2prME"; 2) changing the DENV-4 reference strains that used in plaque reduction neutralization test (PRNT): the new strain C0036 (isolated year 2006) *VS* the old strain 1036 (isolated year 1976).

In vitro protein expression in Vero cells showed that our chimeric D4EDIII-D2prME, D2prME and D4prME DNA vaccines produced the similar levels of E. The immunogenicity was evaluated by three times *in vivo* electroporation of 25 µg chimeric D4EDIII-D2prME or D4prME DNA vaccine in mice. By using an old DENV-4 reference strain 1036, PRNT50 titers were not detectable in any serum samples from mice immunized with both DNA vaccines. Surprisingly, the neutralizing antibody titers were significant increased by changing the reference virus to a new strain C0036. The median PRNT50 induced by the chimeric and original DNA vaccine were 80 and 160, respectively. It is evident that the DENV-4 strain 1036 is not suitable for currently PRNT assay. This study illustrates the need to continuously monitor the dengue viral evolution that might have a profound effect on the design of antigenic antigen using in the DNA vaccine and the choice of the reference strain in the PRNT assay in the future.

Keywords : chimeric DNA vaccine, E domain III, DENV-4, DENV-2, neutralizing antibody

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Chutitorn Ketloy

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CHAPTER I

Executive Summary

Dengue is a mosquito-transmitted viral disease occurring mainly in tropical and subtropical regions. Each year, an estimated 50-100 million cases of dengue occur worldwide and around 250,000-500,000 cases of dengue hemorrhagic fever (DHF) have been reported (1). Dengue infection is caused by any four serotypes of dengue virus (dengue serotype 1-4 virus; designated DENV-1-4) which belong to the genus *Flavivirus* in the family *Flaviviridae* (2). Infection with any four dengue serotypes can produce the illness range from a mild, dengue fever (DF), to the severe disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The disease severity is depended on the subsequent virus infection. The homotypic infection is generally protective. In contrast, the heterotypic infection leads to an increased disease severity as a result of the presence of antibody dependent enhancement (ADE) and cross-reactive memory T lymphocytes (3). Therefore, an effective dengue vaccine should be a tetravalent vaccine.

Although, several dengue vaccine candidates are currently being developed, no licensed dengue vaccine is available. The major obstacles to develop vaccines are the lack of reliable animal model that mimic the dengue disease and the phenomenon of ADE (4). In spite of this, the characteristic of an ideal dengue vaccine should include: a) good safety profile, b) rapid immunization regimen requiring a single vaccine or two that fit in with established vaccine programs, c) long lasting protection to all 4 serotypes (tetravalent), d) no ADE, e) useful for both children and adults, f) inexpensive and affordable, g) easily stored and transported (adapted from (5)).

Nowadays, several approaches are being undertaken for the development of tetravalent dengue vaccines. Of these, live-attenuated vaccine strategy including traditional attenuation, engineered attenuation and chimerization, is the most advanced candidate that demonstrated the high immunogenicity in clinical trials. However, the viral replication interference is a crucial problem when all four DENV serotypes are combined (6-9). The new

non-infectious approach, DNA vaccine strategy, should be overcome this concern since it does not cause interference in antibody response when combined immunization (10). In addition, DNA vaccine has several advantages including safety (no risk for infection), low cost of production, simplicity of construction and stability for storage and handling (11).

In dengue virus vaccine development, DNA vaccine has been reported to be successful in pre-clinical studies in mice and non-human primates. Dengue DNA vaccines encoding the pre-membrance (prM) and envelope (E) genes, were reported to be immunogenic in animal without interference when combining monovalent components (12, 13). Recently, the first proof-of-principle Phase I clinical trial of the prototype DENV-1 DNA vaccine, which was administered intramuscularly using needle-free Biojector 2000, demonstrated the safety and moderate immunogenicity of the dengue DNA vaccine in humans (14).

In our tetravalent dengue DNA vaccine study in mice, which was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, various strategies has been used to improve neutralizing antibody induction including the selected plasmid vector, the codon-optimization of the prM/E sequences, the addition of intracellular signal sequences, and the DNA delivery systems such as needle-free immunization and *in vivo* electroporation. Our tetravalent DNA vaccine candidate, consisting of pCMVkan-based plasmids expressing the prM/E genes of each of the four serotypes of dengue virus, induced neutralizing antibody responses to all dengue serotypes (median PRNT50 titer against DENV-1, -2, -3 are 1:320 and DENV-4 is 1:20) after three-times immunization by *in vivo* electroporation (total 100 μg/dose, 25 μg of each monovalent). It is obvious that our DENV-4 DNA stimulating the lowest titer. Such DENV-4 low immunogenicity has also been observed by others and remains unexplained. The tetravalent DNA vaccine study by Konishi E, based on mixing four monovalent plasmid vaccine encoding the *prM/E* gene, can induce NtAbs to all dengue serotypes but the weakest NtAb was observed in DENV-4 (PRNT70 of pooled serum = 1:10) (13). Recently, Lima DM showed that three time (100 μg) monovalent DENV-4 prM/E DNA immunized mice also developed low titer of neutralizing antibodies (PRNT50 of pooled serum =1:128) (15). In an attempt to enhance the tetravalent DNA vaccine

immunogenicity, Porter KR used Vaxfectin as an adjuvant by mixing in the DNA formulation and evaluated the immunogenicity in monkeys. The addition of Vaxfectin resulted in a significant increase of the NtAb titers against all dengue serotypes. However, anti-DENV-4 NtAb titers are still low in both animals immunized with tetravalent DNA vaccine alone or mixing with Vaxfectin (16). These lines of evidence, therefore, lead us to hypothesize that the antigenicity of DENV-4 viral structure might be a problem of the low immunogenicity.

The current efforts to develop candidate vaccines focus on the critical regions that elicit neutralizing antibodies in E protein. The E protein is the major surface exposed protein of the DENV virion which composed of three discrete domains: a central domain (I), a dimerization domain (II) and an immunoglobulin (Ig) like domain (III). The envelope domain III, referred to as EDIII, consists of 100 amino acids (residues 295-395) of the C-terminus. This domain is known as the receptor recognition and binding domain since it contains multiple serotype-specific neutralizing epitopes (17). Consistent with this, EDIII-based DNA and protein vaccine candidates have been developed and shown their immunogenicity in mice (18-22). However, the comparison study of DENV-2 DNA vaccines showed that levels of neutralizing antibodies were significantly higher in truncate envelope-vaccinated mice than in only EDIII-immunized mice (22).

The main propose of this study is to improve our monovalent DENV-4 DNA vaccine immunogenicity. The antigenic design of a chimeric D4EDIII-D2prME DNA vaccine which used only EDIII of DENV-4 to replace in the prM/E backbone of DENV-2 DNA vaccine was evaluated in a mouse model. Furthermore, the comparison between the old strain DENV-4 reference virus strain 1036 and new strain C0036 in PRNT assay was also investigated in this study.

Research questions

1. Whether candidate chimeric DNA vaccine which encoded humanized DENV-4 envelop glycoprotein domain III of replaced in DENV-2 prM/E backbone (designated “chimeric D4EDIII-D2prME”) can improve the neutralizing antibody against DENV-4 in mice?
2. Whether the PRNT DENV-4 reference virus causes the sub-optimal chimeric and DENV-4 DNA vaccine immunogenicity?

Objectives

1. To improve the neutralizing antibody against DENV-4 by construct candidate chimeric humanized DENV-4 envelop glycoprotein domain III replace in DENV-2 prM/E as a backbone immunized in mice.
2. To evaluate the candidate chimeric and DENV-4 DNA vaccine immunogenicity by changing the DENV-4 reference viruses from DENV-4 strain 1036 (isolated year 1976) to DENV-4 strain C0036 (isolated year 2006).

CHAPTER II

LITERATURE REVIEWS

Dengue virus, DENV, is belong to the *Flavivirus* genus of the *Flaviviriade* family which also includes yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus. There are four antigenically distinct serotypes (DENV1-4) based on neutralization assay. DENV is transmitted to humans by biting of Aedes mosquitoes including the *Aedes aegypti* and *Aedes albopictus (23)*. The prevalence of dengue disease is high especially in the tropical and subtropical regions including the Asia-Pacific region and the Americas. Due to the climate change and increased international travel, dengue disease is becoming one of the most important emerging vector-borne viral diseases with increasing the distribution to new countries. An approximately 50 million dengue infection cases occur annually with around 500,000 cases of severe dengue and 20,000 deaths per year (24).

Characteristics of dengue viral genome

Dengue viral genome is a positive-single strand RNA virus, approximately 10.7 kb and encodes three structural proteins; capsid protein (C), precursor membrane/membrane protein (PrM/M), and envelope protein (E) and seven nonstructural proteins (NS): NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, which are associated with viral replication and disease pathogenesis (25).

The capsid protein stabilizes the viral genome within the viral nucleocapsid. The N-terminus of the C protein act as a nuclear localization sequence which allows C protein translocation into the nucleus and interaction with heterogeneous nuclear ribonucleoprotein (26). The C-terminal of C protein encodes a signal sequence for helping the translocation of prM to ER and then were cleaved by viral serine protease (NS3 and its

co-factor, NS2B) (27). According to the capsid is located inside viral particle, no neutralizing epitope was contained in this region (28).

The prM protein is a primary precursor of M protein which cleaved by host furin in the trans-Golgi network during virion maturation process. prM and M act as a chaperone that helps the folding of E protein during transportation in acidic pH in trans-Golgi network (29, 30). Mice immunized with prM and M proteins had been reported to confer protection.

The E protein is the major protein exposed on the virus surface which functions for viral attachment and endosomal membrane fusion. Therefore, neutralizing epitopes were mostly detected in E protein (31-33). The E protein consists of three distinct structural domains, designed domains I (DI), II (DII) and III (DIII). DI, central β-barrel structure positioned between DII and DIII, play as hinge region which important for fusion. This domain contains predominately non-neutralizing of both virus-specific and cross-reactive epitopes. DII, an elongated domain, contains a dimerization region and a highly conserved “fusion loop”. It participates in structural rearrangements that trigger by acidic pH when the virus fuses with an endosomal membrance (34). Furthermore, several overlapping neutralizing epitopes are found at the tip of DII, in the hinge region between DI and DII. Finally, DIII, an immunoglobulin-like domain, contains the host cell receptor binding site (33, 35) which involved in the immune response. Therefore, various serotype-specific neutralizing epitopes are concentrated in this domain.

The NS1 protein is a glycoprotein that could be found in secreted form but mostly expressed on the surface of infected cells. The functions of surface expressed NS1 have been reported to induce protective antibodies with complement fixing activity, which kills infected target cells (36, 37).

NS2A plays essential role in viral replication by interact with NS3, NS5 and 3’UTR (38) and also able to inhibit interferon (IFN) signaling pathway (39). NS2B is a cofactor of NS3 which together form an active serine protease complex (40). NS3 acts as a multifunctional proteins that required for polyprotein processing, viral replication and viral assembly which included N-terminal protease domain, RNA 5′-triphosphatase, RNA helicase

and RNA-stimulated NTPase domain in the C-terminal region (41-46). Both NS4A and NS4B may be involved in blocking IFN signaling pathway (47, 48). Moreover, NS4B assists viral replication by its association with NS3 (49). NS5 is the largest conserved DENV protein. It encodes two functional enzymes, i.e. methyltransferase (MTase) which can methylate the 5′end of viral RNA (50) and RNA-dependent RNA polymerase (RdRp) (51).

Dengue virus pathogenesis

The pathogenesis of dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) is not fully understood. Various hypotheses have been proposed including viral pathogenesis and immunopathogenesis, which might play important roles in clinical manifestation of DHF/DSS.

The viral pathogenesis caused by the difference of dengue serotype or genotype which might provide to virus variation and influence human disease severity (52). Several factors are involved in immunopathogenesis of dengue disease including the adaptive immune responses and inflammatory mediators.

The adaptive immune responses which known as “antibody-dependent enhancement (ADE)” play essential roles in the dengue immunopathogenesis. ADE phenomenon caused by the presence of preexisting heterologous antibodies which cannot neutralize the recent infecting virus (53). Those sub-neutralizing or non-neutralizing Abs allow viral internalization by Fcγ receptor (FcγR)-dependent (53) or FcγR-independent mechanisms (54) leading to increase viral load (55). This hypothesis was correlated with clinical outcome, 95% of DHF/DSS cases in south-east Asia children are historical related with secondary infected by heterologous DENV serotype (56-58).

Besides the ADE which induced by antibodies, the “original antigenic sin” also play a role in immunopathology. The original antigenic sin is caused by the cross-reactive memory T cells which were preferentially responses to previously infected DENV serotype during secondary infection with low affinity to current infecting serotype resulting in inefficient viral clearance (59, 60).

The inflammatory mediators including cytokines, chemokines, complements, coagulation and endothelial markers are remarkably increased in plasma of the severe dengue patients (61-63). There are mainly produced by monocytes (64), T cells (65), mast cells (66) and neutrophils (67) during the infection.

Dengue Vaccines Development

Ideal dengue vaccine and challenge

The ideal dengue vaccine should provide “tetravalent” protection which induced balance immunity to all four DENV serotypes and reduce the risk of ADE. Moreover, the DENV vaccine should also have a) a good safety profile, b) the cost-effectiveness, c) the rapid immunization regimen (only single or two dose that fit in with established vaccine programs, d) the long lasting protection, e) be suitable for use in children and adults, and f) easily stored and transported (adapted from (5)).

There are two major obstacles for the development of dengue vaccines. The first one is the pathogenesis is complicated and remained unclear. Another one is the lack of an appropriate animal model that reproduces human disease. Mice is commonly used at the beginning of preclinical study due to the reason of cost and convenience. Non-human primate is also used in further step in vaccine development. Although non-human primates can be infected but do not fully develop diseases as seen in human (68). At present, immunocompetent or humanized mice were used in preclinical vaccine evaluation and showed some clinical signs after DENV infection (69-71).

Current DENV vaccines in the pipeline

Although no approved DENV vaccine is available, several approaches have been used to developed dengue vaccine candidates. These include live attenuated virus (LAV), live chimeric virus expressing dengue envelope (E) antigens, purified inactivated virus (PIV), recombinant subunit vaccines and DNA vaccines (72). The most clinically advanced dengue vaccine candidate is live viral vaccine. However, various problems had been reported such as unbalance neutralizing antibody responses of the four serotypes and viral interference when all four serotypes were combined into tetravalent vaccine. Non-viral vaccines are alternative approaches for safety issues. Most of the subunit and DNA vaccines are focused on the E protein and its derivatives.

Live attenuated virus vaccine (LAV)

LAV is the first strategy in DENV vaccine development. Viruses were attenuated by serially passage in primary dog kidney (PDK). Two groups from Mahidol University, Thailand and the Walter Reed Army Research Institute (WRAIR), USA are independently developed tetravalent attenuated DENV vaccines. The Phase I and II clinical trials of Mahidol LAVs has been ceased due to the seroconversion could not induced in all volunteers and some showed improper reactogenicity (73-75). The WRAIR LAVs also showed problems of unbalanced immune response and reactogenicity (76). Recently, new LAVs formulations revealed the safety and immunogenicity in Phase II, however, additional evaluation on the protective efficacy of vaccine are crucial (77).

Live chimeric attenuated virus vaccine

ChimeriVax Dengue tetravalent vaccine (Sanofi Pasteur) is the most advanced DENV vaccines in pipeline which is the first to reach the clinical Phase III studies (ClinicalTrials.gov identifier:NCT01134263). This vaccine employed the YFV 17D as genetic backbone which replacing the prM and E genes of one of the four DENV serotypes (CYD1-4). Pre-clinical studies demonstrated that tetravalent vaccine is immunogenic in monkeys (78).

Although in Phase I studies, the CYD1-4 vaccine indicated their safety, recently Phase II study revealed only 30 percent effectiveness against only DENV-1, 3 and 4 (79). These results raise the number of concerns/questions that needs more testing and modification for clinical trials (80).

A site-directed mutagenesis of the viral genome is another approach for generating the attenuated virus. Deletion of 30 nucleotides (Δ30) in 3'-untranslated region (UTR) of DENV-4 was the first attenuate DENV-4 (designed as DEN4Δ30) (81) and the other serotypes were subsequently performed. Unfortunately, only DENV-1 and DENV-4 were successfully developed and shown its immunogenicity (82-84). Therefore, alternative chimeric virus for DENV-2 and -3 was invented by using the DEN4Δ30 as genetic backbone (designed as DEN2/4Δ30 and DEN3/4Δ30. The mixtures of these monovalent DENV vaccines (DEN1Δ30, DEN-24Δ30, DEN3/4Δ30 and DEN4Δ30) are currently in Phase I clinical studies (85).

Inactivate/Killed virus vaccine

Inactivated vaccine provides some advantages than LAV such as possibility to reverse into virulence virus is very low, induction both cell-mediated and humoral immune responses and more easy to manipulate or combine in tetravalent formulation (86). However, DENV propagation to reach high titer is quite difficult and inactivated DENV vaccination required multiple doses boosting or adjuvants for enhancing immunogenicity. Hence, this vaccine type might be useful in the combination with other vaccine platforms in prime-boost strategy (87, 88).

Subunit protein vaccine

The major immunogen for dengue subunit vaccine is mainly focused on recombinant E protein. There are several dengue E protein expression systems including *E.coli*, yeast and insect cells which has been shown their immunogenicity and protective efficacy in animal models (89-92). Recently, two interesting E protein studies were performed. However, adjuvants are required for enhancing the immunogenicity of recombinant proteins.

First, the truncated 80% E protein (DEN-80E) which was produced by Hawaii Biotech Inc by using Drosophila S2 expression system. Currently, the admixture of tetravalent truncated DEN-80 E proteins with aluminum hydroxide (adjuvant) have advanced into clinical studies (92).

