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Emergence Potential of Sylvatic Dengue Virus Type 4 in the Urban Transmission Cycle is Restrained by Vaccination and Homotypic Immunity

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Abstract

Sylvatic dengue viruses (DENV) are both evolutionarily and ecologically distinct from human DENV and are maintained in an enzootic transmission cycle. Evidence of sylvatic human infections from West Africa and Southeast Asia suggests that sylvatic DENV come into regular contact with humans. Thus, this potential of emergence into the human transmission cycle could limit the potential for eradicating this cycle with vaccines currently in late stages of development.

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We assessed the likelihood of sylvatic DENV-4 emergence in the face of natural immunity to current human strains and vaccination with two DENV-4 vaccine candidates. Our data indicate homotypic neutralization of sylvatic and human DENV-4 strains by human primary convalescent and vaccinee sera but limited heterotypic immunity. These results suggest that emergence of sylvatic strains into the human cycle would be limited by homotypic immunity mediated by virus neutralizing antibodies produced by natural infection or vaccination.

Keywords

Dengue virus (DENV); sylvatic DENV; human DENV; vaccine; antigenic relationships; plaque reduction neutralization test (PRNT)

Introduction

Dengue viruses (DENV) are arthropod-borne viruses (arboviruses) in the genus *Flavivirus* (family *Flaviviridae*) that utilize *Aedes (Stegomyia)* spp., primarily *Ae. aegypti* and to a lesser degree *Ae. albopictus*, as vectors for transmission in urban and peri-urban settings (urban transmission cycle). In Southeast Asia and West Africa, DENV are also transmitted in an enzootic cycle between non-human primates and arboreal *Aedes* spp. mosquitoes. There are four antigenically distinct but genetically related serotypes (DENV-1, -2, -3 and -4) within the dengue (DEN) antigenic complex (Calisher et al., 1989). Unlike most arboviruses, DENV are extremely restricted in their natural vertebrate host range, which most likely includes only primates. Currently, all four DENV serotypes can be found in nearly all urban and peri-urban tropical and subtropical environments where *Ae. aegypti* is present. By current estimates this distribution puts over half of the global human population at risk for infection. The impact of DENV infections on human health is enormous; over 200 million infections and 2 million cases of of dengue hemorrhagic fever (DHF) occur each year, with a case fatality rate of up to 5% (Kyle and Harris, 2008). Most profoundly, the majority of the DEN-associated disease in hyperendemic regions is borne by children (Clark et al., 2005; Mathers et al., 2007; Witayathawornwong, 2005), although recent evidence from Southeast Asia and Latin America suggests that adults are also at high risk (Fox et al., 2011; Guilarde et al., 2008; Hanafusa et al., 2008; Koh et al., 2008; Siqueira et al., 2005; Wichmann et al., 2004), particularly in urban areas that are transitioning or have already transitioned to hyperendemicity.

Phylogenetic (Chen and Vasilakis, 2011; Rico-Hesse, 1990; Twiddy et al., 2003; Vasilakis et al., 2008b; Wang et al., 2000) and ecological studies (Cordellier et al., 1983; Hervy et al., 1984; Monlun et al., 1992; Roche et al., 1983; Rudnick, 1965; Rudnick, 1978, 1984, 1986; Smith, 1956, 1958) indicate that the ancestral sylvatic DENV are both ecologically and evolutionarily independent from the current endemic DENV circulating within urban transmission cycles. However, data from West Africa and Southeast Asia suggest that sylvatic DENV come into regular contact with humans. For example, in West Africa the gallery forest-dwelling mosquito, *Ae. furcifer,* which is highly susceptible to sylvatic DENV infection (Diallo et al., 2005), disperses into villages and may be responsible for sylvatic DENV infection of humans (Diallo et al., 2003; Fagbami et al., 1977). In Southeast Asia, *Ae. albopictus* may transfer sylvatic DENV from the forest into human habitats (Rudnick, 1986). Sylvatic DENV infection can cause human disease in both rural peridomestic and urban settings as documented by spillover epidemics (Carey et al., 1971; Vasilakis et al., 2008c), and human infections in West Africa (Franco et al., 2011; Monlun et al., 1992; Robin et al., 1980; Saluzzo et al., 1986) and Southeast Asia (Cardosa et al., 2009). Clinical illness due to sylvatic DENV not only is indistinguishable from classic dengue fever (DF), but also has the potential to progress to severe disease (Cardosa et al., 2009; Franco et al.,

