ATTENUATION BIOMARKERS OF DENGUE 4 VIRUS STRAIN 1036 PDK40 INFECTIOUS CLONE

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Abstract. Dengue viruses (DENV) transmitted by *Aedes* mosquitoes cause hemorrhagic fever and shock syndrome in tropical and subtropical regions. Live-attenuated (LAV) DEN4V 1036 PDK40 showed small plaque size in LLC-MK2 cells at 37ºC, but not at 39.4ºC and produced low neurovirulence in suckling mice. The major limitations of LAV are genetic instability and reversion. In order to expand the tools available for dengue vaccine development, an infectious clone (IC) DEN4V 1036 PDK40 was constructed from LAV DEN4V 1036 PDK40. IC-DEN4V-1036-PDK40 demonstrated attenuated characteristics, namely, small/pinpoint plaque sizes, temperature sensitivity, low growth efficiency in *Ae. aegypti*, and low neurovirulence in suckling mice. These results indicate IC-DEN4V-1036-PDK40 fulfilled all *in vitro* and *in vivo* safety requirements and should be useful for development as a dengue or a backbone for *Flavivirus*-chimeric vaccines.

Keywords: dengue, vaccine, dengue virus, Gibson assembly, infectious clone, live attenuated vaccine

INTRODUCTION

Dengue virus (DENV) belongs to genus *Flavivirus*, family *Flaviviridae*, with four antigenically distinct DENV serotypes, namely, DEN1V, DEN2V, DEN3V and DEN4, each showing phylogenetically distinct genotype (Klungthong *et al*, 2008). DENV is transmitted to humans through the bite of infected *Aedes* mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus* (Pereira *et al*, 2017). Infection with any of the four DENV causes a wide spectrum of clinical features ranging from nearly asymptomatic, an undifferentiated febrile illness, dengue fever (DF), and dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (Chia *et al*, 2020). DENV annually affects 50-200 million people and leads to about 20,000 deaths (representing about 1-2.5% of severe dengue patients) in tropical and subtropical regions of the world (Guzman *et al*, 2016).

DENV is an icosahedral enveloped virus, 40-50 nm in size, containing an 11-kb positive-sense single-strand RNA (+ssRNA) genome with a single open reading frame (ORF) flanked by 5′- and 3′-untranslated regions, which upon translation is enzymatically cleaved into three structural and seven nonstructural proteins (Guzman *et al*, 2016). The former consists of capsid (C), precursor membrane

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(prM) and envelope (E) proteins, and the latter of NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.

A live-attenuated vaccine (LAV) for DEN2V 16681 PDK53 was previously used for immunization in the Americas (Vaughn *et al*, 1996) and in Thailand (Bhamarapravati and Yoksan, 1989; Rabablert *et al*, 2000). Concerns has been raised on the potential reactogenicity of replicating virus in LAV vaccine, particularly in immunocompromised individuals, as well as of genetic instability leading to a reversion of virulence and heat lability (Kinney and Huang, 2001). In order to address the issue of LAV genetic instability, infectious clones of LAV were constructed for use as a single vaccine or as backbones for chimeric LAV (Kinney *et al*, 1997; Huang *et al*, 2013; Bordat *et al*, 2015). Compared to parental virus, chimeric virus produces significantly smaller plaques, is more temperature sensitive in LLC-MK2 cells, and elicits higher murine neutralizing antibody titers against DEN1V (Huang *et al*, 2000). However, an optimal balance between attenuation and immunogenicity must be taken into consideration (Marchette *et al*, 1990).

In 1976, DEN4V 1036 was isolated from a patient with dengue fever (Angsubhakorn e*t a*l, 1988) and serially passaged in primary dog kidney (PDK) cells (Bhamarapravati and Sutee, 2000). DEN4V 1036 from passage 5 (PDK5) to 50 (PDK 50) showed small plaque sizes; however, DEN4V 1036 PDK5 to PDK30 demonstrated cytopathic effects in LLC-MK2 cells (Halstead and Marchette, 2003). DEN4V 1036 PDK48 was finally chosen for use as a candidate LAV (Bhamarapravati and Sutee, 2000). LAV DEN4V 1036 PDK48 showed small plaque size, temperature sensitivity at 39ºC in LLC-MK2 cells (Bhamarapravati and Sutee, 2000), low neurovirulence in ICR mice (Halstead and Marchette, 2003) and induced immunogenicity in Rhesus and Cynomolgus monkeys (Angsubhakorn *et al*, 1988). In a phase I clinical trial, LAV DEN4V 1036 PDK48 shows safety and induces immunogenicity in Thai volunteers (Bhamarapravati and Sutee, 2000; Rabablert *et al*, 2000).