Second, the domain III of the DENV E protein (EDIII) is a new attractive subunit protein vaccine which had been demonstrated the induction of protective antibody in both mouse (93) and non-human primate model (94) immunized with EDIII protein. The summary of ED III subunit vaccine development within the past 5 year is in the Table 1. In an attempt to develop a tetravalent EDII subunit vaccine, the alignment of amino acid sequence from different isolates of four DENV serotypes was used to create a consensus EDIII (cEDIII) protein. The novel cEDIII with aluminum phosphate adjuvant successfully induced cross-neutralizing Ab against all four DENV serotypes in mice (95) and neutralizing Ab against serotype 2 in non-human primate (96). An engineer recombinant lipoprotein (lapidated EDIII) which is EDIII protein fused with lipid signal peptides was showed to induce higher NtAb than EDIII formulated with alum (97).

Table 1 Currently dengue EDIII-based DNA and subunit protein vaccine candidates within last 5 years

Vaccine types	Developer/ Year	Details	Methodology	Immunogenicity results	Reference
DNA vaccines					
Dengue serotype 2 E and domain III	Azevedo AS. 2011 (Brazil	DENV-2 E DNA vaccines fused to the human tissue plasminogen activator signal sequence (t-PA(using pcDNA3 vector (Invitrogen) -pE1D2 Domain I II III, (80% E) -pE2D2 only E domain III	Two time immunization (i.m) in BALC/ mice on day 0 and 14 1. 100 µg DNA pE1D2 (80%E) 2. 100 µg DNA pE2D2 (EDIII) Challenge intracerebrally with 3.8LD_{50} NGC DENV-2 (mouse brain adapted)	Group 1 $PRNT_{50}$ titers higher than Group 2 (Data not show) Protection rate: DENV-2 Challenge Gr.1 = 100 % (20/20) Gr.2 = 65 % (20/13)	(22)
Subunit Protein Vaccines					
Dengue serotype 2 E and domain III	Izquierdo A. 2012 (Cuba)	Chimeric EDIII-C protein vaccine (DENV-2 Jamica) (Valdes et al., 2009).	Three time immunization (i.p.) of 23 µg of DIII-C-2 protein in mice BALC/c, n=10 using Alum as a adjuvants on day 0, 15, 30 Challenge intracranially (i.c.) with 50 LD_{50} of DEN-1,-2 and -4 on one month after last dose	$PRNT_{50}$ Neutralizing antibody = 36.1 (GMT) DIIIC-2 neutralizing antibodies only against the homologous virus Protection rate: immunized with DIII-C-2 protein DENV-1 Challenge = 6.6 % DENV-2 Challenge = 86.6 % DENV-4 Challenge = 6.6 %	(98)

<table>
<tr><th>Vaccine types</th><th>Developer/ Year</th><th>Details</th><th>Methodology</th><th>Immunogenicity results</th><th>Reference</th></tr>
<tr><td>Dengue serotype 1 E and domain III</td><td>Chiang, C.Y 2012 (China)</td><td>Recombinant dengue-1 envelope protein domain III (DENV-1-ED III) + various adjuvants
1. aluminum phosphate
2. CpG
3. PELC
4. PELC plus CpG</td><td>Three time immunization (S.C.) in mice (BALC/C)10 µg per dose + various adjuvant
1. Aluminum phosphate
2. CpG
3. PELC
4. PELC +CpG</td><td>$FRNT_{50}$ Titer against dengue serotype 1
1. Aluminum phosphate = < 2^3 ($P < 0.05$)
2. CpG = < 2^3 ($P < 0.05$)
3. PELC = < 2^3 ($P < 0.05$)
4. PELC + CpG = $2^{4.6}$ ($P < 0.05$)</td><td>(99)</td></tr>
<tr><td>Dengue serotype 2 E and domain III</td><td>Valdés I. 2011 (Cuba)</td><td>Prime-boost strategy : infective DENV-2 virus and a chimeric E-domain III-capsid) protein (Valdes et al., 2009).</td><td>Prime-boost immunization (S.C.) in green monkeys (n=3)
Prime with infective DENV-2 (4 log10 PFU) and three months later boost with ED-III-C (100 µg) aggregated with random ODNs in Alum</td><td>$PRNT_{50}$ neutralizing antibody (GMT) on day 30,60, 90 after prime on day 0
<table><tr><td>Day 0</td><td>Day 30</td><td>Day 60</td><td>Day 90</td></tr><tr><td><10</td><td>33.6</td><td>109.4</td><td>160.9</td></tr></table>$PRNT_{50}$ neutralizing antibody (GMT) on day 120,150,180,210 and 270 after boost on day 90
<table><tr><td>D120</td><td>D150</td><td>D180</td><td>D210</td><td>D270</td></tr><tr><td>897.4</td><td>880.7</td><td>852.7</td><td>584.9</td><td>341.1</td></tr></table></td><td>(100)</td></tr>
</table>

<table>
<tr><th>Vaccine types</th><th>Developer/ Year</th><th>Details</th><th>Methodology</th><th>Immunogenicity results</th><th>Reference</th></tr>
<tr><td>Dengue tetravalent E domain III</td><td>Chiang, C.Y. 2011 (China)</td><td>Consensus ED III (cED III) and lipidated cED III (LcED)</td><td>Two time immunization (S.C.) in mice (BALC/C) 20 µg of cED III or LcED III</td><td>FRNT$_{40}$ Titer (Log$_2$)<table><tr><td>Virus</td><td>DEN V-1</td><td>DEN V-2</td><td>DEN V-3</td><td>DEN V-4</td></tr><tr><td>cED III</td><td>2</td><td>2.8</td><td>2.6</td><td>2.2</td></tr><tr><td>LcED III</td><td>4.5</td><td>5.2</td><td>5.8</td><td>2.8</td></tr></table></td><td>(101)</td></tr>
<tr><td>Dengue tetravalent E domain III</td><td>Block O.K. 2010 (USA)</td><td>Tetravalent recombinant dengue dIII proteins Using pAcGP67A (Pharmingen, San Diego, CA) as baculovirus transfer vector</td><td>Three time immunization (i.m.) in mice (BALC/c) of individually (10 µg per dose) or in tetravalent combination (5–50µg per dose)
Priming (day 0) in complete Freund’s adjuvant (FCA)
Boost (day 14 and 28) in incomplete Freund’s adjuvant (IFA)Bleeding : Day 42</td><td>PRNT$_{50}$ titer (GMT)
Gr 1 : 10 µg of each a DENV dIII protein mixture (tetravalent)<table><tr><td>Virus</td><td>DEN V-1</td><td>DEN V-2</td><td>DEN V-3</td><td>DEN V-4</td></tr><tr><td>PRNT$_{50}$</td><td>986</td><td>1284</td><td>157</td><td>16</td></tr></table>Gr 2 : 25 µg DENV1 dIII; 5 µg DENV2 dIII; 25 µg DENV3 dIII; and, 50 µg DENV4 dIII<table><tr><td>Virus/ PRNT$_{50}$</td><td>DEN V-1</td><td>DEN V-2</td><td>DEN V-3</td><td>DEN V-4</td></tr><tr><td>Monovalent</td><td>12908</td><td>688</td><td>1753</td><td>18</td></tr><tr><td>Tetravalent</td><td>1196</td><td>3174</td><td>378</td><td>254</td></tr></table></td><td>(19)</td></tr>
</table>

<table>
<tr><th>Vaccine types</th><th>Developer/ Year</th><th>Details</th><th>Methodology</th><th>Immunogenicity results</th><th>Reference</th></tr>
<tr><td>Dengue tetravalent E domain III</td><td>Leng, C.H. 2009 (China)</td><td>Consensus ED III (cED III) with or without aluminum phosphate ($AlPO_4$)</td><td>Three time immunization (S.C.) in mice (BALC/C) 20 µg cED III per dose formulated with or without $AlPO_4$</td><td>$PRNT_{40}$ titers<table><tr><td>Virus</td><td>DEN V-1</td><td>DEN V-2</td><td>DEN V-3</td><td>DEN V-4</td></tr><tr><td>cED III</td><td><1:8</td><td><1:8</td><td><1:8</td><td><1:8</td></tr><tr><td>cED III / $AlPO_4$</td><td>1:16</td><td>1:128</td><td>1:32</td><td>1:8</td></tr></table></td><td>(95)</td></tr>
<tr><td>Dengue tetravalent E domain III</td><td>Etemad, B. (India)</td><td>Chimeric fusion EDIII of the four DENV serotypes</td><td>Four time immunization (i.p.) in mice (BALB/c) 20 µg per dose (3 mice/group) : day 0, 21, 42 and 84
FCA
Montanide ISA720
Alum</td><td>$PRNT_{50}$ titers<table><tr><td>Virus</td><td>DEN V-1</td><td>DEN V-2</td><td>DEN V-3</td><td>DEN V-4</td></tr><tr><td>FCA</td><td>160</td><td>118</td><td>234</td><td>479</td></tr><tr><td>Montanide</td><td>153</td><td>293</td><td>271</td><td>588</td></tr><tr><td>Alum</td><td>197</td><td>363</td><td>47</td><td>546</td></tr></table></td><td>(18)</td></tr>
<tr><td>Dengue serotype 4 E and domain III</td><td>Babu JP. 2008 (India)</td><td>The recombinant D4E-III protein (D4-61NIID strain) using pET 30a+ (Novagen, Madison,WI, USA) vector in various adjuvants</td><td>Three time immunization (S.C.) in mice (BALB/c) 25 µg per dose (6 mice/group) : day 0, 21, and 42
FCA
Montanide ISA720
Alum</td><td>$PRNT_{90}$ titers against DENV-4
FCA = 1:128
Montanide ISA 720= 1: 128
Alum = 1: 64</td><td>(21)</td></tr>
</table>

Vaccine types	Developer/ Year	Details	Methodology	Immunogenicity results	Reference
Dengue serotype 1 E and domain III	Bernardo L 2008 (Cuba, IPK)	Recombinant protein D1E-III (Jamaica strain) + P64k from *Neisseria meningitidis* (Zulueta et al., 2003)	Four time immunization (i.m.) 100 µg per dose on day 0 , 30 , 60 and 90 Group 1 D1E-III + P64K + Freund adjuvant / Alum Group 2 D1ED-III + Freund adjuvant / Alum Only first dose combine with complete Freund adjuvant	Neutralizing titer (ELISA) : 1. Gr.1 group neutralizing titer > Gr.2 2. Gr.1 + Freund adjuvant highest neutralizing antibody compare with other group	(102)

DNA vaccine

DNA vaccine is non-live, non-replicating vaccine platform by using recombinant DNA technology. Gene(s) of interest was/were inserted into expression vector then delivered to host cells to allow target protein(s) expression occur (11). The major target gene of dengue DNA vaccine is similar to subunit vaccine including full-length or partial E protein (80% E and EDIII) (13, 20, 103, 104). Several studies demonstrated that prM is required for accurate processing and structural conformation of E protein, moreover, viral-like particle could be observed when prM and E are co-expressed (29, 30, 105-107). The most advanced dengue DNA vaccine development was conducted by Naval Medical Research Institute, USA which finished phase I clinical trial of monovalent DENV-1 prME in flavivirus naïve volunteers. There is no serious related effected caused by DENV-1 prME DNA vaccine and the vaccine could induced neutralizing antibody in human but the titer is relatively low (103). Further investigation to increase vaccine immunogenicity such as DNA-adjuvant formulation, route of vaccine administration and combination with different vaccine type in prime-boost regimen are currently explored (108).

DNA vaccine provides a number of advantages compared to conventional vaccines. Various genes of interest could be designed, synthesized and inserted into plasmid backbone thus it was further wildly studied in many fields such as immunotherapy, cancer therapy or infectious diseases (11, 109). DNA vaccine is non-replicating agent thus it could overcome the safety issue of live-attenuated vaccine or avoid production error of killed-vaccine (110, 111). Production of DNA vaccine is quite easy and rapid. It could synthesize by PCR reaction and cloning (optimization procedures described below). Economical issue, stability and transportation are other advantages of DNA vaccine. The cost of DNA vaccine is relatively cheaper than other vaccine which used complex techniques or high quality/safety control during production. DNA vaccine is quite stable and not temperature sensitive, no cold-chain require during transportation (11). However, immune responses induced by DNA

vaccine is relatively low when compared to replicating or live vaccine, thus strategies to improve immunogenicity is required (112). Long-term safety issue of DNA vaccine remained elusive. Vaccine integration to host chromosome, anti-DNA antibody induction or resistance to antibiotic used in vaccine construct are the major concerns. Currently, there is no study strongly confirmed that DNA vaccine could integrate to host genome, if possible, the integration rate is lower than spontaneous occurring (113-119). In the context of autoimmunity induction, anti-DNA antibody could not detect in non-human primate or clinical trial and no supportive evidence of autoimmunity related to DNA vaccination (120-123). Antibiotic resistance according to selective marker in plasmid DNA is another concern however, Kanamycin which commonly used for bacterial selection during DNA vaccine construction is not wildly use to cure infected-human currently (11). To date, antibiotic-free selection such as sucrose selection system is available (124, 125). The advantages and disadvantage/concern of DNA vaccine were summarized in table 2.

Table 2 The advantages and disadvantages of DNA vaccine

Advantages	Disadvantages
- Safer than LAV or inactivated vaccine with no risk for infection - Able to induction both CMI and HMI - Ease of development and production - High stability (no cold-chain required during transport and store) - Cost-effectiveness	- Possibility of autoimmunity (antibody against DNA vaccine) - Possibility of antibiotic resistance - The low immunogenicity (requires the strategy to enhance immune response such as the effective DNA delivery systems, immune cells-targeted vaccine, adjuvants or combination with other vaccine platforms which also known as “prime-boost strategy”

Our tetravalent dengue DNA vaccine development was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Several strategies has been used to augment the neutralizing antibody titer in our study including the selected plasmid vector, the codon-optimization of the prM/E sequences, the addition of intracellular signal sequences, and the DNA delivery systems such as needle-free immunization and *in vivo* electroporation. Our tetravalent DNA vaccine candidate, consisting of pCMVkan-based plasmids expressing the prM/E genes of each of the four serotypes of dengue virus, induced neutralizing antibody responses to all dengue serotypes (median PRNT50 titer against DENV-1, -2, -3 are 1:320 and DENV-4 is 1:20) after three-times immunization by *in vivo* electroporation (total 100 μg/dose, 25 μg of each monovalent). The lowest NtAb was observed in DENV-4.

Besides our group, Konishi E from Japan also developed the tetravalent dengue *prM/E* DNA vaccine which showed lower NtAb titer than our candidate vaccine. Although, it cannot be directly compared because of the difference of expression vector, immunization schedule and PRNT assay, the similar encouraging result was observed. The NtAb against all four DENV serotype was induced in mice immunized with tetravalent DNA vaccine, however, the weakest NtAb titer was detected in DENV-4 (PRNT70 of pooled serum = 1:10) (13).

Recently, Lima DM developed a monovalent DENV-4 prM/E DNA vaccine and demonstrated that three time immunization of a large amount of 100 μg monovalent DENV-4 prM/E DNA in mice also generated a low titer of NtAb against DENV-4 (PRNT50 of pooled serum =1:128) (15).

In an attempt to enhance humoral immune responses of DNA vaccines, the lipid-based formulations, vaxfectin, has been widespread used in preclinical studies (126, 127). Porter KR also conducted a non-human primate study to evaluate the formation of Vaxfectin-adjuvanted tetravalent dengue DNA vaccine. The use of Vaxfectin resulted in a significant

increase of NtAb titers against all dengue serotypes. However, the lowest titer of NtAb still obtained against DENV-4 in both animals immunized with tetravalent DNA vaccine alone and tetravalent DNA vaccine formulated with the Vaxfectin (16). The mechanisms of low immunogenicity against DENV-4 are thought to involve the poor antigenicity of DENV-4 viral structure and the dengue reference viruses used in the PRNT assay.

CHAPTER III

MATERIALS AND METHODS

3.1 Design of chimeric D4EDIII-D2prME DNA sequence

3.1.1 DENV-2 and DENV-4 prM+E amino acid sequences collection

The DENV-2 and DENV-4 prM+E consensus amino acid sequence were collected from the published Thailand sequences in GENBANK database during Year 1974-2004, as described below:

3.1.1.1 Thailand DENV-2 prME amino acid: total 124 sequences

NCBI accession number:

- AB194882
- AF195032 - AF195043 12 sequences
- AF264053
- AF410377
- AY577435 - AY577438 4 sequences
- DQ181797 – DQ181901 105 sequences
- NC001474
- NP739582.2
- NP739583.2
- NP739592.2

Total nucleotide sequence = 1983 bp

Total amino acid = 661 amino acids

3.1.1.2 Thailand DENV-4 prME amino acid: total 65 sequences

NCBI accession number:

- AB11108
- AB111087
- AB111089
- AY618935-AY618993 59 sequences
- U18440-U18442 3 sequences

Total amino acid = 661 amino acids

Total nucleotide sequence = 1983 bp

3.1.2 Consensus prME amino acid sequences and humanized codons creation

Consensus prM/E amino acid sequences of DENV-2 and DENV-4 were created from the selected nucleotides and amino acid sequences as indicated in 3.1.1 by using BioEdit Sequence Alignment Editor Program version 7.0.5.3. After both consensuses were created, wild-type nucleotide sequences were changed from native into humanized codons by using a codon usage database (http://www.kazusa.or.jp/codon/).