2011). Because the only means to determine whether the DENV strain causing human infection is from the sylvatic or the urban transmission cycle is by sequencing the virus genome, human infection by sylvatic strains is often misclassified etiologically as dur to urban strains.

Collectively, these observations combined with: (i) experimental evidence in models of human (Vasilakis et al., 2007) and mosquito infection (Diallo et al., 2008; Diallo et al., 2005) (Hanley and Vasilakis, unpublished data) that adaptation is not required for urban transmission; and (ii) the continuing risk of DENV emergence from the sylvatic cycle resulting in human infection, which can manifest as symptomatic dengue disease, suggest that sylvatic dengue spillover may occur at a greater frequency than is currently recognized. A recent study showed broad neutralization by vaccinated monkey serum against a large panel of DENV-1–4, including a DENV-2 sylvatic strain (Barban et al., 2012). Our earlier study (Vasilakis et al., 2008a), evaluating the neutralization capacity of convalescent sera collected from dengue vaccine recipients and from DENV-2 and DENV-3 patients following primary infection, also suggested that the emergence of sylvatic DENV strains into the human transmission cycle would be limited by homotypic humoral immunity.

In this study, to assess the likelihood of current sylvatic DENV-4 re-emergence (emergence of extant sylvatic DENVs into the human transmission cycle) in the face of immunity induced by current urban-circulating DENV strains or vaccine candidates, we tested sera collected from DENV-4 vaccine recipients and convalescent-phase sera from DENV-infected patients against geographically and genetically diverse sylvatic and human DENV-4 strains (Table 1).

Results and discussion

Neutralization studies of human vaccinee sera

For vaccination to achieve effective immunity against DEN disease, the immune response must be effective against all four DENV serotypes and must be long-lived. Natural infection with DENV results in lifelong protection against reinfection with the same serotype (Halstead, 1974; Okuno et al., 1983; Papaevangelou and Halstead, 1977; Rosen, 1986; Sabin, 1952; Tadano et al., 1983) but only in transient cross-protection against heterotypic serotypes (Guirakhoo et al., 2006; Sabin, 1952). Homotypic immunity is mediated by neutralizing antibodies directed mainly against the viral envelope (E) protein (Crill and Roehrig, 2001; Kaufman et al., 1987; Roehrig et al., 1998) and other minor antigens located in the viral membrane [(M) (Kaufman et al., 1989) and nonstructural 1 (NS1) (Schlesinger et al., 1986) proteins] as well as by cell-mediated immunity. However, heterotypic immunity is associated with cross-reactive neutralizing antibodies to E and prM that decline rapidly after infection (Pengsaa et al., 2006; Sabin, 1952).

Previously, we assessed the likelihood of current sylvatic DENV re-emergence in the face of immunity to current vaccine candidates, including the DENV-4 candidate vaccine rDEN4Δ30, by evaluating the neutralizing capacity of sera from DENV4Δ30 vaccinees against reference sylvatic and human DENV-4 strains (Vasilakis et al., 2008a). Here, we assessed the potential of all available sylvatic DENV-4 strains to re-emerge in the face of immunity to an expanded set of human strains, as well as the more attenuated DENV-4 rDEN4Δ30-200,201 and rDEN4Δ30 vaccine candidates, by evaluating the neutralizing capacity of sera from human DENV vaccinees and convalescent patients from Puerto Rico, Mexico, Singapore, Sri Lanka, the West Indies, Honduras, and Thailand.