In order to investigate the usefulness of DENV attenuated infectious clones as components of LAV, an infectious DEN4V 1036 PDK40 clone was constructed and phenotype characterized. The findings should indicate whether this clone could be a safe addition for vaccine development.

MATERIALS AND METHODS

Cell culture and viruses

LLC-MK2 cell line The National Institute for Biological Standards and Control, (NIBSC, Hertfordshire, England) and Vero cell line CCL-81, American Type Culture Collection (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO, NY), 10% fetal bovine serum (FBS) (GIBCO, NY) 2% L-glutamine (Hyclone, South Logan, UT), 1% sodium bicarbonate (Sigma-Aldrich, St Louis, MO), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO, NY) at 37ºC under an atmosphere of 5% CO_2. DEN4V 1036, isolated from an Indonesian child suffering from dengue fever in 1964, was serially passaged 40 times in primary dog kidney (PDK) cells (Yoksan *et al*, 1986). DEN4V 1036 and DEN4V 1036 PDK40 were propagated in monolayer of Vero cells grown in DMEM (GIBCO, NY) supplemented with 2% FBS (GIBCO, NY) at 37ºC under 5% CO_2 atmosphere for five days. Viral titers of DEN4V 1036 and LAV

DEN4V 1036 PDK40 were determined by plaque assay. Viral yields were ~1×10^5 plaque forming units (pfu)/ml.

***Aedes aegypti* rearing**

Ae. aegypti, F100 progeny from eggs oviposited by adults collected as larvae in Chachoengsao Province, Thailand, were fed with sugar meal (10% sucrose + 2% vitamin B) in cage (30×30×30 cm^3) at 28ºC, relative humidity 70-80%, and 16 hours of light and 8 hours of dark. Female three days *Ae. aegypti* were used for replication experiments.

RNA extraction and genome sequencing

Viral RNA was extracted from DEN4V 1036 PDK40 using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized employing Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). Thirteen fragments of cDNA were amplified using a Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) with 13 pairs of DEN4 walking primers (Christenbury *et al*, 2010) in a Tpersonel 48 thermal cycler (Biometra, Göttingen, Germany) as previously described (Chang *et al*, 2018). Amplicons were separated by 1% agarose gel-electrophoresis, stained with SYBR dye (Thermo Fisher Scientific, Waltham, MA), purified using a QIAquick Gel Extraction Kit (Qiagen), then directly sequenced (First BASE Lab, Selangor, Malaysia). DNA sequences were analyzed using BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html), with DEN4V 1036 (GenBank accession no. LQ250785) and DEN4V 1036 PDK48 (GenBank LQ250787) providing reference sequences.

Construction and transfection of infectious clone DEN4V 1036 PDK40

Three DNA fragments of DEN4V genome consisting of (i) fragment 1 (nt 1-4,071), (ii) fragment 2 (nt 4,043-9,064) and (iii) fragment 3 (nt 9,041-10,648) together with a fragment of RBC TA cloning vector (RBC bioscience, New Taipei City, Taiwan) were amplified using primer sets shown in Table 1. T7 promoter sequence (AATACGACTCACTATAGGG) was ligated upstream of fragment 1. M13F and M13R sequences were inserted as overlapping sequences between DEN4V genome and vector. All four fragments were assembled into a circular plasmid (NEBuilder HiFi DNA Assembly mastermix; New England Biolabs, Ipswich, MA) and transfected into *Escherichia coli* NEB10 beta (New England Biolabs, MA) by a heat shock method (Xiao and Lis, 1986). Transformants on Luria-Bertani (LB) agar (Becton Dickinson and Co, Franklin Lakes, NJ) supplemented with 100 μg/ml ampicillin (General Drugs House, Bangkok) were randomly selected and screened for inserts using colony PCR (Agrawal and Roy, 2008) and grown overnight in LB broth (Becton Dickinson and Co, Franklin Lakes, NJ) containing 100 μg/ml ampicillin (General Drugs House, Bangkok). Plasmids were extracted (QIAprep Spin Miniprep Kit; Qiagen), quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and subjected to DNA sequencing (First BASE Lab Selangor). Cloning errors were corrected by PCR-based site-directed mutagenesis (Thermo Fisher Scientific, Waltham, MA). Plasmid DEN4V-1036-PDK40 was linearized with *Sca*I-HF restriction enzyme (New England Biolabs, Ipswich, MA), *in vitro* transcribed employing a T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, Biolabs, WI) at 37ºC for 2 hours, purified and treated with DNase using QIAamp

Table 1
Primers used for amplification of the three DENV4 cDNA fragments (F1-3) a vector fragment (F4).