3.1.3 Chimeric D4EDIII-D2prME DNA sequence creation

Consensus EDIII of DENV-4 was replaced in consensus prM/E of DENV-2 backbone as shown in Figure 1. The Kozak's sequence (ACC/GATGG) and DENV-2 prM leader sequence (Met-Val-Asn-Arg-Arg-Arg-Arg-Ser-Ala-Gly-Met-Ile-Ile-Met-Leu-Ile-Pro-Thr-Val-Met-Ala) were inserted at the N-terminal.

In order to subclone the chimeric nucleotide sequences into pCMVkan expression vector, *Sal*I (GTCGAC) and *Not*I (GCGGCCGC) restriction sites were added at 5' and 3' of its sequence, respectively. Stop codon (TGA) was added at 3' to stop protein translation.

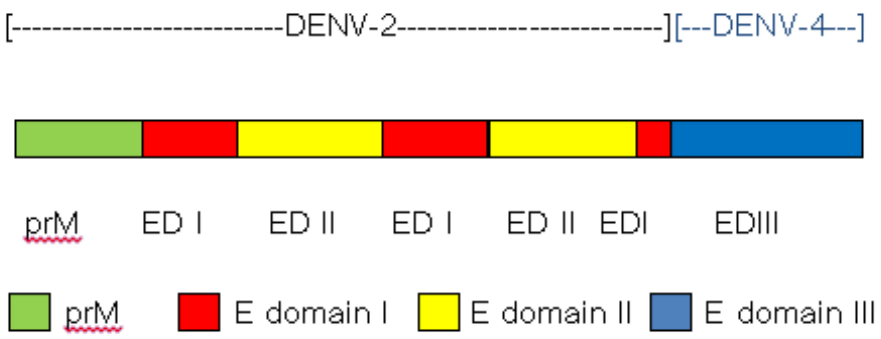

Figure 1 Design of chimeric D4EDIII-D2prME DNA vaccine construct

3.2 Synthesis of the humanized chimeric D4EDIII-D2prME sequence

The completely designed chimeric D4EDIII-D2prME nucleotide sequences were commercially synthesized by GenScript Corp. (NJ, USA) by inserted *prM/E* into pUC57-chimeric D4EDIII-D2prME cloning vector.

3.3 Chimeric D4EDIII-D2prME DNA vaccine construction

3.3.1 Expression vector

pCMVkan was kindly provided by Dr. Barbara K Felber (National Cancer Institute, Frederick, Maryland, USA). This vector contains the cytomegalovirus immediate early promoter, the bovine growth hormone (bGH) polyadenylation signal, and kanamycin resistance gene.

3.3.2 Subcloning procedure

3.3.2.1 Competent cells preparation

Starter *E. coli* (DH5-α) was prepared by slowly thawed frozen glycerol stock on ice and then inoculated into 5 ml of LB medium and incubated in a 37 ^{o}C for overnight with continuously shake, 250 rpm. Next day, starter *E.coli* was inoculated into 50 ml of LB medium (1:500 v/v) at 37^{o}C with continuously shake at 250 rpm about 2-3 hours until reach the $OD_{600\ nm}$ 0.4-0.5. Subsequently, the bacterial cells were incubated on ice for 30 min

then collected by centrifugation at 4,000 rpm (~3,000 x g) for 10 minutes at 4 ^{o}C. The supernatant were discarded, cell pellet was resuspended in 10 ml of cold, sterile 50 mM calcium chloride. The mixture was incubated on ice for 30 min then the bacterial cells were centrifuged at 4,000 rpm (~3,000 x g). The supernatant was discarded and the cell pellet was gently resuspended in 1 ml of cold, sterile 50 mM calcium chloride with 15% sterile glycerol (v/v). *E. coli* (DH5-α) competent cell were aliquot at 100 µl/tube and kept at -80 ^{o}C until use.

3.3.2.2 Plasmid transformation

Competent cells as prepared in 3.3.2.1 were slowly thawed on iced, after competent cells completely thaw, plasmid DNA was put and chilled on ice for 30 min. Then, competent cells-plasmid mixture tube was rapidly put (heat-shock) at 42^{o}C H_2O without shaking, 30 sec. Subsequently, competent cells-plasmid mixture tube was immediately put on ice for 5-10 min. After that, 900 µl of Super Optimal broth with Catabolite repression (SOC) medium was added into tube then shake for 1 hr (37°C) with shaking at 180-200 rpm. Transformants were plate on LB-Kanamycin or or LB-Ampicillin plate (final concentration = 25 µg/ml or 100 µg/ml, respectively) plates. Bacterial colonies were observed after incubated for 16-24 hr, 37^{o}C.

3.3.2.3 Restriction endonuclease reaction

To prepare the target, chimeric D4EDIII-D2prME DNA, DNA fragment and plasmid vector for ligation, the restriction enzymes, Sal I and Not I, were used for digestion. The total concentration of enzyme should be less than 1/10 v/v of total reaction volume. Then, the reaction was incubated at 37^{o}C for 1 hr or followed the manufacturers instructions. The restriction products were analyzed by 1% agarose gel electrophoresis.

3.3.2.4 DNA ligation

Ligation reaction was performed to ligate the target, chimeric D4EDIII-D2prME DNA, DNA fragment into the pCMVkan expression vector with compatible restriction sites by

using T4 DNA ligase (Roche, Germany) and are designed as **"pCMVkan- D4EDIII-D2prME"** (Figure 2). The ligation formula was described below:

$$\frac{\text{vector (50-100 ng)} \times \text{insert length (kb)}}{\text{vector length (kb)}} \times \text{insert:vector ratio} = \text{insert amount (ng)}$$

Ligation reaction was incubated at 16°C, overnight then transformed into competent cells as described in 3.3.2.1.

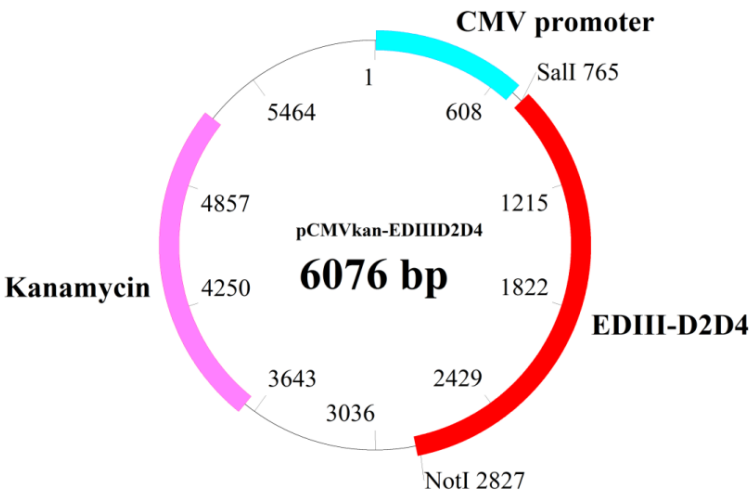

Figure 2 pCMVkan-D4EDIII-D2prME construction

3.3.2.5 Chimeric DNA vaccine inserted-bacteria selection and confirmation

3.3.2.5.1 Selection of DNA vaccine inserted-bacteria by colony PCR

Candidate colonies were selected and inoculated into LB broth with appropriated antibiotic, incubated with continuously shake at 250 rpm, 37°C for 8-16 hr. Bacterial cells were collected by centrifugation, pellets were use as DNA template in PCR reaction.

3.3.2.5.2 Confirmation of positive colony by restriction endonuclease

Positive bacterial colonies by colony PCR were further confirmed by endonuclease restriction. Two approaches were used for analysis.

- Using the enzymes that used for subcloning the target DNA fragment into pCMVkan expression vector (*Sal*I, *Not*I), the expected product size should be equal 2,081 and 4,024 bp.
- Using the enzyme, Sca I, which appear within the target DNA fragment, the expected product size should be equal 6,076 bp.
- Using dual enzymes, Sal I and Sca I, the expected product size should be equal 1,738 and 4,338 bp.

3.3.2.5.3 Nucleotide sequencing analysis

Plasmids or DNA fragments were purified by QIAGEN PCR/gel purification kit. The purified product was used for sequencing reaction by using Sequencing Kit (ABI PRISM dideoxy Dye Terminator Cycle Sequencing Kit, BigDye™ Applied Biosystems, USA).

3.4 Protein Expression

3.4.1 Plasmid DNA transfection

The mammalian cell, African monkey kidney cell mammalian (Vero), was used for evaluating the protein expression of our chimeric DNA vaccine construct, pCMVkan-D4EDIII-D2prME**,** in comparison with the non-chimeric DNA vaccine construct; pCMVkan-D2prME and pCMVkan-D4prME. Vero cells were plate on six well plate (Costar, NY, USA) at $4x10^5$ cells/well total volume 2 mL culture medium (10% FBS MEM) and incubated 24 hours. Lipofectamine 2000^{Tm} was used for the plasmid DNA transfection as following protocol, 4 µg of each plasmid DNA were diluted in 250 µl Opti-MEM Medium (Invitrogen, USA) and diluted lipofectamine in 250 µl Opti-MEM Medium. The mixtures of DNA plasmid with lipofectamine were incubated at room temperature for 30 minutes and then added into Vero

cells by slowly drop on it. Transfected cells were incubated at 37^{o}C, 5% CO_2 for 24 hour. The protein expression was analyzed by indirect immunofluorescence.

3.4.2 Indirect immunofluorescence assay

According to the limitation of the monoclonal antibodies which are specific for the DENV-2 E domain I or II and the DENV-4 E domain III, the protein expression can be indirectly confirmed by using the available monoclonal antibodies as follows:

- anti-DENV-2 E domain III antibody (clone 3H5)
- anti-DENV-4 E domain I, II antibody (clone 1H10)
- anti-flavivirus E antibody (clone 4G2)

All monoclonal antibodies were kindly provided by Dr.Chunya Puttikhunt (National Center for Genetic Engineering and Biotechnology (BIOTEC).

The 24 hr post-transfected cells were fixed on ice-cold acetone for a few minutes and then incubated with different monoclonal antibodies described above for 1 hour, 37 ^{o}C. Rabbit anti mouse Immunoglobulins-FITC (Dako, Denmark) and goat anti- mouse IgG-Alexa-fluor™ 568 (Molecular probe, OR, USA) were use as secondary antibody for E protein detection. Vero nuclei were counter stained with 4, 6-diamino-2-phenylindole hydrochloride (DAPI) (Sigma–Aldrich, MO, USA). Stained cells were visualized under confocal microscope.

3.5 DNA vaccine preparation

The candidate chimeric DNA vaccine construct which confirmed by various strategies was further produced in large scale. Single positive clone of each construct was firstly cultured as a starter of 5-10 ml of LB medium containing 100 mg/ml Kanamycin and incubated overnight at 37 ^{o}C with vigorous shaking for 6-8 hr. The starter culture was diluted 1/500-1/1000 in LB medium and inoculated in 2.5 liters LB medium containing 100 mg/ml Kanamycin. The bacterial cells were grown at 37 ^{o}C for 16-18 h with vigorous shaking at 250 rpm, then centrifuged at 6000 x g for 15 min at 4 ^{o}C and discarded the supernatant. QIAGEN® plasmid Giga purification kit was used to preparation of plasmid following

QIAprep® Endo-free gigaprep handbook (QIAGEN, USA). All reagents and containers used in this step were endotoxin-free. Purified plasmid DNA was aliqouted and stored at -20 °C until use.

3.6 Mice Immunization

ICR mice, age 3–6 weeks, were purchased from the National Laboratory Animal Centre, Bangkok, Thailand. Five mice/group were immunized 3 times with 25 µg of indicated plasmid DNA as shown in Table III by using *in vivo* electroporation (Ichor Medical System, San Diego, CA) on both thighs at two weeks interval. The negative control mice were immunized with 16.5 µg of empty pCMVkan which contain same molecular ratio of vector to the vaccine constructs. Then, the mice were bled at week 4 after the last injection for evaluating the neutralizing antibody titer. The immunization schedule was shown in figure 3.

Table 3 Groups of dengue DNA vaccine immunized mice

Group		Dose / DNA vaccines	Number of mice
1	Test	25 µg pCMVkan-D4EDIII-D2prME	5
2	Comparison group	25 µg pCMVkan-D4prME	5
3	Negative control	16.5 µg pCMVkan-empty	5

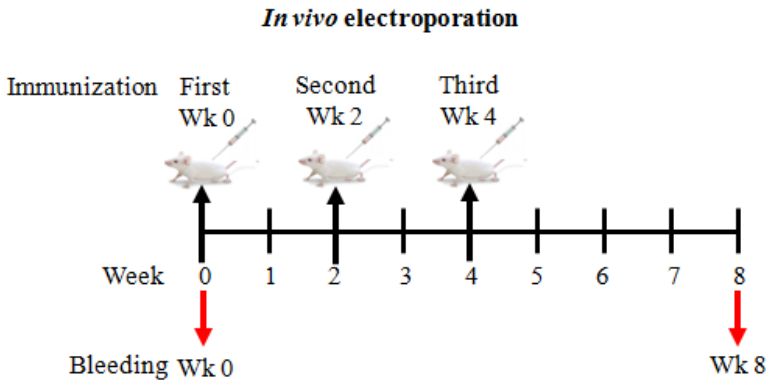

Figure 3 Mice immunization schedule

3.7 Plaque reduction neutralizing test (PRNT)

The plaque reduction neutralizing test was employed in the assessment of neutralizing antibody against DENV-4 strain 1036, DENV-4 strain C0036 (new isolated) and DENV-2 strain 16681. Briefly, sera were inactivated at 56°C for 30 min, serially two-fold diluted in 10% MEM (1:10-1:1280), and then incubated at 37°C for 1 hour with an equal volume of DENV-4 or -2 dilution containing about 500 PFU/mL. The virus-antibody mixes were incubated on LLC-MK_2 monolayers for 1 hour. Following virus absorption, cells were overlaid with 0.15 mL of the first overlay medium containing 1.8% low-melting agarose (Invitrogen). After 4-5 days of incubation at 37°C/5%CO_2, cells were stained with 1.5 mL of the second overlay medium containing 4% [w/v] neutral red. Plaques were counted following an incubation of 24 hours at 37°C. The neutralizing antibody titer were expressed as the maximum serum dilution yielding a 50% reduction in plaque number by comparing when infected LLC-MK_2 with medium-virus mixture which calculated by the given formula.

$$\text{Percent plaque reduction} = \left[100 - \left(\frac{\text{plaque number when incubated with serum}}{\text{plaque number when incubated without serum}} \right) \right] \text{X } 100$$

3.8 Characterization of the nucleotide sequences of the DENV-4 reference viruses; strain 1036 *VS* C0036

According to the nucleotide sequence encoding the prM and E of both DENV-4 reference strain 1036 and DENV-4 strain C0036 are not available in Genbank database, their prM and E of both DENV-4 were identified and compared their homology in this study.

3.8.1 DENV-4 RNA extraction

DENV-4 RNA was extracted as follows, 500 µl of DENV-infected Vero cell culture supernatant was centrifuged at 13,000 rpm for 90 min. at 4 °C, then discarded supernatant. DENV particles were lyses by adding 200 µl lysis buffer, Amplicor monitor test, Roche, NJ, USA and incubated at 60 °C for 10 min. Two hundred of cold isopropanol was added and vortexed then centrifuged at 13,000 rpm for 20 min. Discarded the supernatant, pellet was washed with 400 µl cold 70% ethanol then centrifuged at 13,000 rpm, 10 min. The supernatant was discarded and let the RNA pellet dried at RT. The pellet was reconstituted with 20 µl of diluents and stored at -70 °C until use.

3.7.2 DENV-4 *prM/E* nucleotide sequencing

As DENV-4 strain 1036 were not available from Genbank database, thus the set of DENV-4 walking primers was designed by based on the DENV-4 accession no. AY618992 (Thailand isolated year 2001). Figure 4 and Table 4 were shown the map and list of the DENV-4 walking primers.

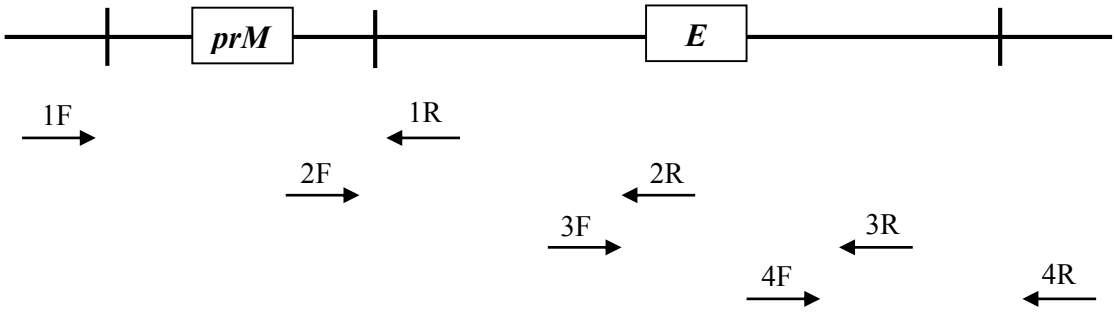

Figure 4 Map of the prM/E walking primers using for DENV-4 nucleotides sequencing

Table 4 List of the walking primers using for DENV-4 prM/E sequencing

Primer	Sequence 5'-3'
DENV-4_1F	ACTGGATTCAGGAAGGAGATAGG
DENV-4_1R	GCATCGCATTCCGTAGGATGG
DENV-4_2F	TTTCTAGCACATGCCATAGGAAC
DENV-4_2R	TTCATCAGAATCATCTCATTAAA
DENV-4_3F	ATGGAGTGACAGCCACGATAAC
DENV-4_3R	CTGTTGGTATTCTCAGCAAAAGG
DENV-4_4F	AGATGGCAGAAACACAGCATGG
DENV-4_4R	CTCGCTGGGGACTCTGGTTG

Briefly for the assay, the DENV-4 RNA was reverse transcribed into complementary DNA (cDNA) and then amplified by PCR with the DENV-4_1F and DENV-4_4R primers. The PCR products were subjected for nucleotide sequencing by the walking primers as describe above. Then, the full length of prME were translated to amino acid and compared to detect their homology.