Sera collected at 42 days post-vaccination from 7 and 10 placebo-controls participating in the clinical trials evaluating rDEN4Δ30-200,201 and rDEN4Δ30, respectively, were unable

to neutralize any DENV tested ($PRNT_{60}$ < 20). Sera collected from all rDEN4Δ30-200,201 vaccinees were previously shown to neutralize the parent DENV-4 strain 814669 (McArthur et al., 2008) and were not retested in the current study. Overall, sera from 16/19 rDEN4Δ30-200,201 vaccinees neutralized some or all of the DENV-4 strains. Weak to modest ($PRNT_{60}$ = 20 – 160) homotypic neutralization was exhibited by vaccinee sera (Table 2). Sera from 13/19 vaccinees neutralized all of the DENV-4 tested, while sera from three additional vaccinees neutralized all viruses tested except for the India G11337 (3 test sera 214.01.14, 214.01.16, 214.01.18) and the H241 strains (test serum 214.01.14). Weak to modest ($PRNT_{60}$ = 20 – 160) homotypic neutralization responses were exhibited by sera collected at 42 days post vaccination (Table 2). The levels of homotypic neutralization further decreased 180 days post vaccination ($PRNT_{60}$ = 20 – 40) (data not shown). Similarly, sera collected from 21/22 persons vaccinated with rDEN4Δ30 neutralized some (14) or all (7) of the DENV-4 tested, and several sera from other persons were unable to neutralize human or sylvatic DENV-4 strains (Table 3). The homotypic neutralization responses exhibited by sera collected 42 days after vaccination ranged from weak to strong ($PRNT_{60}$ = 20 – >640) (Table 3) (sera collected 180 days post vaccination were not tested) and were collectively more potently neutralizing than sera from the rDEN4Δ30-200,201 vaccinees (mean log titers 1:40 and 1:28, respectively, P=0.0163), consistent with greater attenuation of the rDEN4Δ30-200,201 virus. Sera from all but two persons vaccinated with either rDEN4Δ30-200,201 or rDEN4Δ30 failed to neutralize heterotypic human DENV-1 – 3 or sylvatic DENV-2 ($PRNT_{60}$ < 20) (Table 2 – 3). Serum from patient JHU02 exhibited a strong heterotypic response to the sylvatic DENV-2 DKD811 strain (Cardosa et al., 2009). Interestingly serum from one vaccinated subject (JHU 22) exhibited a modest to relatively strong heterotypic neutralizing response to DENV-1 and - 3 ($PRNT_{60}$ = 40 – >640) and a robust neutralization titer (>1280) to DENV-2, suggesting a secondary antibody response (previous DENV-2 infection), or possibly an inapparent West Nile virus infection and ‘original antigenic sin’ (Halstead et al., 1983; Kuno et al., 1993). However, there were stringent eligibility criteria for this trial, including absence of prior exposure to flaviviruses (Durbin et al., 2006), and this volunteer was previously found to be seronegative by PRNT to DENV-1 – 4, yellow fever (YFV) and WNV and by hemagglutination-inhibition assay to Saint Louis encephalitis virus (SLEV), WNV, and Japanese encephalitis virus (JEV) prior to vaccination. Furthermore, it is very unlikely that this volunteer (JHU 22) would have been exposed to any flavivirus other than WNV because he/she was a Baltimore resident with no travel history to any DENV-endemic countries. We reported a similar observation previously (Vasilakis et al., 2008a). The broad heterotypic response after vaccination or previous, unknown exposure could be due to antigenic mismatch between the vaccine and DENV strains used for the PRNT (Lanata et al., 2012; Sabchareon et al., 2012).