Fragment number	Primer name	Sequence (5′→3′)	Amplicon size (bp)	Position in DEN4V genome
F1	M13F-T7-1F	TGTAAAACGACGGCCAG**TAATACGACTCACTATAGGG**AGTTGTTAGTCTGTG	4,071	1-4071
	1036 4071R	TGAGTGCTGTTATTTCTACCCAATG		
2	1036 4043F	GTCTCATTGGGTAGAAATAACAG	5,022	4043-9064
	1036 9064R	TCTCTGCCAAACCAGTGATCTTC		
3	1036 9041F	TGAAGATCACTGGTTTGGCAGAG	1,608	9041-10648
	10648-M13-R	CAGGAAACAGCTATGACC*AGTACT* AGAACCTGTTGGATCAAC		
4	M13R-F	GGTCATAGCTGTTTCCTG	2,725	
	M13F-R	ACTGGCCGTCGTTTTACA		

Note T7 promoter sequence (bold): restriction site of *Sca*I (underlined italic).

Viral RNA Mini Kit and RNaseFree DNase Set (Qiagen), and modified with m^7Gppp5′N employing a Vaccinia Capping System (New England Biolabs, Ipswich, MA). Then one μg of 5′-capped RNA was transfected into Vero cells (1 × 10^5 cells) using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). Following an overnight incubation at 37ºC, DMEM containing 2% FBS (GIBCO, NY) was added and DEN4V-infected cells and supernatant were collected at day-10 post-transfection. Mock-transfected cells were treated as above but without plasmid. Rescued DEN4V, named IC-DEN4V-1036-PDK40, in supernatant was quantified by plaque assay (Tsai *et al*, 2015) and quantitative (q)RT-PCR (Alm *et al*, 2015).

Detection of DEN4V antigen by indirect immunofluorescence assay (IFA)

IC-DEN4V-1036-PDK40- and mock-infected Vero cells were spotted on slides, fixed with cold acetone and treated with mouse anti-DEN4V monoclonal antibody (1-H-10; ATCC, Manassas, VA) [1:100 dilution in phosphate-buffered saline pH 7.4 (PBS)], followed by goat fluorescein isothiocyanate-conjugated anti-mouse monoclonal antibody (Thermo Fisher Scientific, Waltham, MA) (1:50 dilution in PBS). Slides were mounted with 50% buffered glycerol and examined under a UV microscope (series BX60; Olympus, Hachioji-shi, Japan, 40x magnification).

Plaque assay of DEN4V-infected LLC-MK2 cells

Monolayer of LLC-MK2 cells was cultured in a six-well plate. IC-DEN4V 1036-PDK40-containing supernatant was 10-fold serially diluted with DMEM containing 2% FBS (GIBCO, NY) and 200 μl aliquot of each dilution was added to LLC-MK2 cells and incubated at 37ºC for 90 minutes. Negative control cells were treated with DMEM/2% FBS. Media

containing DMEM (GIBCO, NY), 2% carboxymethyl cellulose (Sigma-Aldrich St Louis, MO) and 0.02% neutral red was overlaid onto each well, which was incubated at 37ºC under 5% CO_2 atmosphere for a further seven days and plaque size and number were recorded as pfu/ml.

Temperature sensitivity of DEN4V replication in LLC-MK2 cells

Temperature sensitivity of viral replication was determined as published previously (Kinney *et al*, 1997). In brief, monolayer of LLC-MK2 cells were infected with DEN4V 1036 or IC-DEN4V-1036-PDK40-containing supernatant and incubated for eight days under 5% CO_2 atmosphere at 37ºC and 39ºC before viral titers were quantified by plaque assay as described above. Temperature sensitivity was defined by a viral titer reduction ≥100 folds at 39ºC compared to that at 37ºC (named shut-off temperature phenotype).

Replication kinetics in Vero cells

DEN4V 1036 or IC-DEN4V-1036-PDK40 added to Vero cells (1×10^6 cells) at a multiplicity of infection (MOI) of 0.01 were incubated at 37ºC under 5% CO_2 for 90 minutes. Then 4 ml aliquot of DMEM/2% FBS (GIBCO, NY) was added and further incubated at 37ºC. Supernatant was collected daily for eight days and kept at -80ºC until assayed. Viral titer was quantified by plaque assay in LLC-MK2 cells as described above.