3.9 Statistical analysis

The comparisons of neutralizing antibody titer ($PRNT_{50}$) between groups of immunized mice were performed by two-tailed nonparametric Mann-Whitney test using GraphPad Prism software version 5.0 (La Jolla, USA). Values were considered significant at $p<0.05$.

CHAPTER IV

RESULTS

4.1 Design of chimeric D4EDIII-D2prME DNA sequence

The DENV-2 and DENV-4 prME consensus amino acid sequence were generated from 1 24 and 65 sequences of DENV-2 and DENV-4, respectively, from Thailand during 1974-2004, GENBANK database. Consensus EDIII of DENV-4 (highlighting text) were replaced in consensus prM/E of DENV-2 backbone. Twenty one amino acid of DENV-2 prME signal sequence (bold alphabets) and stop codon (indicated by *) was added at N-terminus and C-terminus, respectively. The chimeric D4EDIII-D2prME DNA sequence is listed below.

Chimeric D4EDIII-D2prME DNA sequence

DENV-2 signal sequence

MVNRRRRSAGMIIMLIPTVMAFHLTTRNGEPHMIVGIQEKGKSLLFKTEDGVNMCTLMAMDL
GELCEDTITYKCPLLRQNEPEDIDCWCNSTSTWVTYGTCTTTGEHRREKRSVALVPHVGMG
LETRTETWMSSEGAWKHAQRIETWILRHPGFTIMAAILAYTIGTTHFQRVLIFILLTAVAPSMT
MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYCIEA
KLTNTTTESRCPTQGEPSLKEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFTCKK
NMEGKIVQPENLEYTIVVTPHSGEEHAVGNDTGKHGKEIKVTPQSSITEAELTGYGTVTMEC
SPRTGLDFNEMVLLQMENKAWLVHRQWFLDLPLPWLPGADKQESNWIQKETLVTFKNPHA
KKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYTMCSGK
FSIDKEMAETQHGTIVVRVKYEGTGAPCKVPIEIRDVNKEKVVGRIISSTPFAENTNSVTNIEL
EPPFGDSYIVIGVGDSALTLHWFRKGSSIGQMFESTYRGAKRMAILGDTAWDFGSLGGVFT
SIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRSTSLSVSLVLVGIVTIYLGVMVQA
*

Pale = ED III of DENV-4 that different from EDIII of DENV-2

Dark = ED III of DENV-4 that similar to EDIII of DENV-2

4.2 Chimeric D4EDIII-D2prME DNA vaccine construction

Chimeric D4EDIII-D2prME gene which successfully subcloned into pCMVKan expression vector and transformed into the *E.Coli* DH5-α were subsequently selected by colony PCR and analyzed by restriction endonuclease.

The candidate chimeric D4EDIII-D2prME DNA vaccine was digested with different restriction enzymes as shown in Figure 5

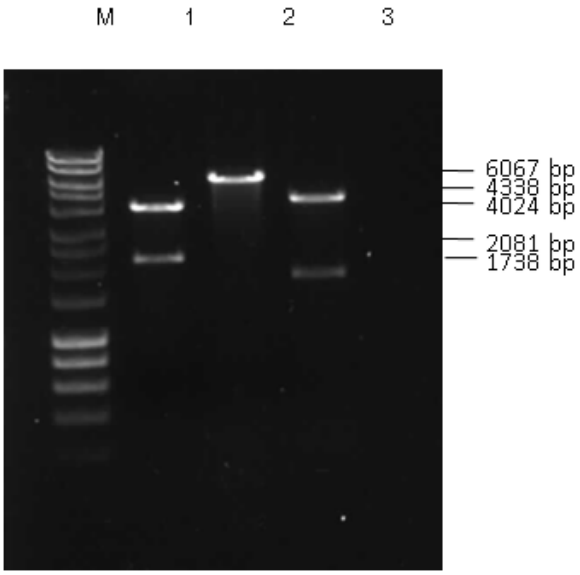

Figure 5 1 % Agarose gel electrophoresis of pCMVkanD4EDIII-D2prME DNA analyzed by restriction endonucleases

Lane 1 : digested with Sal I /Not I Product size : 2,081 bp and 4,024 bp

Lane 2 : digested with Sal I Product size : 6,076 bp

Lane 3 : digested with Sal I/ Sca I Product size : 1,738 bp and 4,338 bp

Marker : Hyper ladder 1 Kb marker

The candidate chimeric D4EDIII-D2prME DNA vaccine which confirmed by various strategies was further produced in large scale for mice immunization. Prior to use the candidate chimeric DNA vaccine was confirmed by nucleotide sequencing. The comparison between the consensus D4EDIII-D2prME and candidate DNA vaccine showed no mutations found in candidate DNA vaccine constructs after large scale production.

4.3 Protein expression analysis

Chimeric D4EDIII-D2prME, D4prME or D2prME DNA vaccine were transfected into Vero cells, intracellular protein expression were determined after transfection 24 hrs by indirect detection with the different serotype-specific E domain monoclonal antibodies (Figure 6). Based on the 200X magnification power image, the chimeric DNA vaccine transfected cells are capable to express ED III of DENV-4 and ED II of DENV-2 which directly detected by a cross-reactive moAb to ED III of all four dengue serotype (clone 2H12) and an ED II flavivirus group specific moAb (clone 4G2), respectively. However, since the chimeric DNA vaccine used ED III of DENV-4 replaced in prM/E of DENV-2 DNA backbone and the specific E moAb are limited, the indirect confirmation was done by using the anti-DENV-4 ED I and II (clone 1H10) and anti-DENV-2 ED III (clone 3H5). As expected, no immunofluorescence signal from both moAb was observed in the chimeric DNA transfected cells. The D4prME and D2prME DNA transfection which are the positive controls demonstrated their specific E protein expression when using the specific moAb, clone 1H10 and 3H5, respectively. The negative control, empty pCMV vector transfection showed no expression of E. These results demonstrated that the candidate chimeric DNA vaccine could express the expected E proteins after entered the mammalian cells.

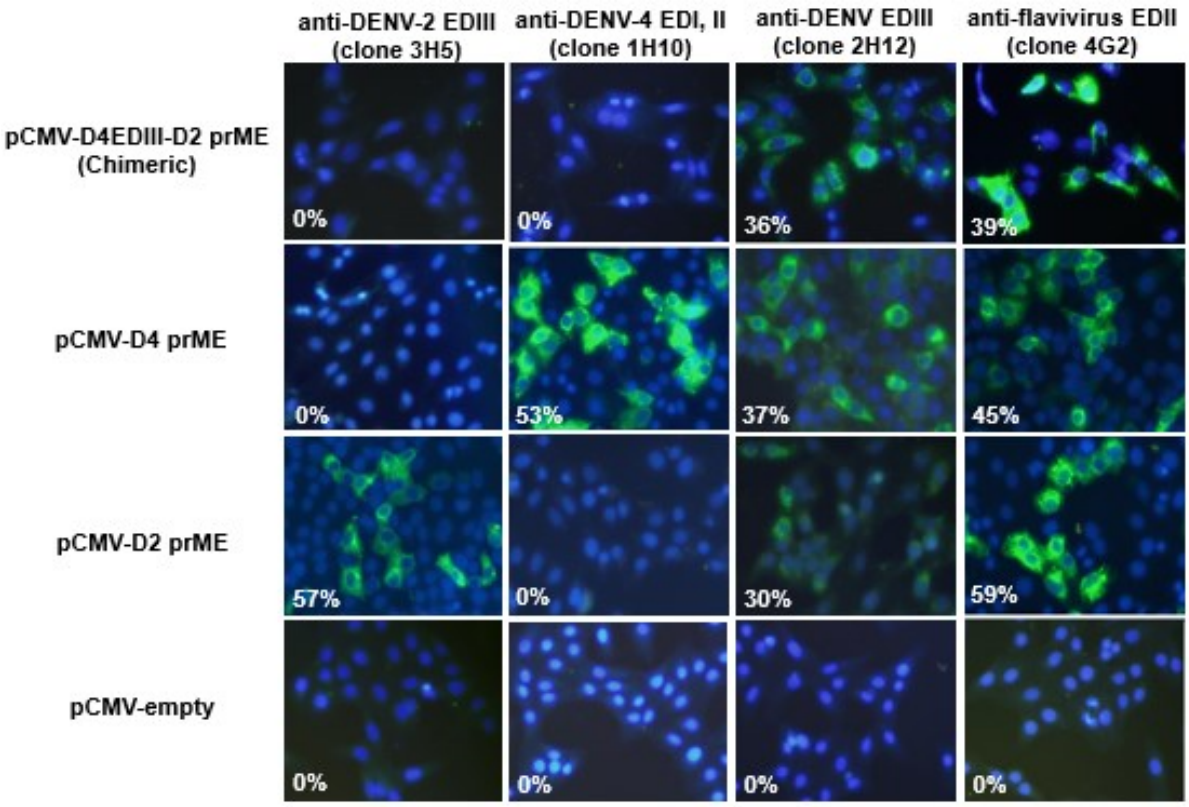

Figure 6 Intracellular dengue E protein expression. Vero cells were transfected with indicated dengue DNA vaccine. The different dengue serotype-specific E domains are shown in green (FITC), whereas DNA staining is shown in blue (DAPI). Merge images show a merge of the green and blue staining.

4.4 Vaccine immunogenicity in mice

The immunogenicity of the candidate chimeric DNA vaccine was evaluated in mice in comparison with the original D4prME DNA vaccine. ICR mice were immunized with 25 μg of either chimeric D4EDIII-D2prME or D4prME DNA, three times at two weeks interval using *in vivo* electroporation. Four weeks after the last immunization, individual mouse serum was assayed for neutralizing antibody against viruses currently used in DENV vaccine development or reference labs; DENV-2 strain 16681 and DENV-4 strain 1036, and used more recent DENV-4 (strain C0036) for comparison purpose (Table 5).

Table 5 PRNT50 titers of indicated DNA vaccine constructs against DENV-4 and DENV-2 reference viruses

Vaccine construct / No. of mice	PRNT50 titers against DENV-4 and -2 reference strains		
	DENV-4 strain 1036 (isolated year 1976)	DENV-4 strain C0036 (isolated year 2006)	DENV-2 strain 16681
25 µg pCMVkan-D4EDIII-D2prME			
No.1	<10	80	40
No.2	<10	160	160
No.3	<10	40	40
No.4	<10	40	40
No.5	<10	160	160
25 µg pCMVkan-D4prME			
No.1	<10	320	20
No.2	<10	160	40
No.3	<10	20	20
No.4	<10	<10	20
No.5	<10	160	40
16 µg pCMVkan-empty			
No.1	<10	<10	<10
No.2	<10	<10	<10
No.3	<10	<10	<10
No.4	<10	<10	<10
No.5	<10	<10	<10

By using DENV-4 strain 1036 as a reference virus, PRNT50 titers < 10 (undetectable) were observed in all individual mice immunized with either new chimeric D4EDIII-D2prME or original D4prME DNA vaccine as shown in Figure 7.

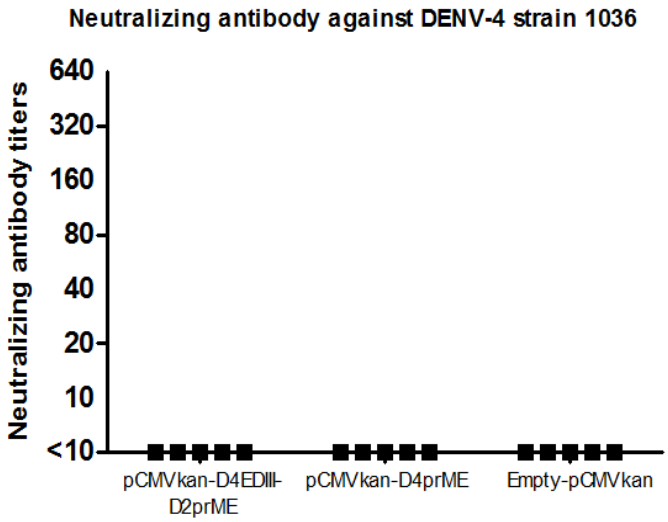

Figure 7 Neutralizing antibody against DENV-4 strain 1036 in individual mice immunized with 25 µg pCMVkan-D4EDIII-D2prME, 25 µg pCMVkan-D4prME and 16 µg pCMVkan-empty (n=5/group). Horizontal lines represent the median PRNT50 titer in each group of mice. $p < 0.05$ was considered statistically significant.

By using the DENV-4 reference virus strain 1036 in PRNT assay, both a new design of the chimeric pCMVkan-D4EDIII-D2prME and the original D4prME DNA vaccines showed the undetectable NtAb against DENV-4. Then, the neutralizing antibody levels were re-measured by replacing a reference virus with a more recent isolated DENV-4 strain C0036. The comparable of the NtAb titers against two reference strain of DENV-4 were shown in Figure 8.

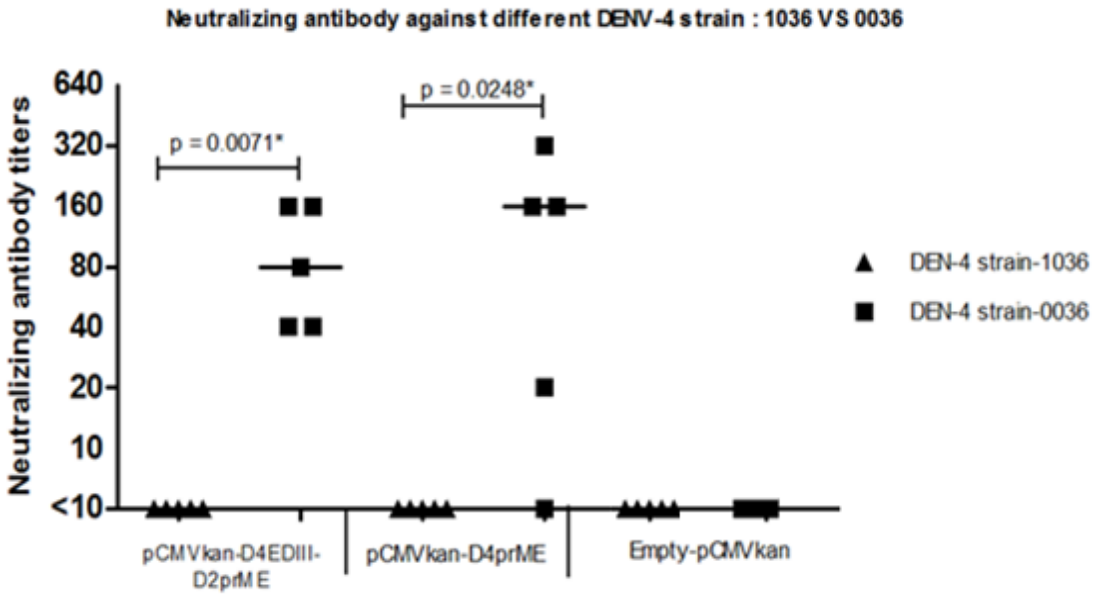

Figure 8 Neutralizing antibody against DENV-4 strain 1036 (closed-triangle) and C0036 (closed-square) in individual mice immunized with 25 μg pCMVkan-D4EDIII-D2prME, 25 μg pCMVkan-D4prME and 16 μg pCMVkan-empty (n=5/group). Horizontal lines represent the median PRNT50 titer in each group of mice. $p < 0.05$ was considered statistically significant.

By using strain C0036 as a reference DENV-4 virus, NtAb were detectable in most of individual mice. NtAb levels against DENV-4 in mice immunized with either new chimeric D4EDIII-D2prME or original D4prME DNA vaccine were significantly increased (p = 0.0071 and 0.0248, respectively) compared to those measured by using strain 1036 (Figure 8). The median PRNT50 induced by the chimeric and original DNA vaccine were 80 and 160, repectively. However, the statistic significant difference between both groups was not observed due to the small sample size and the high variation in NtAb titers of individual mice within the group. The negative control, when mice were immunized with the pCMVkan-empty, there was no any NtAb detected.

According to the chimeric DNA vaccine construct, the cross-reactive neutralizing antibody to dengue serotype 2 was further evaluated in both DNA vaccine immunized mice by using DENV-2 strain 16681. Interestingly, pCMVkan-D4EDIII-D2prME could generate slightly higher NtAb than pCMVkan-D4prME (the median PRNT50= 40 and 20, respectively), however the difference is not statistically significant (Figure 9).

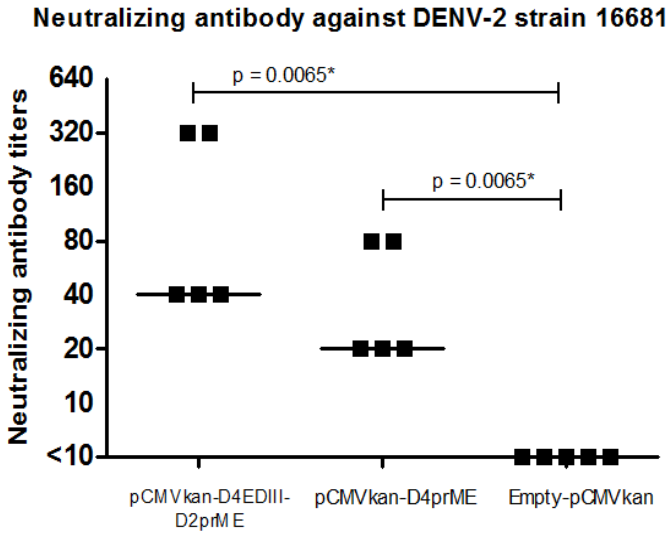

Figure 9 Cross-reactive neutralizing antibody against DENV-2 strain 16681 in individual mice immunized with 25 µg pCMVkan-D4EDIII-D2prME, 25 µg pCMVkan-D4prME and 16 µg pCMVkan-empty (n=5/group). Horizontal lines represent the median PRNT50 titer in each group of mice. $p < 0.05$ was considered statistically significant.