The vaccinee sera offer a unique opportunity to explore the relationship between neutralization and DENV genetic diversity. Because the sequences are known for the vaccine strain and for the endemic and sylvatic strains used in the assays, it is possible to test the relationship between differences in neutralization titers of sera and genetic differences among the viruses used in the assays. Using published sequences for the rDEN4Δ30 vaccine strain and the endemic and sylvatic strains (Table 1), genetic p-distances between the amino acid sequences of the structural proteins were calculated. As expected, the structural proteins of rDEN4Δ30 were much more similar to those of the endemic DENV-4 strains than to that of the sylvatic E glycoprotein, with nearly 100% amino acid sequence identity between the vaccine strain and its parent 814669, followed by INH6412 (99.4%), Haiti73 (99.3%), IndiaG11337 (97.3%) and H241 (97.0%). The sylvatic strains’ structural proteins were all 95% identical to the vaccine strain. These distances were plotted against the log $PRNT_{60}$ titers for each vaccine recipient and subjected to regression analysis. For the rDEN4Δ30 recipients, 18 of the 22 sera demonstrated a positive relationship between the genetic relatedness of the neutralized virus to the vaccine strain and

the neutralization titer and, of these, 11 of the 18 were statistically significant ($P<0.05$). The only sera that failed to show a relationship were JHU 5 and JHU 11 (non-neutralizing sera), the weakly neutralizing sera JHU 2, and the potently neutralizing sera JHU 6.

Of note, the less potently neutralizing rDEN4Δ30-200, 201 vaccinee sera did not show a consistent relationship between genetic distance and neutralization titer. Of the 19 sera tested, only 11 showed a positive relationship between neutralization titer and genetic distance, and of these 11, only 2 had a statistically significant correlation (214.01.01 and 214.01.13). This likely is because of the overall lower antibody titers induced by these more attenuated vaccines. This explanation is supported by the observation that these vaccinees showed reduced homotypic neutralization at 180 days post vaccination.

Neutralization studies of human primary convalescent sera

We also assessed the ability of convalescent sera collected from primary DENV-4 pastients to neutralize sylvatic and endemic DENV-4, (Table 4), including (i) sera obtained 9–21 days (Puerto Rico and Singapore) or up to a year (Mexico) after onset (Table 4), as part of routine national surveillance programs, (ii) very late convalescent sera (defined as those collected >2 years after documented DENV infection) obtained as part of an ongoing dengue in traveler study (French West Indies and Honduras) and, (iii) anonymous sera from a Sri Lankan blood bank (length of convalescence unknown) and a reference serum collection (Thailand). Primary DENV infections were defined by the capture IgM:IgG ratio during the acute phase of disease (0–5 days after onset of symptoms) (Falconar et al., 2006) or by a monotypic neutralization profile on late convalescent sera. Because a limited volume of DENV-4 primary convalescent sera was available, we were unable to evaluate their neutralization capacity with a large collection of DENV-4 strains. Of the early convalescent Puerto Rico sera, all but one (PR-A02 with India G11337 strain), robustly neutralized of both endemic and sylvatic DENV-4 ($PRNT_{60}$ 40 – >1280) (Table 5). Sera obtained from Puerto Rico and Singapore exhibited weak to modest heterotypic neutralization of endemic DENV-1 – 3 or sylvatic DENV-1 and -2 ($PRNT_{60}$ = 20 – 320), except serum PR-A04, which strongly neutralized the sylvatic DENV-1 strain ($PRNT_{60}$ >1280). Several sera exhibited no heterotypic virus neutralization of endemic DENV-1 – 2 or sylvatic DENV-2 strains ($PRNT_{60}$ < 20) (Table 5). Notably, both sera from Mexico weakly neutralized both endemic and sylvatic DENV-4 ($PRNT_{60}$ 20 – 40), but not endemic DENV-1 – 3 or sylvatic DENV-1 and -2 ($PRNT_{60}$ < 20), whereas one serum exhibited a relatively strong virus neutralizing activity against sylvatic DENV-1 ($PRNT_{60}$ = 160)(Table 5). Because these sera were collected several months after infection (Table 4), the lack of heterotypic neutralization is consistent with transient heterotypic cross-protection after infection. The relatively robust heterotypic responses observed in PR-A04 and MX-01 samples suggest that the patients had a previously undiagnosed, heterologous DENV infection. This concept was proposed by Halstead (Halstead et al., 1983) as a mechanism of DENV immune response involved in sequential infections. In such instances, the immune response to a secondary infection is dominated by the proliferation of cross-reacting memory cells induced by the primary infection, which may be of lower affinity for the secondary challenging antigen.