Replication in *Ae. aegypti*

Female three-day old *Ae. aegypti* (n = 300) intrathoracically inoculated with 0.34 μl of DEN4V 1036 or IC-DEN4V-1036-PDK40 (100 pfu/dose) were reared at 28ºC for 14 days. Negative control mosquitoes were inoculated with DMEM/2% FBS (GIBCO, NY). Mosquitoes (n = 10) were harvested at Day 1, 3, 5, 7, 9, 11, and 14 post-incubation (dpi) and kept at -80ºC until assayed. Viral titer from mosquitoes were quantified by qRT-PCR as described below.

DEN4V quantification by TaqMan qRT-PCR

QRT-PCR was performed as previously described (Johnson *et al*, 2005). Each mosquito was crushed in 100 μl of PBS using a micropestle, centrifuged at 13,000 *g* for 10 minutes at 4ºC. Viral RNA was extracted (QIAamp viral RNA mini kit; QIAGEN) and amplified using KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix Kit (KAPA biosystems, Wilmington, MA) with DEN4V-specific forward primer (5'-TTGTCC TAATGATGCTGGTCG-3') and reverse primer (5'-TCCACCTGAGA CTCCTTCCA) and TaqMan probe (Cy5-TTCCTACTCCTACGCATCGCATTCCG-BHQ3) (GenScript Biotech Corporation Piscataway, NJ). Thermocycling was conducted in Chromo4 thermal cycler (Bio-Rad, Benicia, CA) as follows: 42ºC for 5 minutes, 95ºC for 5 minutes; followed by 40 cycles of 95ºC for 5 seconds, and 60ºC for 30 seconds. Each assay was performed in triplicate. Viral titer was quantified by comparison with DEN4V 1036 PDK40 standard curve and is reported as copies/ml.

Neurovirulence in suckling mice

Neurovirulence in suckling mice was evaluated as previously described (Kinney *et al*, 1997). In short, 3-day-old ICR mice (n = 15) were intracerebral inoculated with 20 μl of DEN4V 1036 or IC-DEN4V-1036-PDK40 (10^4 pfu/dose). For mock infection, mice were injected with DMEM/2% FBS (GIBCO, NY). Mice were observed for 21 days and average survival time and rate were calculated using Kaplan-Meier survival estimate (Rich *et al*, 2010).

The research protocol was approved by The Institutional Animal Care and Use Committee, the National Laboratory Animal Center, Mahidol University (protocol no. MUVS-2017-10-48).

RESULTS

IC-DEN4V 1036 PDK40 construct

IC-DEN4V 1036 PDK40 was constructed, firstly, using Gibson's assembly method (Fig 1) (Gibson *et a*l, 2009) to assemble three DEN4V 1036 PDK40 cDNA fragments and RBC TA cloning vector (Fig 2a) into a circular plasmid that was then used to transform *E. coli* NEB10 beta. Insert (10,648 bp) (Fig 2b) in recombinant plasmid was sequenced, revealing four missense mutations, which were subsequently corrected by site-directed mutagenesis to agree with that of PDK48 (GenBank accession no. LQ250787) (data not shown). Secondly, recombinant plasmid was linearized with *Sca*I-HF, insert sequence transcribed *in vitro* using T7 RNA polymerase, m^7Gppp5′ capped and transfected into Vero cells mediated by Lipofectamine 3000. At Day 10 post-transfection, presence of DENV in Vero cells were demonstrated by immunofluorescence [infected cells exhibiting bright green apple cytoplasmic fluorescence (Fig 3a)]. Rescued IC-DEN4V 1036 PDK40 in supernatant quantified by qRT-PCR revealed a viral titer of 1×10^5 copies/ml (Fig 3b).

Phenotypic characteristics of IC-DEN4V 1036 PDK40-infection *in vitro* and *in vivo*

IC-DEN4V-1036-PDK40 produced pinpoint plaques (≤1 mm) in LLC-MK2 cells, while DEN4V 1036 produced medium (2-3 mm) and large plaques (≥5 mm) (Fig 4). The infectious clone also exhibited a temperature sensitive phenotype demonstrated by complete