4.5 Characterization of the nucleotide sequences of the DENV-4 reference viruses; strain 1036 *VS* C0036

The full length *prME* nucleotide sequences of DENV-4 reference viruses; strain 1036 and C0036, were analyzed as described in 3.8. The translated prME amino acid sequences are shown as below.

4.5.1 DENV-4 strain 1036 prME amino acid sequence

FHLSTRDGEPLMIVAKHERGRPLLFKTTEGINKCTLIAMDLGEMCEDTVTYKCPLL
VNTEPEDIDCWCNLTSTWVMYGTCTQSGERRREKRSVALTPHSGMGLETRAETW
MSSEGAWKHAQRVESWILRNPGFALLAGFMAYMIGQTGIQRTVFFVLMMLVAPS
YGMRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAK
EVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRTDVVDRGWGNGCG
LFGKGGVVTCAKFSCSGKITGNLVQIENLEYTVVVTVHNGDTHGVGNDTSNHGVT
ATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMKMKKKTWLVHKQWFLDLP
LPWTAGADTSEVHWNYKERMVTFKVPHAKRQDVTVLGSQEGAMHSALAGATEV
DSGDGNHMFAGHLKCKVRMEKLRIKGMSYTMCSGKFSIDKEMAETQHGTTVVKV
KYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAENTNSATNIELEPPFGDSYIVIGV
GNSALTLHWFRKGSSIGKMFESTYRGAKRMAILGETAWDFGSVGGLFTSWGKAV
HQVFGSVYTTMFGGVSWMIRILIGFLVLWIGTNSRNTSMAMTCIAVGGITLFLGFT
VQA

4.5.2 DENV-4 strain C0036 prME amino acid sequence

FHLSTRDGEPLMIVAKHERGRPLLFKTTEGINKCTLIAMDLGEMCEDTVTYKCPLLVN
TEPEDIDCWCNLTSAWVMYGTCTQSGERRREKRSVALTPHSGMGLETRAETWMSS
EGAWKHAQRVETWILRNPGFALLAGFMAYMIGQTGIQRTVFFILMMLVAPSYGMRCV
GVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTAKEVALLRTYCI
EASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRGWGNGCGLFGKGGVVTCA
KFSCLGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTATITPRSPSVEVKL

PDYGELTLDCEPRSGIDFNEMILMKMKTKTWLVHKQWFLDLPLPWTTGADTLEVHW
NHKERMVTFKVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCK
VRMEKLRIKGMSYTMCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVN
KEKVVGRIISSTPFAENTNSVTNIELEPPFGDSYIVIGVGDGALTLHWFRKGSSIGKMF
ESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYTTMFGGVSWMVRILI
GLLVLWIGTNSRNTSMAMSCIAVGGITLFLGFTVHA

Pairwise alignment of E amino acid sequence between DENV-4 strain 1036 and strain C0036 showed only 96.6% (475/495 amino acid) homology, this due to the different strain of viruses (see appendix 1). The E amino acid homology was also compared between the original D4prME DNA and both DENV-4 reference strains and was shown in table 6 (see appendix 2, 3). The results demonstrated that the original candidate D4prME DNA vaccine is quite similar to new isolated DENV-4 strain C0036 with 99.2% amino acid homology.

As chimeric D4EDIII-D2prME vaccine used only consensus EDIII of DENV-4 replaced in prME of DENV-2 backbone, the EDIII homology of D4 vaccine candidate was further analyzed with two DENV-4 reference strains and showed the highly match (99.2%) with the new DENV-4 strain C0036 (isolated year 2006). Further multiple alignment was investigated, the newly isolated Thailand DENV-4 in Year 2011 (accession no. APS033) was included for analysis. The consistent results were observed and emphasized that our designed D4prME vaccine candidate is similar to DENV-4 strain C0036 (see appendix 4).

Table 6 Pairwise alignment results of the original candidate D4prME DNA vaccine and DENV-4 reference viruses (only “E” and “EDIII” amino acid sequence)

Aligned D4prME DNA with	**Amino acid homology (%)**	
	all E	**Only E domain III**
DENV-4 strain 1036	96.8 % (479/495)	95.0 % (95/100)
DENV-4 strain C0036	99.2 % (491/495)	99.0 % (99/100)

CHAPTER V

DISCUSSION AND CONCLUSION

At present, dengue disease is a major and emergent public health concern due to its expanding distribution and also to an increased incidence of epidemics (128). There are several dengue vaccine candidates in clinical and preclinical development. However, no commercial dengue vaccine is available. The potent dengue vaccine have to protect against all serotypes of dengue virus or “tetravalent”.

At present, our prototype of tetravalent dengue DNA vaccines has been developed and demonstrated the induction of neutralizing antibodies responses to all serotypes in mice. Our DNA vaccine composed of four separately plasmids containing the prM/E gene of each serotype. Our candidate vaccines showed the encouraging results compared with the previous study by Konishi E, et al, however, it could not be directly compared due to the different of immunization schedule, route of DNA delivery, expression vector and the PRNT assay. Overall, the NtAb titers induced by our TDNA were relatively higher than Konishi E (13). Surprisingly, the similar low level of NtAb against DENV-4 was observed in both studies. Other laboratories also faced with this problem (15, 16).

In this study, we had tried to improve our monovalent DENV-4 DNA vaccine immunogenicity. The new design of antigenic gene was performed for the monovalent DENV-4 DNA vaccine. The chimeric DNA vaccine “designated as chimeric D4EDIII-D2prME” has been designed by focused on the EDIII of the DENV-4 because this domain contains multiple type- and sub-type-specific neutralizing epitopes and choose DENV-2 prM/E DNA vaccine as a DNA backbone due to the highest NtAb stimulation compared to other serotypes.

Since the monoclonal specific antibodies which specific to different E domains of both dengue serotype 2 and 4 are limited, the indirect detection with the available monoclonal antibodies was used. We demonstrated the correct chimeric E protein expression from the chimeric DNA transfected mammalian cells as follows both ED III of DENV-4 (inserted domain) and ED II of DENV-2 (backbone) 1) can be directly detected by anti-DENV EDII (2H12) and anti-flavivirus EDII (4G2), respectively, and 2) cannot be stained with anti-DENV-2 ED III and anti-DENV-4 ED I, II, respectively.

Interestingly, the NtAb against dengue serotype 4 from mice immunized with both chimeric D4EDIII-D2prME and D4prME DNA vaccines were observed when the DENV4 reference viruses in PRNT assay has been changed from strain 1036 to strain C0036. Our finding is consistent with the study of Dr.Butsaya Thaisomboonsuk and colleagues, AFRIMS, Thailand. They reported that using of strain 1036 reflected sub-optimal NtAb titers in dengue-infected clinical specimens compared with more recent isolated Thailand DENV-4 strain C0036. Since there is no significant difference of NtAb against DENV-4 strain C0036 between the chimeric D4EDIII-D2prME and original D4prME DNA vaccines, we can conclude that the new antigenic design is not benefit to improve the immunogenicity against DENV-4 as expect.

The DNA vaccine and target virus mismatch might be used to explain the undetectable of NtAb against DENV-4 when strain 1036 was used in PRNT. DENV-4 strain 1036 was isolated since year 1976 from dengue fever patient in Indonesia (belongs to genotype 2), in contract, DENV-4 strain C0036 was recently isolated in Thailand, year 2006 (belongs to genotype 1) (129, 130). The difference of E amino acid sequence between strain 1036 and C0036 was compared and found 96% homology (475/495 amino acid). Further analysis of the homology of E and only E domain III amino acid sequence between the original D4prME vaccine construct and reference viruses used in PRNT assay were investigated. Our consensus E and ED III sequence of vaccine construct is closely similar to DENV-4 strain C0036 with the 99.2% and 99.0% homology, respectively, while showed only

96.8% and 95% homology to strain 1036. From 16 amino acid difference in truncated E protein between our original D4prME vaccine and DENV-4 stain 1036, only one mismatch (E329) at the critical neutralizing epitope had been reported in the lateral ridge of E domain III (131). At this E329 position, both chimeric D4EDIII-D2prME and D4prME vaccine contained Threonine (T), a polar side-chain whereas DENV-4 strain 1036 contained Alanine (T), non-polar side-chain. The difference in amino acid property might change the protein conformation. Therefore, the antibody produced from mice immunized with both vaccines could not recognize this region efficiency. Consequently, DENV-4 strain C0036, which contained Threonine at position E329 was highly neutralized by antibody induced by both vaccines. Furthermore, the other mismatch might be involved, further investigation is required.

Of note, the cross-neutralizing to the DENV-2 serotype was found in both chimeric D4EDIII-D2prME and original D4prME DNA, this partly according to the E DI and E DII of DENV-2 were used as a DNA backbone in the chimeric DNA. Moreover, it might be imply that the fusion loop of domain II which contained highly conserved residues might be involved in the induction of the cross-reactive antibodies as shown by Lai that the mostly cross-reactive anti-E antibodies recognized the ED II epitope (132).

In summary, the continuously monitor the dengue viral evolution should be concerned in order to design a vaccine and select the suitable reference viruses strain for vaccine efficacy evaluation especially in the current endemic area.

REFERENCES

1. Gubler DJ, editor. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century.2002.

2. Murphy BR, Whitehead SS. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol. 2011;29:587-619.

3. Halstead SB, O'Rourke EJ. Antibody-enhanced dengue virus infection in primate leukocytes. Nature. 1977;265(5596):739-41.

4. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis. 2000;181(1):2-9.

5. Webster DP, Farrar J, Rowland-Jones S. Progress towards a dengue vaccine. Lancet Infect Dis. 2009;9(11):678-87.

6. Lang J. Development of Sanofi Pasteur tetravalent dengue vaccine. Rev Inst Med Trop Sao Paulo. 2012;54 Suppl 18:S15-7.

7. Osorio JE, Brewoo JN, Silengo SJ, Arguello J, Moldovan IR, Tary-Lehmann M, et al. Efficacy of a tetravalent chimeric dengue vaccine (DENVax) in Cynomolgus macaques. Am J Trop Med Hyg. 2011;84(6):978-87.

8. Raviprakash K, Apt D, Brinkman A, Skinner C, Yang S, Dawes G, et al. A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques. Virology. 2006;353(1):166-73.

9. Anderson KB, Gibbons RV, Edelman R, Eckels KH, Putnak RJ, Innis BL, et al. Interference and facilitation between dengue serotypes in a tetravalent live dengue virus vaccine candidate. J Infect Dis. 2011;204(3):442-50.

10. Donnelly JJ, Wahren B, Liu MA. DNA vaccines: progress and challenges. J Immunol. 2005;175(2):633-9.

11. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? Nat Rev Genet. 2008;9(10):776-88.

12. Simasathien S, Watanaveeradej V. Dengue vaccine. J Med Assoc Thai. 2005;88 Suppl 3:S363-77.

13. Konishi E, Kosugi S, Imoto J. Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. Vaccine. 2006;24(12):2200-7.

14. Beckett CG, Tjaden J, Burgess T, Danko JR, Tamminga C, Simmons M, et al. Evaluation of a prototype dengue-1 DNA vaccine in a Phase 1 clinical trial. Vaccine. 2010.

15. Lima DM, de Paula SO, Franca RF, Palma PV, Morais FR, Gomes-Ruiz AC, et al. A DNA vaccine candidate encoding the structural prM/E proteins elicits a strong immune response and protects mice against dengue-4 virus infection. Vaccine. 2011;29(4):831-8.

16. Porter KR, Ewing D, Chen L, Wu SJ, Hayes CG, Ferrari M, et al. Immunogenicity and protective efficacy of a vaxfectin-adjuvanted tetravalent dengue DNA vaccine. Vaccine. 2012;30(2):336-41.

17. Megret F, Hugnot JP, Falconar A, Gentry MK, Morens DM, Murray JM, et al. Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein. Virology. 1992;187(2):480-91.

18. Etemad B, Batra G, Raut R, Dahiya S, Khanam S, Swaminathan S, et al. An envelope domain III-based chimeric antigen produced in Pichia pastoris elicits neutralizing antibodies against all four dengue virus serotypes. The American journal of tropical medicine and hygiene. 2008;79(3):353-63.

19. Block OK, Rodrigo WW, Quinn M, Jin X, Rose RC, Schlesinger JJ. A tetravalent recombinant dengue domain III protein vaccine stimulates neutralizing and enhancing antibodies in mice. Vaccine. 2010;28(51):8085-94.

20. Ramanathan MP, Kuo YC, Selling BH, Li Q, Sardesai NY, Kim JJ, et al. Development of a novel DNA SynCon tetravalent dengue vaccine that elicits immune responses against four serotypes. Vaccine. 2009;27(46):6444-53.

21. Babu JP, Pattnaik P, Gupta N, Shrivastava A, Khan M, Rao PV. Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. Vaccine. 2008;26(36):4655-63.

22. Azevedo AS, Yamamura AM, Freire MS, Trindade GF, Bonaldo M, Galler R, et al. DNA vaccines against dengue virus type 2 based on truncate envelope protein or its domain III. PLoS One. 2011;6(7):e20528.

23. Simmons CP, Farrar JJ, Nguyen v V, Wills B. Dengue. The New England journal of medicine. 2012;366(15):1423-32.

24. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: a continuing global threat. Nature reviews Microbiology. 2010;8(12 Suppl):S7-16.

25. Clyde K, Kyle JL, Harris E. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. Journal of virology. 2006;80(23):11418-31.

26. Chang CJ, Luh HW, Wang SH, Lin HJ, Lee SC, Hu ST. The heterogeneous nuclear ribonucleoprotein K (hnRNP K) interacts with dengue virus core protein. DNA and cell biology. 2001;20(9):569-77.

27. Lorenz IC, Allison SL, Heinz FX, Helenius A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J Virol. 2002;76(11):5480-91.

28. Bulich R, Aaskov JG. Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies. J Gen Virol. 1992;73 (Pt 11):2999-3003.

29. Guirakhoo F, Bolin RA, Roehrig JT. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology. 1992;191(2):921-31.

30. Konishi E, Mason PW. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. J Virol. 1993;67(3):1672-5.

31. Putnak R, Feighny R, Burrous J, Cochran M, Hackett C, Smith G, et al. Dengue-1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicits virus-neutralizing antibody in mice and protects them from virus challenge. Am J Trop Med Hyg. 1991;45(2):159-67.

32. Anderson R, King AD, Innis BL. Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. J Gen Virol. 1992;73 (Pt 8):2155-9.

33. Chen Y, Maguire T, Marks RM. Demonstration of binding of dengue virus envelope protein to target cells. J Virol. 1996;70(12):8765-72.

34. Guirakhoo F, Heinz FX, Kunz C. Epitope model of tick-borne encephalitis virus envelope glycoprotein E: analysis of structural properties, role of carbohydrate side chain, and conformational changes occurring at acidic pH. Virology. 1989;169(1):90-9.

35. Huerta V, Chinea G, Fleitas N, Sarria M, Sanchez J, Toledo P, et al. Characterization of the interaction of domain III of the envelope protein of dengue virus with putative receptors from CHO cells. Virus Res. 2008;137(2):225-34.

36. Cardiff RD, Lund JK. Distribution of dengue-2 antigens by electron immunocytochemistry. Infect Immun. 1976;13(6):1699-709.

37. Schlesinger JJ, Brandriss MW, Putnak JR, Walsh EE. Cell surface expression of yellow fever virus non-structural glycoprotein NS1: consequences of interaction with antibody. J Gen Virol. 1990;71 (Pt 3):593-9.

38. Mackenzie JM, Khromykh AA, Jones MK, Westaway EG. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology. 1998;245(2):203-15.

39. Liu WJ, Wang XJ, Mokhonov VV, Shi PY, Randall R, Khromykh AA. Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. J Virol. 2005;79(3):1934-42.

40. Niyomrattanakit P, Winoyanuwattikun P, Chanprapaph S, Angsuthanasombat C, Panyim S, Katzenmeier G. Identification of residues in the dengue virus type 2 NS2B cofactor that are critical for NS3 protease activation. Journal of virology. 2004;78(24):13708-16.

41. Bartholomeusz AI, Wright PJ. Synthesis of dengue virus RNA in vitro: initiation and the involvement of proteins NS3 and NS5. Arch Virol. 1993;128(1-2):111-21.

42. Li H, Clum S, You S, Ebner KE, Padmanabhan R. The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. J Virol. 1999;73(4):3108-16.

43. Wengler G. The carboxy-terminal part of the NS 3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. Virology. 1991;184(2):707-15.

44. Wengler G. The NS 3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. Virology. 1993;197(1):265-73.

45. Matusan AE, Kelley PG, Pryor MJ, Whisstock JC, Davidson AD, Wright PJ. Mutagenesis of the dengue virus type 2 NS3 proteinase and the production of growth-restricted virus. J Gen Virol. 2001;82(Pt 7):1647-56.

46. Xu T, Sampath A, Chao A, Wen D, Nanao M, Chene P, et al. Structure of the Dengue virus helicase/nucleoside triphosphatase catalytic domain at a resolution of 2.4 A. J Virol. 2005;79(16):10278-88.

47. Munoz-Jordan JL, Laurent-Rolle M, Ashour J, Martinez-Sobrido L, Ashok M, Lipkin WI, et al. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. Journal of virology. 2005;79(13):8004-13.

48. Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A. Inhibition of interferon signaling by dengue virus. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(24):14333-8.