The late convalescent sera DT100 (West Indies, 4 years post acute infection) and DT102 (Honduras, 2 years post acute infection) showed that durable and broad homotypic neutralization can persist several years after infection. The blood donor sera DV002 and GS58, both from Sri Lanka, showed a less robust but equally broad neutralization against both endemic and sylvatic viruses. The DENV-4 reference sera, SS06/105, 302 and SS07/333 were interesting in that they were broadly neutralizing but had as much as a 16-fold difference in neutralization titers between endemic and sylvatic DENV-4. This suggests that, while natural infection induces broad intraserotypic neutralization, there can be

significant variation within that serotype, underscoring the role of genotype-specific epitopes in antibody mediated protection.

Our results demonstrate the ability of convalescent sera collected after primary DENV-4 infection as well as from DENV-4-vaccinees to neutralize genetically diverse DENV-4. The observed protection is mainly due to homotypic immunity, and consistent with Sabin's observations, we observed only a transient heterotypic immunity. Sera from human vaccinees collected at 42 and 180 days following vaccination with rDEN4Δ30-200,201 or at day 42 after rDEN4Δ30 vaccination exhibited limited to no heterotypic immunity. Furthermore, our rDEN4Δ30-200,201 data corroborate the reduced levels of neutralization titers observed previously, attributed to the greater attenuation of this candidate (McArthur et al., 2008).

Several investigators have suggested that amino acid differences in the lateral ridge of domain III of the E glycoprotein (Cockburn et al., 2012; Endy et al., 2004; Wahala et al., 2010; Zulueta et al., 2006) are responsible for variable intra-serotypic neutralization. For the viruses in this study, there are thirteen variable amino acid positions on the E glycoprotein that are conserved between the endemic and sylvatic strains. Five are in domain I, only one occurs in domain II, and the remaining eight residue differences are in domain III, of which only two occur along the lateral ridge (A329T and V382A). It is unlikely that these two polymorphisms alone would account for differences in neutralization. Moreover, recently published research suggests the flavivirus targets of human antibodies are likely to be non-EDIII or quaternary epitopes that potentially involve residues in multiple domains in variable neutralization (de Alwis et al., 2012).

As noted above, within-serotype neutralization varied broadly. For the rDEN4Δ30 vaccinee sera, neutralization titer was significantly correlated with genetic distances between the vaccine strain and the tested strains in half the sera tested. Additionally, the $PRNT_{60}$ titers of recent convalescent sera against endemic and sylvatic strains did not vary significantly for most samples.

Collectively, our data suggest that re-emergence of sylvatic DENV-4 into the human transmission cycle may be limited by homotypic humoral immunity, induced either by vaccination or prior natural DENV-4 infection. The potential licensing of effective DENV vaccines raises the prospect of control or even eradication of the human transmission cycle, which relies solely on human-mosquito-human transmission. However, sylvatic transmission cycles are not amenable to control by human vaccination and epidemiological evidence suggests that sylvatic DENV come into regular contact with humans and can cause severe disease (Cardosa et al., 2009; Franco et al., 2011) or transient spill-over to urban settings (Carey et al., 1971; Vasilakis et al., 2008c). Furthermore, recent experimental evidence indicates a low to non-existent adaptive barrier for the emergence of sylvatic DENV into the human population (Vasilakis et al., 2007), implying that re-emergence is a clear and present danger. Therefore, even as efforts to control circulation of human dengue intensify in a manner analogous to historic efforts to control urban yellow fever, reduction and ultimate eradication of dengue from human populations solely due to vaccination campaigns could be, at best, short-lived. The urban yellow fever experience suggests that successful eradication of dengue will hinge on sustained vaccination of susceptible populations, including those who are at risk from the introduction of sylvatic DENV, with potently immunogenic and long-lasting vaccines, as well as control of the urban mosquito vectors.