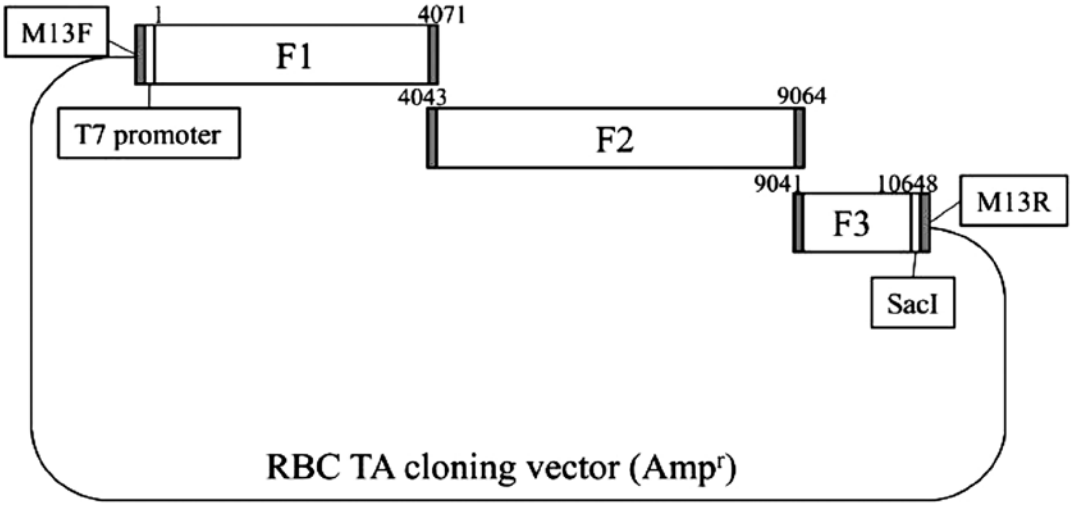

Fig 1-Schematic diagram of Gibson's construction of IC-DEN4V-1036-PDK40 infectious clone. Three fragments of dengue 4 virus (DEN4V) cDNA and one fragment of BBA TA cloning vector were assembled to construct recombinant pDEN4V-1036-PDK40. Full-length DEN4V insert is under control of T7 promoter for *in vitro* transcription. Gray bar: overlapping region between fragments.

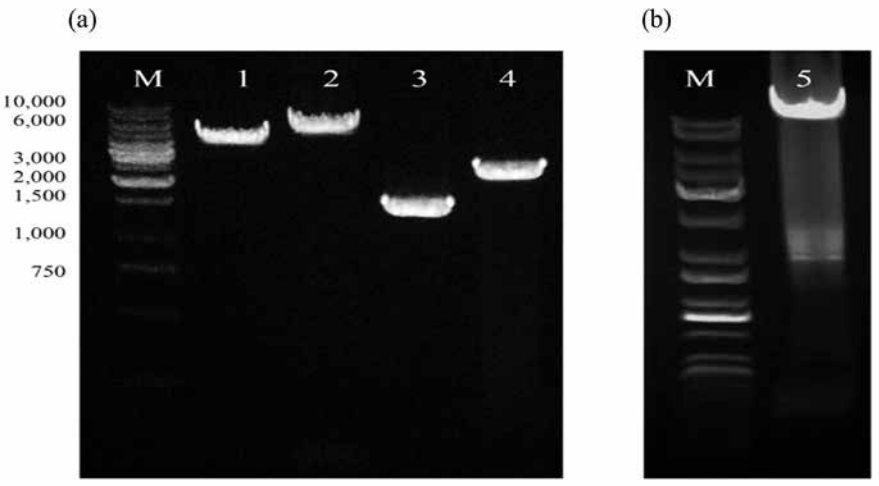

Fig 2-Two percent agarose gel-electrophoresis of (a) three cDNA fragments of dengue 4 virus (DEN4V) and one fragment of BBA TA cloning vector, and (b) linearized insert of recombinant pDEN4V-1036-PDK40. DEN4V fragments were amplified and that of BBA TA cloning vector using RT- and uniplex-PCR respectively. (a) Lane 1, DEN4V fragment 1 (4,071 bp); Lane 2, DEN4V fragment 2 (5,022 bp); Lane 3; DEN4V fragment 3 (1,608 bp); Lane 4, BBA TA vector fragment 4 (2,725 bp). (b) Lane 5, IC-DEN4V-1036-PDK40 (10,648 bp). Lane M, 100-10,000 bp DNA size markers.