49. Umareddy I, Chao A, Sampath A, Gu F, Vasudevan SG. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. The Journal of general virology. 2006;87(Pt 9):2605-14.

50. Egloff MP, Decroly E, Malet H, Selisko B, Benarroch D, Ferron F, et al. Structural and functional analysis of methylation and 5'-RNA sequence requirements of short capped RNAs by the methyltransferase domain of dengue virus NS5. Journal of molecular biology. 2007;372(3):723-36.

51. Ackermann M, Padmanabhan R. De novo synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase. The Journal of biological chemistry. 2001;276(43):39926-37.

52. Leitmeyer KC, Vaughn DW, Watts DM, Salas R, Villalobos I, de C, et al. Dengue virus structural differences that correlate with pathogenesis. Journal of virology. 1999;73(6):4738-47.

53. Halstead SB. Neutralization and antibody-dependent enhancement of dengue viruses. Advances in virus research. 2003;60:421-67.

54. Huang KJ, Yang YC, Lin YS, Huang JH, Liu HS, Yeh TM, et al. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. Journal of immunology. 2006;176(5):2825-32.

55. Littaua R, Kurane I, Ennis FA. Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection. J Immunol. 1990;144(8):3183-6.

56. Nguyen TH, Lei HY, Nguyen TL, Lin YS, Huang KJ, Le BL, et al. Dengue hemorrhagic fever in infants: a study of clinical and cytokine profiles. J Infect Dis. 2004;189(2):221-32.

57. Thein S, Aung MM, Shwe TN, Aye M, Zaw A, Aye K, et al. Risk factors in dengue shock syndrome. Am J Trop Med Hyg. 1997;56(5):566-72.

58. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg. 1988;38(1):172-80.

59. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, Vasanawathana S, Avirutnan P, Jairungsri A, et al. T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? J Immunol. 2006;176(6):3821-9.

60. Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, et al. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med. 2003;9(7):921-7.

61. Srikiatkhachorn A, Green S. Markers of dengue disease severity. Current topics in microbiology and immunology. 2010;338:67-82.

62. Espada-Murao LA, Morita K. Dengue and soluble mediators of the innate immune system. Tropical medicine and health. 2011;39(4 Suppl):53-62.

63. Wan SW, Lin CF, Yeh TM, Liu CC, Liu HS, Wang S, et al. Autoimmunity in dengue pathogenesis. Journal of the Formosan Medical Association = Taiwan yi zhi. 2013;112(1):3-11.

64. Anderson R, Wang S, Osiowy C, Issekutz AC. Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. Journal of virology. 1997;71(6):4226-32.

65. Hatch S, Endy TP, Thomas S, Mathew A, Potts J, Pazoles P, et al. Intracellular cytokine production by dengue virus-specific T cells correlates with subclinical secondary infection. The Journal of infectious diseases. 2011;203(9):1282-91.

66. King CA, Anderson R, Marshall JS. Dengue virus selectively induces human mast cell chemokine production. Journal of virology. 2002;76(16):8408-19.

67. Juffrie M, van Der Meer GM, Hack CE, Haasnoot K, Sutaryo, Veerman AJ, et al. Inflammatory mediators in dengue virus infection in children: interleukin-8 and its relationship to neutrophil degranulation. Infection and immunity. 2000;68(2):702-7.

68. Rothman AL. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol.11(8):532-43.

69. Shresta S, Kyle JL, Snider HM, Basavapatna M, Beatty PR, Harris E. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J Virol. 2004;78(6):2701-10.

70. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E. Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol. 2006;80(20):10208-17.

71. Mota J, Rico-Hesse R. Humanized mice show clinical signs of dengue fever according to infecting virus genotype. J Virol. 2009;83(17):8638-45.

72. Murrell S, Wu SC, Butler M. Review of dengue virus and the development of a vaccine. Biotechnology advances. 2011;29(2):239-47.

73. Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, et al. Safety and immunogenicity of a three dose regimen of two tetravalent live-attenuated dengue vaccines in five- to twelve-year-old Thai children. The Pediatric infectious disease journal. 2004;23(2):99-109.

74. Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, et al. Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. The American journal of tropical medicine and hygiene. 2002;66(3):264-72.

75. Sanchez V, Gimenez S, Tomlinson B, Chan PK, Thomas GN, Forrat R, et al. Innate and adaptive cellular immunity in flavivirus-naive human recipients of a live-attenuated dengue serotype 3 vaccine produced in Vero cells (VDV3). Vaccine. 2006;24(23):4914-26.

76. Sun W, Edelman R, Kanesa-Thasan N, Eckels KH, Putnak JR, King AD, et al. Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. The American journal of tropical medicine and hygiene. 2003;69(6 Suppl):24-31.

77. Thomas SJ, Eckels KH, Carletti I, De La Barrera R, Dessy F, Fernandez S, et al. A phase II, randomized, safety and immunogenicity study of a re-derived, live-attenuated dengue virus

vaccine in healthy adults. The American journal of tropical medicine and hygiene. 2013;88(1):73-88.

78. Guirakhoo F, Pugachev K, Zhang Z, Myers G, Levenbook I, Draper K, et al. Safety and efficacy of chimeric yellow Fever-dengue virus tetravalent vaccine formulations in nonhuman primates. Journal of virology. 2004;78(9):4761-75.

79. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, et al. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet. 2012;380(9853):1559-67.

80. Halstead SB. Dengue vaccine development: a 75% solution? Lancet. 2012;380(9853):1535-6.

81. Men R, Bray M, Clark D, Chanock RM, Lai CJ. Dengue type 4 virus mutants containing deletions in the 3' noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. Journal of virology. 1996;70(6):3930-7.

82. Blaney JE, Jr., Hanson CT, Firestone CY, Hanley KA, Murphy BR, Whitehead SS. Genetically modified, live attenuated dengue virus type 3 vaccine candidates. The American journal of tropical medicine and hygiene. 2004;71(6):811-21.

83. Blaney JE, Jr., Hanson CT, Hanley KA, Murphy BR, Whitehead SS. Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2. BMC infectious diseases. 2004;4:39.

84. Whitehead SS, Falgout B, Hanley KA, Blaney Jr JE, Jr., Markoff L, Murphy BR. A live, attenuated dengue virus type 1 vaccine candidate with a 30-nucleotide deletion in the 3' untranslated region is highly attenuated and immunogenic in monkeys. Journal of virology. 2003;77(2):1653-7.

85. Durbin AP, Kirkpatrick BD, Pierce KK, Schmidt AC, Whitehead SS. Development and clinical evaluation of multiple investigational monovalent DENV vaccines to identify components for inclusion in a live attenuated tetravalent DENV vaccine. Vaccine. 2011;29(42):7242-50.

86. Aihara H, Takasaki T, Toyosaki-Maeda T, Suzuki R, Okuno Y, Kurane I. T-cell activation and induction of antibodies and memory T cells by immunization with inactivated Japanese encephalitis vaccine. Viral Immunology. 2000;13(2):179-86.

87. *Barrett DWCBaADT*. The Infectious Agent. In: Halstead SB, editor. Tropical Medicine: Science and Practice. Dengue. 5. Singapore.: Imperial College Press; 2008. p. 29-73.

88. Simmons M, Burgess T, Lynch J, Putnak R. Protection against dengue virus by non-replicating and live attenuated vaccines used together in a prime boost vaccination strategy. Virology.396(2):280-8.

89. Clements DE, Coller BA, Lieberman MM, Ogata S, Wang G, Harada KE, et al. Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. Vaccine. 2010;28(15):2705-15.

90. Guzman MG, Rodriguez R, Rodriguez R, Hermida L, Alvarez M, Lazo L, et al. Induction of neutralizing antibodies and partial protection from viral challenge in Macaca fascicularis immunized with recombinant dengue 4 virus envelope glycoprotein expressed in Pichia pastoris. The American journal of tropical medicine and hygiene. 2003;69(2):129-34.

91. Kelly EP, Greene JJ, King AD, Innis BL. Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. Vaccine. 2000;18(23):2549-59.

92. Coller BA, Clements DE, Bett AJ, Sagar SL, Ter Meulen JH. The development of recombinant subunit envelope-based vaccines to protect against dengue virus induced disease. Vaccine. 2011;29(42):7267-75.

93. Chin JF, Chu JJ, Ng ML. The envelope glycoprotein domain III of dengue virus serotypes 1 and 2 inhibit virus entry. Microbes and infection / Institut Pasteur. 2007;9(1):1-6.

94. Hermida L, Bernardo L, Martin J, Alvarez M, Prado I, Lopez C, et al. A recombinant fusion protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. Vaccine. 2006;24(16):3165-71.

95. Leng CH, Liu SJ, Tsai JP, Li YS, Chen MY, Liu HH, et al. A novel dengue vaccine candidate that induces cross-neutralizing antibodies and memory immunity. Microbes and infection / Institut Pasteur. 2009;11(2):288-95.

96. Chen HW, Liu SJ, Li YS, Liu HH, Tsai JP, Chiang CY, et al. A consensus envelope protein domain III can induce neutralizing antibody responses against serotype 2 of dengue virus in non-human primates. Archives of virology. 2013;158(7):1523-31.

97. Chen HW, Liu SJ, Liu HH, Kwok Y, Lin CL, Lin LH, et al. A novel technology for the production of a heterologous lipoprotein immunogen in high yield has implications for the field of vaccine design. Vaccine. 2009;27(9):1400-9.

98. Izquierdo A, Valdes I, Gil L, Hermida L, Gutierrez S, Garcia A, et al. Serotype specificity of recombinant fusion protein containing domain III and capsid protein of dengue virus 2. Antiviral Res. 2012;95(1):1-8.

99. Chiang CY, Huang MH, Hsieh CH, Chen MY, Liu HH, Tsai JP, et al. Dengue-1 envelope protein domain III along with PELC and CpG oligodeoxynucleotides synergistically enhances immune responses. PLoS Negl Trop Dis. 2012;6(5):e1645.

100. Valdes I, Gil L, Romero Y, Castro J, Puente P, Lazo L, et al. The chimeric protein domain III-capsid of dengue virus serotype 2 (DEN-2) successfully boosts neutralizing antibodies generated in monkeys upon infection with DEN-2. Clin Vaccine Immunol. 2011;18(3):455-9.

101. Chiang CY, Liu SJ, Tsai JP, Li YS, Chen MY, Liu HH, et al. A novel single-dose dengue subunit vaccine induces memory immune responses. PloS one. 2011;6(8):e23319.

102. Bernardo L, Izquierdo A, Alvarez M, Rosario D, Prado I, Lopez C, et al. Immunogenicity and protective efficacy of a recombinant fusion protein containing the domain III of the dengue 1 envelope protein in non-human primates. Antiviral Res. 2008;80(2):194-9.

103. Beckett CG, Tjaden J, Burgess T, Danko JR, Tamminga C, Simmons M, et al. Evaluation of a prototype dengue-1 DNA vaccine in a Phase 1 clinical trial. Vaccine.29(5):960-8.

104. Raviprakash K, Kochel TJ, Ewing D, Simmons M, Phillips I, Hayes CG, et al. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. Vaccine. 2000;18(22):2426-34.

105. Konishi E, Yamaoka M, Kurane I, Mason PW. A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine. 2000;18(11-12):1133-9.

106. Allison SL, Stadler K, Mandl CW, Kunz C, Heinz FX. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J Virol. 1995;69(9):5816-20.

107. Sugrue RJ, Fu J, Howe J, Chan YC. Expression of the dengue virus structural proteins in Pichia pastoris leads to the generation of virus-like particles. J Gen Virol. 1997;78 (Pt 8):1861-6.

108. Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. Nat Rev Microbiol. 2007;5(7):518-28.

109. Abdulhaqq SA, Weiner DB. DNA vaccines: developing new strategies to enhance immune responses. Immunol Res. 2008;42(1-3):219-32.

110. Ruprecht RM. Live attenuated AIDS viruses as vaccines: promise or peril? Immunol Rev. 1999;170:135-49.

111. Offit PA. The Cutter incident, 50 years later. N Engl J Med. 2005;352(14):1411-2.

112. Casimiro DR, Chen L, Fu TM, Evans RK, Caulfield MJ, Davies ME, et al. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-

defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J Virol. 2003;77(11):6305-13.

113. Kurth R. Risk potential of the chromosomal insertion of foreign DNA. Ann N Y Acad Sci. 1995;772:140-51.

114. Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, et al. Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. Intervirology. 2000;43(4-6):273-81.

115. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, et al. Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. Intervirology. 2000;43(4-6):258-72.

116. Temin HM. Overview of biological effects of addition of DNA molecules to cells. J Med Virol. 1990;31(1):13-7.

117. Pal R, Yu Q, Wang S, Kalyanaraman VS, Nair BC, Hudacik L, et al. Definitive toxicology and biodistribution study of a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 (HIV-1) vaccine in rabbits. Vaccine. 2006;24(8):1225-34.

118. Sheets RL, Stein J, Manetz TS, Duffy C, Nason M, Andrews C, et al. Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. Toxicol Sci. 2006;91(2):610-9.

119. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, et al. Plasmid DNA vaccines: assay for integration into host genomic DNA. Dev Biol (Basel). 2000;104:33-43.

120. Klinman DM, Takeshita F, Kamstrup S, Takeshita S, Ishii K, Ichino M, et al. DNA vaccines: capacity to induce auto-immunity and tolerance. Dev Biol (Basel). 2000;104:45-51.

121. MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, et al. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J Infect Dis. 1998;178(1):92-100.

122. Le TP, Coonan KM, Hedstrom RC, Charoenvit Y, Sedegah M, Epstein JE, et al. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. Vaccine. 2000;18(18):1893-901.

123. MacGregor RR, Boyer JD, Ciccarelli RB, Ginsberg RS, Weiner DB. Safety and immune responses to a DNA-based human immunodeficiency virus (HIV) type I env/rev vaccine in HIV-infected recipients: follow-up data. J Infect Dis. 2000;181(1):406.

124. Luke J, Carnes AE, Hodgson CP, Williams JA. Improved antibiotic-free DNA vaccine vectors utilizing a novel RNA based plasmid selection system. Vaccine. 2009;27(46):6454-9.

125. Luke JM, Vincent JM, Du SX, Gerdemann U, Leen AM, Whalen RG, et al. Improved antibiotic-free plasmid vector design by incorporation of transient expression enhancers. Gene Ther. 2011;18(4):334-43.

126. Hartikka J, Bozoukova V, Ferrari M, Sukhu L, Enas J, Sawdey M, et al. Vaxfectin enhances the humoral immune response to plasmid DNA-encoded antigens. Vaccine. 2001;19(15-16):1911-23.

127. Reyes L, Hartikka J, Bozoukova V, Sukhu L, Nishioka W, Singh G, et al. Vaxfectin enhances antigen specific antibody titers and maintains Th1 type immune responses to plasmid DNA immunization. Vaccine. 2001;19(27):3778-86.

128. Wilder-Smith A, Schwartz E. Dengue in travelers. The New England journal of medicine. 2005;353(9):924-32.

129. Vasilakis N, Weaver SC. The history and evolution of human dengue emergence. Advances in virus research. 2008;72:1-76.

130. Weaver SC, Vasilakis N. Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2009;9(4):523-40.

131. CB H. Identification of epitopes on dengue virus type 4 envelop glycoprotein involved in neutralization by antibodies. Doctoral dissertation, School of Life Sciences, Queenland University. 2006.

132. Lai CY, Tsai WY, Lin SR, Kao CL, Hu HP, King CC, et al. Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. Journal of virology. 2008;82(13):6631-43.