Material and Methods

Ethical considerations

Written consent to participate in the study was obtained from each subject enrolled in the dengue vaccine studies. All data were handled anonymously and confidentially. The studies were conducted at the Center for Immunization Research (CIR) at the John Hopkins Bloomberg School of Public Health under an investigational new drug application for both rDEN4Δ30-200,201 (BB-IND 12670) and rDEN4Δ30 (BB-IND 12977). The University of Texas Medical Branch Institutional Review Board reviewed the study as protocol UTMB-IRB#10-047. Human sera from Singapore were from the early dengue infection and outcome study (Low et al., 2006), and were collected following written informed consent and approved by the National Healthcare Group Domain Specific Review Board (DSRB 5B/05/013). Human sera from Puerto Rico, and Mexico, obtained from routine surveillance, and sera from US travelers were de-identified and approved for this research study under IRB exemption 4797 at the CDC, CI986-L62 Mexico and 08-0895 UNC, respectively.

Cell Cultures

Monolayer cultures Vero cells (obtained from American Type Culture Collection, Bethesda, MD) were grown at 37°C in Dulbecco's minimal essential medium (DMEM) (4.5 g/L D-Glucose) with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). C6/36 mosquito cells (a generous gift from Ilya Frolov) were grown at 28°C in Dulbecco's minimal essential medium (DMEM) (4.5 g/L D-Glucose) with 10% heat-inactivated FBS, 1% P/S and 1% tryptose phosphate broth (TPB).

Viruses

DENV-1,-2, -3 and -4 isolates of low passage history (Table 1), were obtained from the World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch. The Dominica strain, 814669, from which both tested DENV-4 vaccine candidates were derived, was provided by Dr. Anna Durbin. Viral isolates were passaged once in C6/36 cultures to obtain high titer stocks. Supernatants were clarified from cellular debris by low-spin centrifugation (630 × g, 20 min, 4°C) stabilized with the addition of 10X SPG (2.18 M Sucrose, 0.038M KH_2PO_4, 0.072M K_2HPO_4 and 0.054M L-glutamate), and stored at −;80°C.

Vaccine Viruses

The rDEN4Δ30 vaccine candidate was derived from the DENV-4 strain 814669 (Dominica 1981) by the removal of 30-nucleotides (nt) from the 3' UTR of the genome (Durbin et al., 2001). The rDEN4Δ30-200,201 vaccine virus was derived from the rDEN4Δ30 vaccine virus by paired charge-to-alanine mutagenesis. (Hanley et al., 2004; McArthur et al., 2008). The rDEN4Δ30-200,201 virus was generated as described previously in Hanley etal. (Hanley et al., 2004).

Vaccinee sera

Serum samples were collected as part of previously described DENV vaccine trials (Durbin et al., 2011; McArthur et al., 2008). Healthy adult male and non-pregnant female volunteers were recruited from metropolitan Baltimore, Maryland. Informed consent was obtained from each enrolled healthy volunteer, between 18 – 50 years of age, in accordance with the Code of Federal Regulations (CFR21, Part 50). Each enrolled volunteer met the following eligibility criteria: normal findings during physical examination; negative for antibodies to all DENV, yellow fever virus, West Nile virus, St. Louis encephalitis virus, Japanese encephalitis virus, human immunodeficiency virus, and hepatitis C virus; negative for

hepatitis B surface antigen; normal values for complete blood count (CBC), and urinalysis. Additional safety-related exclusion criteria were also applied. Female volunteers were required to have a negative urine pregnancy test at least three days prior to vaccination and on the day of vaccination and to agree to use contraception or abstain from sexual intercourse for the duration of the study. Sera provided for this study represent collections at day 42 post vaccination as described in McArthur etal (McArthur et al., 2008).