inability to replicate in LLC-MK2 cells at 39°C while DEN4V 1036 had a 90% reduction of viral titer at 39°C compared to 37°C. IC-DEN4V-1036-PDK40 and DEN4V 1036 showed a peak titer of $2.3x10^7$ and $3.4x10^7$ pfu/ml at 7 dpi of Vero cells respectively, indicating that IC-DEN4V-1036-PDK40 and DEN4V 1036 exhibited similar replication pattern (Fig 5a). In *Ae. aegypti*, qRT-PCR revealed IC-DEN4V-1036-PDK40 peak titer of $1.3x10^7$ copies/ml at 11 dpi, which declined to $5.3x10^5$ copies/ml at 14 dpi, while DEN4V 1036 peak titer was $5.6x10^7$ copies/ml at 14 dpi, indicating a lower replication efficiency of the infectious clone (Fig 5b). The average survival time of IC-DEN4V-1036PDK40-infected suckling mice was 19 days and survival rate of 87% compared to 15 days and 47% respectively in DEN4V 1036 infection, attesting to the attenuated status of IC-DEN4V-1036PDK40 (data not shown). Infected mice presented inactivity, loss of appetite, tremor, unresponsiveness and eventually death.

DISCUSSION

In order to characterize a virus at the molecular and biological levels, it is necessary to produce an infectious clone. Current molecular tools that permit rapid genetic analysis should be employed for quality control to document the genetic authenticity of vaccine viruses. IC-DEN4V 1036 PDK40 constructed using a Gibson's assembly method contained four missense mutations that had to be corrected by site-directed mutagenesis. Phenotypic characteristics of DEN4V 1036 PDK40 infectious clone confirmed it attenuated status.

Plaque size is primary selection criterion in DENV vaccine development

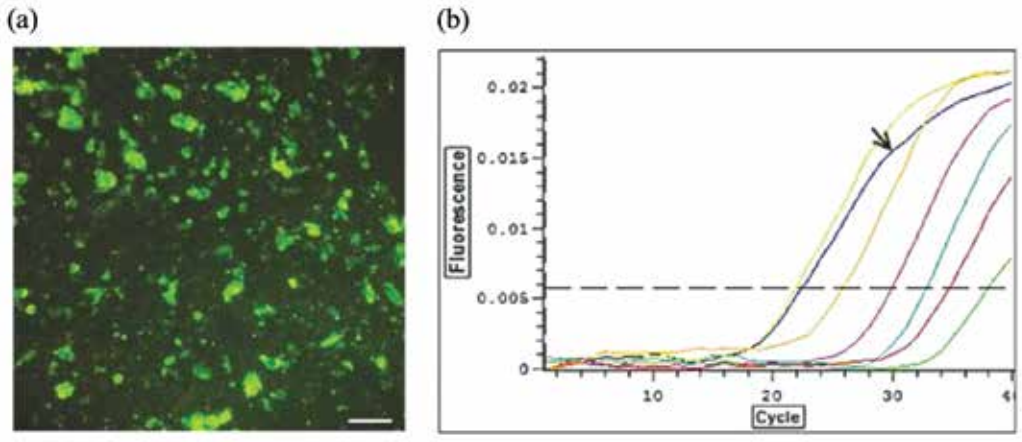

Fig 3-Detection of dengue 4 virus (DEN4V) in Vero cells at day 10 post-transfection by (a) immunofluorescence assay (IFA) and (b) quantitative (q)RT-PCR). (a) IC-DEN4V-1036-PDK40-infected Vero cells were fixed with cold acetone and treated with mouse anti-DEN4V monoclonal antibody followed by goat fluorescein isothiocyanate-conjugated anti-mouse monoclonal antibody and examined under a UV microscope (40x magnification). Scale bar = 100 μm. (b) DEN4V in infected cell supernatant was quantified using TaqMan RT-PCR performed for 40 cycles. Arrow indicates DEN4V-positive supernatant sample (black line). Amplification curves of known amounts of DEN4V are in yellow (1×10^5 copies/ml), orange (1×10^4 copies/ml), pink (1×10^3 copies/ml), blue (1×10^2 copies/ml), and red (1×10^1 copies/ml). Dash horizontal line indicates fluorescence detection threshold.