APPENDIX

1. Pairwise alignment of DENV-4 1036 and DENV-4 C0036 (only E amino acid were compared)

Identity: 478/495 (**96.6%**)

Similarity: 483/495 (97.6%)

Gaps: 0/495 (0.0%)

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D4_1036E           1 MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTA     50
                     |||||||||||||||||||||||||||||||||||||||||||||||.||||
D4_C0036E          1 MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTA     50

D4_1036E          51 KEVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDVVDRG    100
                     ||||||||||||||||||||||||||||||||||||||||||||||:||||
D4_C0036E         51 KEVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRG    100

D4_1036E         101 WGNGCGLFGKGGVVTCAKFSCSGKITGNLVQIENLEYTVVVTVHNGDTHA    150
                     ||||||||||||||||||||.||||||||||||||||||||||||||||||
D4 C0036E        101 WGNGCGLFGKGGVVTCAKFSCLGKITGNLVQIENLEYTVVVTVHNGDTHA    150

D4_1036E         151 VGNDTSNHGVTATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMK    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036E        151 VGNDTSNHGVTATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMK    200

D4 1036E         201 MKKKTWLVHKQWFLDLPLPWTAGADTSEVHWNYKERMVTFKVPHAKRQDV    250
                     ||.||||||||||||||||.||||.||||||:||||||||||||||||||
D4_C0036E        201 MKTKTWLVHKQWFLDLPLPWTTGADTLEVHWNHKERMVTFKVPHAKRQDV    250

D4_1036E         251 TVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036E        251 TVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT    300

D4_1036E         301 MCPGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGR    350
                     ||.|||||||||||||||||||||.||||||||||||||||||||||||||
D4_C0036E        301 MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGR    350

D4_1036E         351 IISSTPLAENTNSATNIELEPPFGDSYIVIGVGNSALTLHWFRKGSSIGK    400
                     ||||||.|||||||.||||||||||||||||:.|||||||||||||||||||
D4_C0036E        351 IISSTPFAENTNSVTNIELEPPFGDSYIVIGVGDGALTLHWFRKGSSIGK    400

D4_1036E         401 MFESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYTTMFGG    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036E        401 MFESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYTTMFGG    450

D4_1036E         451 VSWMIRILIGFLVLWIGTNSRNTSMAMTCIAVGGITLFLGFTVQA    495
                     ||||:|||||.||||||||||||||:|||||||||||||||.|
D4_C0036E        451 VSWMVRILIGLLVLWIGTNSRNTSMAMSCIAVGGITLFLGFTVHA    495
```

2. Pairwise alignment of the original D4prME candidate vaccine and DENV-4 strain 1036

Length: 495

Identity: 479/495 (**96.8%**)

Similarity: 484/495 (97.8%)

Gaps: 0/495 (0.0%)

```
D4prME            1 MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTA     50
                    |||||||||||||||||||||||||||||||||||||||||||||||.||||
D4_1036           1 MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTA     50

D4prME           51 KEVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRG    100
                    ||||||||||||||||||||||||||||||||||||||||||||.|:||||
D4_1036          51 KEVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRTDVVDRG    100

D4prME          101 WGNGCGLFGKGGVVTCAKFSCSGKITGNLVQIENLEYTVVVTVHNGDTHA    150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||.
D4_1036         101 WGNGCGLFGKGGVVTCAKFSCSGKITGNLVQIENLEYTVVVTVHNGDTHG    150

D4prME          151 VGNDTSNHGVTATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMK    200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
D4_1036         151 VGNDTSNHGVTATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMK    200

D4prME          201 MKTKTWLVHKQWFLDLPLPWTAGADTSEVHWNHKERMVTFKVPHAKRQDV    250
                    ||.|||||||||||||||||||||||||:||||||||||||||||||||
D4_1036         201 MKKKTWLVHKQWFLDLPLPWTAGADTSEVHWNYKERMVTFKVPHAKRQDV    250

D4prME          251 TVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT    300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
D4_1036         251 TVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT    300

D4prME          301 MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGR    350
                    ||||||||||||||||||||||||||||.||||||||||||||||||||||
D4_1036         301 MCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGR    350

D4prME          351 IISSTPFAENTNSVTNIELEPPFGDSYIVIGVGDSALTLHWFRKGSSIGK    400
                    ||||||.|||||.||||||||||||||||:||||||||||||||||||||
D4_1036         351 IISSTPLAENTNSATNIELEPPFGDSYIVIGVGNSALTLHWFRKGSSIGK    400

D4prME          401 MFESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYTTMFGG    450
                    ||||||||||||||||||||||||||||.||.||||||||||||||||||
D4_1036         401 MFESTYRGAKRMAILGETAWDFGSVGGLFTSWGKAVHQVFGSVYTTMFGG    450

D4prME          451 VSWMVRILIGLLVLWIGTNSRNTSMAMSCIAVGGITLFLGFTVHA    495
                    ||||:|||||.|||||||||||||:|||||||||||||||.|
D4_1036         451 VSWMIRILIGFLVLWIGTNSRNTSMAMTCIAVGGITLFLGFTVQA    495
```

High light indicated the amino acid mismatch within E protein.

3. Pairwise alignment of the original D4prME candidate vaccine and DENV-4 strain C0036

Length: 495

Identity: 491/495 (**99.2%**)

Similarity: 491/495 (99.2%)

Gaps: 0/495 (0.0%)

```
D4prME              1 MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTA     50
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036/06         1 MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTA     50

D4prME             51 KEVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRG    100
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036/06        51 KEVALLRTYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRG    100

D4prME            101 WGNGCGLFGKGGVVTCAKFSCSGKITGNLVQIENLEYTVVVTVHNGDTHA    150
                      ||||||||||||||||||||.||||||||||||||||||||||||||||||
D4_C0036/06       101 WGNGCGLFGKGGVVTCAKFSCLGKITGNLVQIENLEYTVVVTVHNGDTHA    150

D4prME            151 VGNDTSNHGVTATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMK    200
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036/06       151 VGNDTSNHGVTATITPRSPSVEVKLPDYGELTLDCEPRSGIDFNEMILMK    200

D4prME            201 MKTKTWLVHKQWFLDLPLPWTAGADTSEVHWNHKERMVTFKVPHAKRQDV    250
                      ||||||||||||||||||||.||||.||||||||||||||||||||||||||
D4_C0036/06       201 MKTKTWLVHKQWFLDLPLPWTTGADTLEVHWNHKERMVTFKVPHAKRQDV    250

D4prME            251 TVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT    300
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036/06       251 TVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT    300

D4prME            301 MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGR    350
                      |||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036/06       301 MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGR    350

D4prME            351 IISSTPFAENTNSVTNIELEPPFGDSYIVIGVGDSALTLHWFRKGSSIGK    400
                      ||||||||||||||||||||||||||||||||||.|||||||||||||||
D4_C0036/06       351 IISSTPFAENTNSVTNIELEPPFGDSYIVIGVGDGALTLHWFRKGSSIGK    400

D4prME            401 MFESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYTTMFGG    450
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
D4_C0036/06       401 MFESTYRGAKRMAILGETAWDFGSVGGLLTSLGKAVHQVFGSVYTTMFGG    450

D4prME            451 VSWMVRILIGLLVLWIGTNSRNTSMAMSCIAVGGITLFLGFTVHA    495
                      |||||||||||||||||||||||||||||||||||||||||||||
D4 C0036/06       451 VSWMVRILIGLLVLWIGTNSRNTSMAMSCIAVGGITLFLGFTVHA    495
```

High light indicated the amino acid mismatch within E protein.

4. Multiple alignment of E amino acid among the original D4prME candidate vaccine, DENV- 4 strain 1036, DENV-4 strain C0036 and newly isolated Thailand DENV-4 (Year 2011)

```
D4C0036/06      MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTAKEVALLRTYC
D4_TH11         MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTAKEVALLRTYC
D4prME          MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELIKTTAKEVALLRTYC
D4_1036         MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAKEVALLRTYC
                ****************************************** **************

D4C0036/06      IEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRGWGNGCGLFGKGGVVTCAKFS
D4_TH11         IEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRGWGNGCGLFGKGGVVTCAKFS
D4prME          IEASISNITTATRCPTQGEPYLKEEQDQQYICRRDMVDRGWGNGCGLFGKGGVVTCAKFS
D4_1036         IEASISNITTATRCPTQGEPYLKEEQDQQYICRTDVVDRGWGNGCGLFGKGGVVTCAKFS
                ******************************** *:************************

D4C0036/06      CLGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTATITPRSPSVEVKLPDYGE
D4_TH11         CSGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTATITPRSPSVEVKLPDYGE
D4prME          CSGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTATITPRSPSVEVKLPDYGE
D4_1036         CSGKITGNLVQIENLEYTVVVTVHNGDTHGVGNDTSNHGVTATITPRSPSVEVKLPDYGE
                * ***************************.******************************

D4C0036/06      LTLDCEPRSGIDFNEMILMKMKTKTWLVHKQWFLDLPLPWTTGADTLEVHWNHKERMVTF
D4_TH11         LTLDCEPRSGIDFNEMILMKMKTKTWLVHKQWFLDLPLPWTAGADTLEVHWNHKERMVTF
D4prME          LTLDCEPRSGIDFNEMILMKMKTKTWLVHKQWFLDLPLPWTAGADTSEVHWNHKERMVTF
D4_1036         LTLDCEPRSGIDFNEMILMKMKKTWLVHKQWFLDLPLPWTAGADTSEVHWNYKERMVTF
                **********************.******************:**** ***** :*******

D4C0036/06      KVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT
D4_TH11         KVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT
D4prME          KVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT
D4_1036         KVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT
                ************************************************************

                                      E329
D4C0036/06      MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGRIISSTPFAEN
D4_TH11         MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGRIISSTPFAEN
D4prME          MCSGKFSIDKEMAETQHGTTVVKVKYEGTGAPCKVPIEIRDVNKEKVVGRIISSTPFAEN
D4_1036         MCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAEN
                ****************************:****************************:***

D4C0036/06      TNSVTNIELEPPFGDSYIVIGVGDGALTLHWFRKGSSIGKMFESTYRGAKRMAILGETAW
D4_TH11         TNSVTNIELEPPFGDSYIVIGVGESALTLHWFRKGSSIGKMFESTYRGAKRMAILGETAW
D4prME          TNSVTNIELEPPFGDSYIVIGVGDSALTLHWFRKGSSIGKMFESTYRGAKRMAILGETAW
D4_1036         TNSATNIELEPPFGDSYIVIGVGNSALTLHWFRKGSSIGKMFESTYRGAKRMAILGETAW
                ***.******************:.**********************************

D4C0036/06      DFGSVGGLLTSLGKAVHQVFGSVYTTMFGGVSWMVRILIGLLVLWIGTNSRNTSMAMSCI
D4_TH11         DFGSVGGLLTSLGKAVHQVFGSVYTTMFGGVSWMVRILIGLLVLWIGTNSRNTSMAMSCI
D4prME          DFGSVGGLLTSLGKAVHQVFGSVYTTMFGGVSWMVRILIGLLVLWIGTNSRNTSMAMSCI
D4_1036         DFGSVGGLFTSWGKAVHQVFGSVYTTMFGGVSWMIRILIGFLVLWIGTNSRNTSMAMTCI
                ********:** ********************:******:****************:**