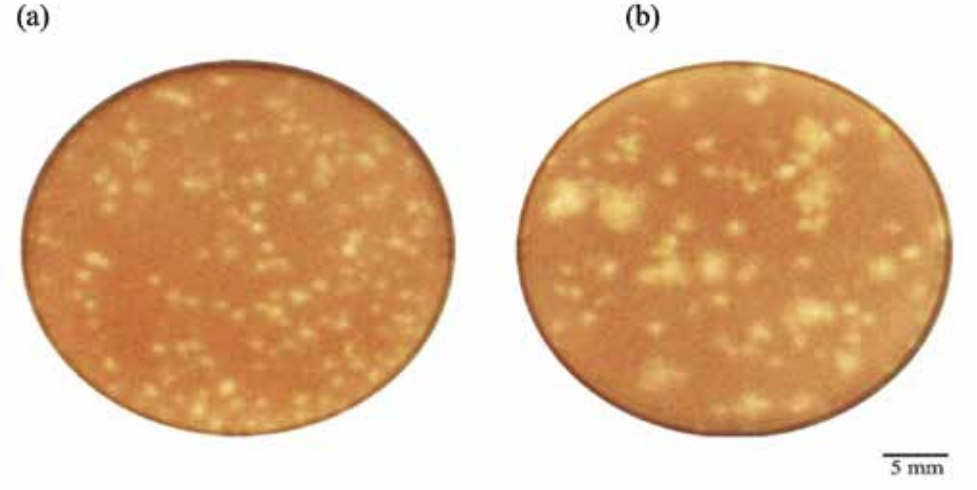

Fig 4-Plaque sizes of (a) IC-DEN4V-1036-PDK40 and (b) DEN4V 1036 in LLC-MK2 cells. IC-DEN4V 1036-PDK40- or DEN4V 1036-containing culture supernatant was added to monolayer of LLC-MK2 grown in Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum, 2% L-glutamine, 1% sodium bicarbonate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C under a 5% CO_2 atmosphere for 90 minutes, then overlaid with DMEM containing 2% carboxymethyl cellulose and neutral red and further incubated for seven days.

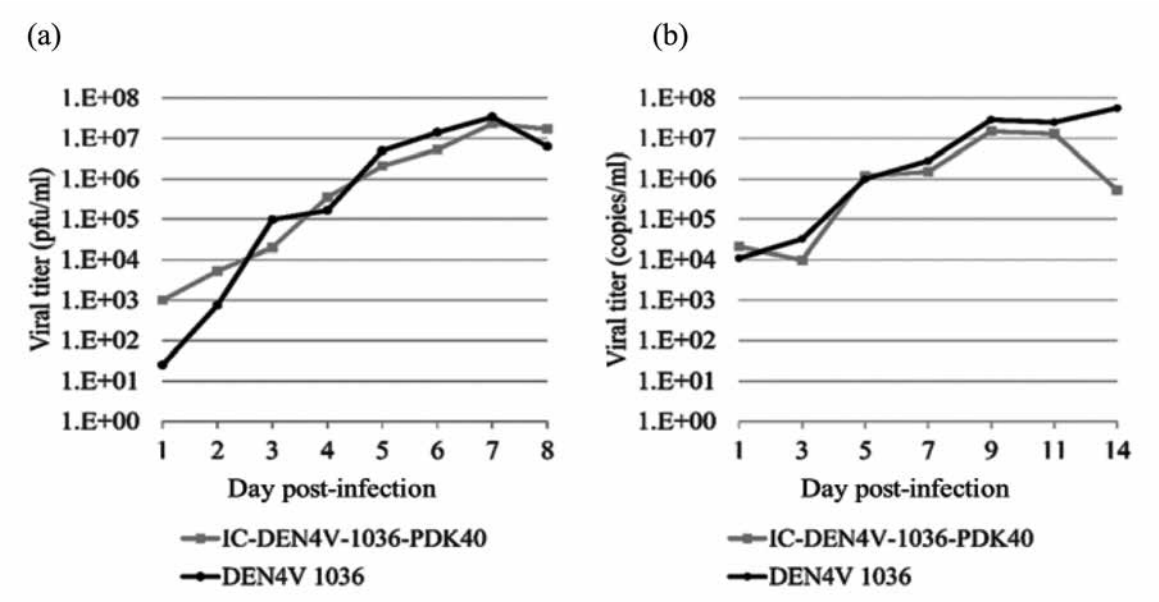

Fig 5-Kinetics of IC-DEN4V-1036-PDK40 and DEN4V 1036 replication in (a)Vero cells and (b) *Aedes aegypti*. (a) Dengue virus (DENV)-infected Vero cells were incubated in supplemented Dulbecco's modified Eagle medium for eight days and supernatant viral titer was determined by a plaque assay as described in legend to Fig 4. (b) Female three-day old *Ae. aegypti* (n = 30) intrathoracically were inoculated with 100 pfu/dose dengue virus and reared at 28ºC for 14 days. Viral titer in mosquitoes (n = 10 harvested on Days 1, 3, 5, 7, 9, 11, and 14 post-incubation were quantified by TaqMan qRT-PCR as described in legend to Fig 3. Y-axis indicates $\log_{10}$(mean value) of experiment conducted in triplicate.

(Eckels *et al*, 1976), where smaller plaque sizes indicate attenuated virus (Goh *et al*, 2016). Smaller plaque size from IC-DEN4V-1036-PDK40 is consistent with previous reports in which serial passages of DEN4V 1036 in PDK cells result in small plaque size in PDK5 to PDK 10, PDK20 and PDK30, but not PDK50 (Yoksan *et al*, 1986; Halstead and Marchette, 2003).

Temperature sensitivity at 39-41ºC is another crucial biomarker for LAV DENV vaccine (Eckels *et al*, 1976) as DENV infected patients commonly present with temperatures of 39-41°C. It was reassuring IC-DEN4V-1036-PDK40 failed to replicate in LLC-MK2 cells as previous reported LAV DEN4 (Yoksan *et al*, 1986; Halstead and Marchette, 2003).

Vero cell line is widely accepted for use in vaccine development as propagation of live-attenuated viruses in these cells maintains the desirable biologic properties of viruses derived during serial passage in PDK cells (Halstead and Marchette, 2003). Replication of IC-DEN4V-1036-PDK40 in Vero cells was comparable to DEN4V 1036 at the same MOI. Rapid replication of LAV in HuH-7 cells was previously observed with DEN2V 16681 PDK53 (Kinney *et al*, 1997; Goh *et al*, 2016). On the other hand, LAV DEN3V 16562 PGMK30 has a lower replication efficiency than DEN3V 16562 in HuH-7 cells (Goh *et al*, 2016). The cause for association between replication

efficiency and attenuation is still unclear, but a high replication efficiency of IC-DEN4V-1036-PDK40 in Vero cells would be of advantage for vaccine production as high viral titers can be achieved.

Replication efficiency in the mosquito vector determines the capability of DENV to infect the host. Crippled replication of LAV DENV in mosquito is a desirable trait to ensure a low probability of transmission of LAV virus (Kinney and Huang, 2001). Route of inoculation is also an importance factor in LAV DENV replication in mosquito vector. Artificial oral transmission models showed a restricted capability to infect and disseminate LAV DENV in *Ae. aegypti* and *Toxorynchites splendens* (Khin *et al*, 1994; Jirakanjanakit *et al*, 1999). Reduced efficiency of IC-DEN4V-1036-PDK40 replication in *Ae. aegypti* via intrathoracic inoculation is consistent with that of LAV DEN2V 16681 PDK53 (Khin *et al*, 1994), LAV DEN3V 16562 PGMK30 FRhL3 (Jirakanjanakit *et al*, 1999) and chimeric yellow fever/DENV (CYD) 1-4 (Johnson *et al*, 2004).

All *Flavivirus* vaccine candidates should be evaluated for neurovirulence in suckling mice to provide a surrogate predictive of tests in monkeys (Monath *et al*, 2005). Improved survival rate and time of IC-DEN4V-1036-PDK40 compared to DEN4V 1036 inoculated suckling mice was consistent with the report of Yoksan *et al* (1986) using LAV DEN4V 1036 PDK40.

Each DENV serotype consists of 4-5 genotypes (Klungthong *et al*, 2008), and thus co-circulation of different serotypes provides a broad spectrum of disease symptoms and host immunity. DEN4V genotype I is predominant in Thailand over many decades [Genbank data (http://www.ncbi.nih.nlm.gov/)].

Many strains of DEN4V genotype II have been isolated from both humans and *Ae. aegypti* mosquitoes in Thailand since 2013 (Kittichai *et al*, 2015), and phylogenetic analysis revealed DEN4V 1036 is categorized as genotype II strain (Gallichotte *et al*, 2018; Yoksan *et al*, 2018).

Ideally, a dengue vaccine candidate should provide sufficient protection against every DENV serotype/genotype. Of note, a tetravalent vaccine chimeric yellow fever/DENV (CYD) 1-4 elicited seroneutralizing antibodies protecting *Cynomolgus macaque* against all known DENV serotypes and genotypes irrespective of geographic origin or year of isolation (Barban *et al*, 2012).

In summary, infectious dengue virus clone IC-DEN4V-1036-PDK40 shows attenuated properties, namely, small plaque size, temperature sensitivity, low growth in *Ae. aegypti* and low neurovirulence in suckling mouse model. This infectious may be used as vaccine or a backbone for construction of chimera vaccine. Nevertheless, further studies are required on IC-DEN4V1036-PDK40 immunogenicity *in vivo* (adult mice and monkey), identification of mutations responsible for attenuation and construction of IC-DEN4V-PDK40-based DENV chimera.

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