D4C0036/06      AVGGITLFLGFTVHA
D4_TH11         AVGGITLFLGFTVHA
D4prME          AVGGITLFLGFTVHA
D4_1036         AVGGITLFLGFTVQA
                *************:*
```

→ ← : E Domain III

เอกสารแนบหมายเลข 3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   1.1 Manuscript is under submission:

   Prompetchara E, Ketloy C, Keelapand P, Sittisombut N, Ruxrungtham K. Induction of neutralizing antibody response against four dengue viruses in mice by intramuscular electroporation of tetravalent DNA vaccines.

   1.2 Manuscript is under preparation:

   Ketloy C, Aungkundee T, Prompetchara E Keelapand P, Sittisombut N, Ruxrungtham K. Immunogenicity in mice of a chimeric dengue DNA vaccine based on DENV-4 E domain III replaced in DENV-2 prM/E backbone.

2. การนำผลงานวิจัยไปใช้ประโยชน์

   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
     มีการนำผลงานวิจัยไปใช้การพัฒนาการเรียนการสอน ในรายวิชา Advance Cellular and Molecular Biology และสร้างนักวิจัยระดับปริญญาโท ในหลักสูตรวิทยาศาสตรมหาบัณฑิต สาขาวิทยาศาสตร์การแพทย์ จุฬาลงกรณ์มหาวิทยาลัย

3. อื่นๆ: การเสนอผลงานในที่ประชุมวิชาการ แบบ poster presentation เรื่อง “Strategies to improve the immunogenicity of prM/E dengue virus type-4 DNA vaccine” ที่งานประชุม “นักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว.” วันที่ 16-18 ตุลาคม 2556 ณ โรงแรมเดอะรีเจ้นท์ ชะอำบีช รีสอร์ท หัวหิน ชะอำ จังหวัดเพชรบุรี

Induction of Neutralizing Antibody Response against Four Dengue Viruses in Mice by Intramuscular Electroporation of Tetravalent DNA Vaccines

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*Both contributed equally as a first author.

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Abstract

DNA vaccine against dengue is an interesting strategy for a prime/boost approach. This study evaluated neutralizing antibody (NAb) induction of a dengue tetravalent DNA (TDNA) vaccine candidate administered by intramuscular-electroporation (IM-EP) and the benefit of homologous TDNA boosting in mice. Consensus humanized pre-membrane (*prM)* and envelope (*E*) of each serotypes, based on isolates from year 1962-2003, were separately cloned into a pCMVkan expression vector. ICR mice, five-six per group were immunized for three times (2-week interval) with TDNA at 100 µg (group I; 25 µg/monovalent) or 10 µg (group II; 2.5 µg/monovalent). In group I, mice received a TDNA boosting 13 weeks later. Plaque reduction neutralization tests (PRNT) were performed at 4 weeks post-last immunization**.** Both 100 µg and 10 µg doses of TDNA induced high NAb levels against all DENV serotypes. The median PRNT50 titers were comparable among four serotypes of DENV after TDNA immunization. Median PRNT50 titers ranged 240-320 in 100 µg and 160-240 in 10 µg groups (p=ns). A time course study of the 100 µg dose of TDNA showed detectable NAb at 2 weeks after the second injection. The NAb peaked at 4 weeks after the third injection then declined over time but remained detectable up to 13 weeks. An additional homologous TDNA boosting significantly enhanced the level of NAb from the nadir for at least ten-fold (p<0.05)**.** Tetravalent dengue prME DNA vaccine candidate, with a very high homology DNA sequence to recent dengue viral isolates, induced good neutralizing antibody responses in mice; and the tetravalent dengue DNA/DNA prime/boost strategy is promising and warrants further evaluation in non-human primates.

Introduction

Dengue virus (DENV) infection is a major public health problem in tropical and sub-tropical countries. Approximately 2.5 billion people are at risk and 50-100 million infections occur annually worldwide [1]. Unfortunately, licensed vaccine to prevent dengue is currently unavailable although various vaccine development strategies have been investigated [2-9]. As four antigenic-related serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) commonly co-circulate, thus an effective vaccine must cover all serotypes. Safety, balance between immunogenicity and attenuation, and "interference" among DENV serotypes represent problems for live, attenuated dengue vaccine candidates [10-14] [15,16]. DNA vaccine is an alternative strategy to overcome the concern of these problems together with its many advantages [[17,18]. In addition, while a live-attenuated dengue vaccine requires at least 6 months apart from the last immunization to be able to re-immunize as an effective boosting dose [19] [20]. DNA vaccine may have an advantage to be able to boost much sooner.

In this study, we constructed TDNA dengue vaccine candidates encoding the prM and E proteins with a very high homology DNA sequence to recent dengue viral isolates; and examined their immunogenicity following repeated IM-EP in mice. We also investigated whether an additional boosting with the homologous TDNA when the NAb was declining, a TDNA-prime/TDNA-boost strategy, would further enhance the neutralizing antibody responses.

Materials and Methods

Cells and viruses

Monkey kidney-derived cell lines (Vero and LLC-MK_2 cells), DENV-1: strain 16007, DENV-2: strain 16681, DENV-3: strain 16562 and DENV-4: strain 1036 and C0036 were kindly provided by Drs. Ananda Nisalak and Robert Gibbons, Armed Forces Research Institute of Medical Sciences, Thailand. Vero and LLC-MK_2 were propagated in minimum essential medium supplemented with 10% fetal bovine serum (FBS) and in Medium 199 supplemented with 20% FBS, respectively (all reagents were from Gibco) and incubated at 37°C, 5% CO_2. Dengue viral stocks were propagated in Vero cells and stored in -80 °C. Virus titers were determined by a plaque assay on LLC-MK_2 cell monolayer.

TDNA construction

Full length humanized codon of consensus *prM/E* were generated from dengue viral sequences deposited in Genbank during 1962-2003 then commercially synthesized GeneArt (Germany). The Kozak sequence and *prM* signal sequence were inserted at the N-terminus. The expression cassettes were subcloned into pCMVkan expression vector [21] and designated as pCMVkanD1prME, pCMVkanD2prME, pCMVkanD3prME and pCMVkanD4prME (Figure 1A). All recombinant plasmid constructs were transformed into *Escherichia coli* DH5αF' (Invitrogen) and confirmed by nucleotides sequence analysis.

Protein expression

Vero cells were separately transfected with individual recombinant plasmid constructs (pCMVkanD1prME-pCMVkanD4prME) using lipofectamine 2000 (Invitrogen). At 24 hr post-transfection, cells were fixed, permeabilized and stained with flavivirus-reactive anti-E antibody (clone 4G2) [22] and anti-DENV-NS1 antibody (clone DN3, Abcam). Rabbit-anti-mouse IgG-FITC (Dako) and goat-anti-mouse IgG-

Alexa-fluor (Molecular Probe) were used as secondary Ab for detection of anti-E and anti-NS1, respectively. Cell nuclei were counter stained with 4, 6-diamino-2-phenylindole hydrochloride (DAPI) (Sigma–Aldrich). Stained cells were visualized under fluorescence microscope. Western blot was used for detection of E protein expression in cells culture supernatant at 24 hr post-transfection or infection by using 4G2 mAb. Rabbit-anti-mouse IgG conjugated with horseradish peroxidase (KPL) was used as secondary Ab and detected by chemiluminescence substrate (Immobilon™ western, Millipore) then exposed to an X-ray film. Vero cells infected with DENV-2 (strain 16681) at the multiplicity of infection of 0.5 or transfected with empty pCMVkan expression vector were employed as positive and negative controls, respectively.

Mice experiments

ICR mice at 4-6 weeks of age were procured from the National Laboratory Animal Center, Mahidol University, Thailand. Mice were immunized with DNA constructs by intramuscular *in vivo* electroporation, IM-EP (Ichor Medical Systems) at the tibialis muscle as previously described [23]. Immunization and bleeding procedures were performed under isoflurane-induced anesthesia. All experimental procedures were approved by the Committee of Animal Care and Use of Faculty of Medicine, Chulalongkorn University (approval no. 05/54).

Five-six mice/group were immunized with TDNA cocktail at a total of 100 μg (25 μg of each the monovalent preparation) or 10 μg (2.5 μg each) per dose for 3 times at a 2-week interval using IM-EP. Mice were bled at 4 weeks after the last immunization and the sera were individually examined for NAb activity against each of the four dengue serotypes.

In the prime-boost study, six mice were immunized with 100 μg of the TDNA cocktail (25 μg of each the monovalent preparation) for 3 times at a 2-week interval and then boosted with 100 μg of the TDNA cocktail on week 17. Mice were bled at week 4, 6, 8, 10, 17 and 20 after the first immunization.

Plaque reduction neutralization test (PRNT)

NAb titer was determined by PRNT as previously described [24]. Briefly, mice sera were inactivated at 56 °C, 30 min and serially diluted with MEM supplemented with 10% FBS. Diluted sera were mixed with equal volume of target virus (30-50 PFU/well) and incubated at 37 °C for 1 hr. Virus-serum mixture was transferred onto LLC-MK2 monolayer and allowed to absorb for 1 hr at room temperature. Cells were overlaid with first overlayer medium containing FBS, amino acid, vitamin, L-glutamine, 0.9% low-melting point agarose (Invitrogen), Hank's BSS and $NaHCO_3$. After 4-5 days of incubation in 37 °C, 5% CO_2, the secondary overlayer containing 4% v/v neutral red (Sigma-Aldrich) was added. Plaques were counted after 24 hr of additional incubation. The highest serum dilution that resulted in 50% or more reduction of the average number of plaques as compared with the virus control wells was considered as the neutralizing endpoint titer (PRNT50).

Statistic analysis

The comparisons of NAb (PRNT50) between experimental groups or at different time-points were performed with the Mann-Whitney test. $p<0.05$ was considered significant.

Results

In vitro protein expression analysis

At 24 hr post transfection, E protein, but not NS1, expression was detected in the cytoplasm of Vero cells transfected with each of the recombinant dengue prME DNA constructs (Figure 1B). Vero cells that were infected with dengue viruses showed both cytoplasmic E and NS1 protein expression. These two proteins were not detected in mock-infected Vero cells. Extracellular E protein, approximately 55 kDa in size, was detected after 24 hr post transfection in immunoblot analysis using the mAb 4G2 (Figure 1C) for all constructs, but not the empty expression vector.

Induction of neutralizing antibody response in mice

Mice immunized for three times with either 100 μg or 10 μg of total TDNA by IM-EP showed high levels of NAb against all four DENV serotypes. At 100 μg/dose of TDNA, the induced NAb titers against four dengue serotypes were comparable. The median PRNT50 titers against DENV-1, DENV-2, DENV-3 and DENV-4 (strain C0036) were 240, 320, 240 and 320, respectively. Slightly lower levels of NAb were detected in mice after three injections of 10 μg TDNA/dose. An interesting finding was observed during the PRNT testing of sera against DENV-4. When strain 1036 was used initially as target in the PRNT, the NAb titers were significantly lower than those of other serotypes. When a more recent isolate, C0036, was employed, the magnitude of NAb titers improved significantly; the median NAb titers were 320 and 240 in the 100 μg/dose and 10 μg/dose TDNA groups, respectively (Figure 2). A similar observation had been reported previously with recent clinical samples from DENV-4-infected individuals [74].

Kinetics of NAb response following a prime-boost TDNA immunization

In mice that were immunized for three times with 100 μg/dose of TDNA, NAb response were detected against all DENV serotypes at week 2 after the second immunization, reaching the peak levels at week 4 after the third dose (Figure 3). The NAb gradually declined over time but remained detectable at week 13 after the third dose. An additional TDNA boost injected at 3 months after the third dose resulted in a significant increase of the NAb titers (Figure 3). The median NAb titers increased by 16-fold (p=0.012), 12-fold (p=0.005), 10.7-fold (p=0.005) and 21-fold (p=0.007) against DENV-1, DENV-2, DENV-3, and DENV-4, respectively, when compared with the baseline levels at week 13 after the third dose.

Discussion

In this study, the recombinant dengue PrME DNA vaccine candidates, when immunized by intramuscular electroporation (IM-EP) as a tetravalent cocktail, generated good neutralizing antibody against all serotypes of DENV. With DNA/DNA prime/boost approach, the median NAb titers after the 4^{th} TDNA immunization at 1280, 640, 640 and 1280 against DENV1, 2, 3 and 4, respectively Our study confirms there is no interference observed when TDNA was administered in mice [5]. We also demonstrated the potential of IM-EP in DNA vaccine dose reduction. Ten microgram of TDNA (2.5 μg/monovalent vaccine) generated the similar levels median NAb titers against all DENV when compared with the 100 μg TDNA dose. Whether such an efficient delivery system can significantly reduce the cost of DNA vaccine and can

183 increase the accessibility of dengue vaccine in most resource-limited countries and
184 warrants further investigation.

185 The dengue-specific NAb induced by this TDNA vaccine candidates was
186 detectable at least up to approximately 3 months from the last immunization. The
187 other study demonstrated that NAb induced by DNA vaccine encoding prME can
188 persist until 30 weeks [5]. In addition, our study demonstrated that homologous DNA
189 vaccine itself can be efficiently used as a boosting vaccine. This approach has been
190 shown to be promising in a cancer DNA vaccine regimen [25]. It may be explained
191 by previous observations that DNA vaccine is capable of induction both memory B
192 and helper T cells [26,27]. Further study is warranted in non-human primate and
193 thereafter in clinical study whether this tetravalent dengue DNA vaccine can really
194 be used in a sequential boosting regimen i.e. before a rainy season in order to
195 enhance the recall the memory cells and NAb responses similar to the seasonal
196 influenza vaccine approach [28-31].

197 Nonetheless, the magnitudes of NAb responses cannot be compared
198 between studies dues to the difference in the neutralizing antibody assay
199 methodologies and reported unit,. Ramanathan et al. reported the range of NAb
200 responses of 400-1000 unit/ml when mice immunized with 10 μg of synthetic
201 consensus EDIII by using IM-EP [32]. Konishi et al. reported PRNT70 NAb titers of
202 40-80 in mice after 2 doses of 100 μg tetravalent DNA immunization by needle-free
203 injector. [5] In this study, the DNA/DNA prime/boost strategy induced median NAb
204 titers at 1280, 640, 640 and 1280 against DENV1, 2, 3 and 4, after the 4^{th} TDNA
205 immunization respectively. The induced NAb levels in this study were promising
206 when compared to the previous reports which showed protective efficacy in animal

after viral challenge.[33]. A recent study by Porter et al. demonstrated that tetravalent prME DNA formulated with Vaxfectin–adjuvant with NAb titers before challenge against DENV-2 at 105-390 significantly reduced viremia compared to control group [34] and this vaccine formulation is ongoing in phase I trial.

Recent evidences have raised the relevant of dengue viral strains to be used in vaccine design and PRNT-50 assay. A concern in matching between the strain used for constructing a vaccine and the recent circulating virus has been raised from a recent phase 2b study conducted in Thailand [35]. A very low protection rate against DENV-2 (9.2%) was observed. The authors hypothesized that the mismatch between the DENV-2 CYD vaccine and circulating DENV-2 might be the cause of failure to protect against DENV-2 infection. In addition, the impact of more recent versus old dengue viral isolates on the PRNT50 results has also been observed by Thaisomboonsuk et al. [36]. They found that when DENV-4 strain 1036 (genotype-2, isolated in 1976, Indonesia) was used to test with DENV-infected serum samples collected during the 2005-2006 endemic season, the detected PRNT50 titers were less than expected. In contrast, the titers increased by 4.2 folds when a more recent Thailand DENV-4 isolate strain C0036 (genotype-1, isolates in 2006, Thailand) was used.

Interestingly, our observation on dengue virus serotype 4 has further supported the critical consideration of viral strain to be used for vaccine design and for PRNT50 assay while developing a vaccine. The synthesized consensus dengue *prME* DNA sequence used in our candidate TDNA vaccine were based on the sequences of dengue viral isolates reported to the GenBank up to 2003, whereas the old DENV-4 strain 103 isolated from Indonesia in 1976 was used to test the post-

immunized murine sera. The PRNT50 titers were found to be very low; but a significantly increase of the titer in approaching to those other serotype NAb titers when the newer viral isolates strain C0036 (Thailand 2006) was used for the assay (see Figure 2). To investigate how close of our candidate DNA vaccine to the recent dengue isolates is, analyses the homology of E protein amino acid sequence were performed between the D4prME vaccine construct and DENV-4 strain 1036 or strain C0036. The D4prME in the DNA vaccine is only 96.8% (479/495 amino acid) homology to strain 1036 (Indonesia 1976), in contrast it is 99.2% (491/495 amino acid) homology to strain C0036 (Thailand 2006). The 16 amino acids difference within E protein between the D4prME vaccine construct and DENV-4 strain 1036 might be sufficient to alter the neutralization capacity of NAb, of which requires further investigation. This finding supports the use of most recent dengue viral strain for both vaccine immunogen design and neutralization assays to avoid such mismatching outcomes. However, this may affect the use of such vaccine in other geographical areas.

Taken together, this study has shown that the tetravalent dengue prME DNA vaccine, with a very high homology DNA sequence to recent dengue viral isolates, induced good neutralizing antibody responses in mice; and the tetravalent dengue DNA/DNA prime/boost strategy is promising and is currently being evaluated in non-human primates.

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Figures legend

Figure 1. (A) Dengue DNA vaccine construction. Humanized sequence of the consensus prME genes from each dengue serotypes were cloned into pCMVkan expression vector. ss: signal sequence; HCMV: human CMV promoter. **(B) Intracellular dengue proteins expression.** Vero cells were transfected with indicated dengue DNA vaccine constructs or infected with DENV. The transfected or infected cells were stained with DAPI, anti-flaviviruses E mAb (4G2) or anti-DENV NS1 antibody, and analyzed using fluorescence microscopy. **(C) Immunoblot analysis of secreted E protein**. Cell culture supernatants were collected at 24 hr post transfection or infection, and analyzed by employing anti-flavivirus E antibody (clone 4G2). Lanes 1-5, recombinant plasmid pCMVkanD1, -2, -3, -4 prME and pCMVkan empty vector; lane 6, DENV-2 strain 16681. M: protein marker.

Figure 2: NAb responses against reference viruses used in PRNT in mice immunized with TDNA. NAb activities in sera of individual mouse following an IM-EP immunization with 100 µg/dose (opened diamond) and 10 µg/dose (grey square) for three times were determined at week 4 after the third dose. Horizontal lines

represent the median PRNT50 titer for each group of mice (n = 5-6). ns: no statistically significant difference.

Figure 3: Kinetics of NAb responses following TDNA prime-boost immunization. NAb titers of individual mice sera (n = 6), against each of the four dengue serotypes were shown separately. Open circle and grey square represent the median PRNT50 titer with inter-quartile ranges for the TDNA group and the empty vector group, respectively. Arrows represent the injection of each dose of TDNA .

* indicates $p<0.05$.

References

1. WHO (2009) Dengue: guidelines for diagnosis, treatment, prevention and control: new edition. Geneva: World Health Organization.
2. Simasathien S, Thomas SJ, Watanaveeradej V, Nisalak A, Barberousse C, et al. (2008) Safety and immunogenicity of a tetravalent live-attenuated dengue vaccine in flavivirus naive children. Am J Trop Med Hyg 78: 426-433.
3. Guirakhoo F, Pugachev K, Zhang Z, Myers G, Levenbook I, et al. (2004) Safety and efficacy of chimeric yellow Fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J Virol 78: 4761-4775.
4. Apt D, Raviprakash K, Brinkman A, Semyonov A, Yang S, et al. (2006) Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen. Vaccine 24: 335-344.
5. Konishi E, Kosugi S, Imoto J (2006) Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. Vaccine 24: 2200-2207.
6. Holman DH, Wang D, Raviprakash K, Raja NU, Luo M, et al. (2007) Two complex, adenovirus-based vaccines that together induce immune responses to all four dengue virus serotypes. Clin Vaccine Immunol 14: 182-189.

7. Beckett CG, Tjaden J, Burgess T, Danko JR, Tamminga C, et al. (2011) Evaluation of a prototype dengue-1 DNA vaccine in a Phase 1 clinical trial. Vaccine 29: 960-968.
8. Morrison D, Legg TJ, Billings CW, Forrat R, Yoksan S, et al. (2010) A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naive adults. J Infect Dis 201: 370-377.
9. Clements DE, Coller BA, Lieberman MM, Ogata S, Wang G, et al. (2010) Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. Vaccine 28: 2705-2715.
10. Guy B, Barban V, Mantel N, Aguirre M, Gulia S, et al. (2009) Evaluation of interferences between dengue vaccine serotypes in a monkey model. Am J Trop Med Hyg 80: 302-311.
11. Kitchener S, Nissen M, Nasveld P, Forrat R, Yoksan S, et al. (2006) Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults. Vaccine 24: 1238-1241.
12. Durbin AP, Kirkpatrick BD, Pierce KK, Elwood D, Larsson CJ, et al. (2013) A single dose of any of four different live attenuated tetravalent dengue vaccines is safe and immunogenic in flavivirus-naive adults: a randomized, double-blind clinical trial. J Infect Dis 207: 957-965.
13. Anderson KB, Gibbons RV, Edelman R, Eckels KH, Putnak RJ, et al. (2011) Interference and facilitation between dengue serotypes in a tetravalent live dengue virus vaccine candidate. J Infect Dis 204: 442-450.
14. Sun W, Cunningham D, Wasserman SS, Perry J, Putnak JR, et al. (2009) Phase 2 clinical trial of three formulations of tetravalent live-attenuated dengue vaccine in flavivirus-naive adults. Hum Vaccin 5: 33-40.
15. Swaminathan S, Khanna N, Herring B, Mahalingam S (2013) Dengue vaccine efficacy trial: does interference cause failure? Lancet Infect Dis 13: 191-192.
16. Halstead SB (2013) Identifying protective dengue vaccines: Guide to mastering an empirical process. Vaccine: "In press".
17. Kutzler MA, Weiner DB (2008) DNA vaccines: ready for prime time? Nat Rev Genet 9: 776-788.

18. Raviprakash K, Porter KR, Kochel TJ, Ewing D, Simmons M, et al. (2000) Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. J Gen Virol 81: 1659-1667.
19. Thomas SJ, Eckels KH, Carletti I, De La Barrera R, Dessy F, et al. (2013) A phase II, randomized, safety and immunogenicity study of a re-derived, live-attenuated dengue virus vaccine in healthy adults. Am J Trop Med Hyg 88: 73-88.
20. Simmons M, Burgess T, Lynch J, Putnak R (2010) Protection against dengue virus by non-replicating and live attenuated vaccines used together in a prime boost vaccination strategy. Virology 396: 280-288.
21. Patel V, Valentin A, Kulkarni V, Rosati M, Bergamaschi C, et al. (2010) Long-lasting humoral and cellular immune responses and mucosal dissemination after intramuscular DNA immunization. Vaccine 28: 4827-4836.
22. Henchal EA, Gentry MK, McCown JM, Brandt WE (1982) Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am J Trop Med Hyg 31: 830-836.
23. Pulsawat P, Pitakpolrat P, Prompetchara E, Kaewamatawong T, Techakriengkrai N, et al. (2013) Optimization of a Der p 2-based prophylactic DNA vaccine against house dust mite allergy. Immunol Lett 151: 23-30.
24. Thomas SJ, Nisalak A, Anderson KB, Libraty DH, Kalayanarooj S, et al. (2009) Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: How alterations in assay conditions impact performance. Am J Trop Med Hyg 81: 825-833.
25. Brave A, Hallengard D, Gudmundsdotter L, Stout R, Walters R, et al. (2009) Late administration of plasmid DNA by intradermal electroporation efficiently boosts DNA-primed T and B cell responses to carcinoembryonic antigen. Vaccine 27: 3692-3696.
26. Mattapallil JJ, Douek DC, Buckler-White A, Montefiori D, Letvin NL, et al. (2006) Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. J Exp Med 203: 1533-1541.
27. Pasetti MF, Ramirez K, Resendiz-Albor A, Ulmer J, Barry EM, et al. (2009) Sindbis virus-based measles DNA vaccines protect cotton rats against respiratory measles: relevance of antibodies, mucosal and systemic antibody-

secreting cells, memory B cells, and Th1-type cytokines as correlates of immunity. J Virol 83: 2789-2794.
28. Eick-Cost AA, Tastad KJ, Guerrero AC, Johns MC, Lee SE, et al. Effectiveness of Seasonal Influenza Vaccines against Influenza-Associated Illnesses among US Military Personnel in 2010-11: A Case-Control Approach. PLoS One 7: e41435.
29. Johns MC, Eick AA, Blazes DL, Lee SE, Perdue CL, et al. Seasonal influenza vaccine and protection against pandemic (H1N1) 2009-associated illness among US military personnel. PLoS One 5: e10722.
30. Vesikari T, Pellegrini M, Karvonen A, Groth N, Borkowski A, et al. (2009) Enhanced immunogenicity of seasonal influenza vaccines in young children using MF59 adjuvant. Pediatr Infect Dis J 28: 563-571.
31. Cowling BJ, Ng S, Ma ES, Cheng CK, Wai W, et al. Protective efficacy of seasonal influenza vaccination against seasonal and pandemic influenza virus infection during 2009 in Hong Kong. Clin Infect Dis 51: 1370-1379.
32. Ramanathan MP, Kuo YC, Selling BH, Li Q, Sardesai NY, et al. (2009) Development of a novel DNA SynCon tetravalent dengue vaccine that elicits immune responses against four serotypes. Vaccine 27: 6444-6453.
33. Lima DM, de Paula SO, Franca RF, Palma PV, Morais FR, et al. (2011) A DNA vaccine candidate encoding the structural prM/E proteins elicits a strong immune response and protects mice against dengue-4 virus infection. Vaccine 29: 831-838.
34. Porter KR, Ewing D, Chen L, Wu SJ, Hayes CG, et al. (2012) Immunogenicity and protective efficacy of a vaxfectin-adjuvanted tetravalent dengue DNA vaccine. Vaccine 30: 336-341.
35. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet 380: 1559-1567.
36. Thaisomboonsuk B, Jarman R, Gibbons RV, Nisalak A, Klungthong C, et al. (2012) Considerations for changing PRNT Dengue 4 reference viruses: sub-optimal immunity to documented infections. International Journal of Infectious Diseases 16, Supplement 1: e113-e114.