Human primary convalescent sera

Sera from convalescent patients after primary infection with DENV-4 obtained 9–21 days (Puerto Rico and Singapore) or up to a year (Mexico) after the onset of symptoms (Table 4), were obtained from routine surveillance, and were de-identified and approved for this research study under IRB exemption 4797 at the CDC. Late convalescent sera were collected as part of an ongoing dengue in traveler study under University of North Carolina (UNC) IRB approval 08-0895. Anonymous blood bank and reference sera were kindly provided by the lab of Aravinda de Silva, UNC School of Medicine, Department of Microbiology.

PRNT and Immunostaining

PRNTs were performed in 12-well, Vero-microplate-cell cultures, using a fixed virus inoculum [~800 focus forming units (FFU)] against varying serum dilutions (1:10--1:640). Serum samples were diluted in minimal essential medium (MEM), containing 2% fetal bovine serum. Virus was mixed with an equal volume of each serum dilution and the mixture was incubated 1hr at 37°C. Then, 250 μL of the serum-virus mixture was placed into Vero cultures and incubated 1hr at 37°C. A 1.5 mL volume of 4% methycellulose in OPTIMEM-I overlay was placed in each well and the plates were incubated at 37°C for 4–5 days. The plates were then fixed with 1:1 methanol:acetone and foci were stained immunologically and counted to determine the level of virus neutralization, as described previously (Vasilakis et al., 2008a; Vasilakis et al., 2007). The PRNT titers were scored as reciprocal of the highest dilution of serum that inhibited 60% of foci.

Statistical analyses

All statistical comparisons and regression analyses were calculated in JMP v7.0 (Cary, NC). Mean log titers were compared by one-way ANOVA. $P<0.05$ was considered significant in all comparisons. Correlations between $PRNT_{60}$ titers and genetic P-distances were tested using a standard least squares model. P-distances between DENV-4 structural protein amino acid sequences were calculated with MEGA V5.0 (Tamura *et al.* 2011) default settings.

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REFERENCES

Barban V, Munoz-Jordan JL, Santiago GA, Mantel N, Girerd Y, Gulia S, Claude JB, Lang J. Broad neutralization of wild-type dengue virus isolates following immunization in monkeys with a tetravalent dengue vaccine based on chimeric yellow fever 17D/dengue viruses. Virology. 2012; 429:91–98. [PubMed: 22542002]

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. The Journal of general virology. 1989; 70(Pt 1):37–43. [PubMed: 2543738]

Cardosa J, Ooi MH, Tio PH, Perera D, Holmes EC, Bibi K, Abdul Manap Z. Dengue virus serotype 2 from a sylvatic lineage isolated from a patient with dengue hemorrhagic Fever. PLoS neglected tropical diseases. 2009; 3:e423. [PubMed: 19399166]

Carey DE, Causey OR, Reddy S, Cooke AR. Dengue viruses from febrile patients in Nigeria, 1964–68. Lancet. 1971; 1:105–106. [PubMed: 4099602]

Chen R, Vasilakis N. Dengue - Quo tu et quo vadis? Viruses. 2011; 3:1562–1608. [PubMed: 21994796]

Clark DV, Mammen MP Jr, Nisalak A, Puthimethee V, Endy TP. Economic impact of dengue fever/dengue hemorrhagic fever in Thailand at the family and population levels. The American journal of tropical medicine and hygiene. 2005; 72:786–791. [PubMed: 15964964]

Cockburn JJ, Navarro Sanchez ME, Fretes N, Urvoas A, Staropoli I, Kikuti CM, Coffey LL, Arenzana Seisdedos F, Bedouelle H, Rey FA. Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody. Structure. 2012; 20:303–314. [PubMed: 22285214]

Cordellier R, Bouchite B, Roche JC, Monteny N, Diaco B, Akoliba P. Circulation selvatique du virus Dengue 2, en 1980, dans les savanes sub-soudaniennes de Cote d'Ivoire. Cah. ORSTOM. ser Ent. Med. et Parasitol. 1983; 21:165–179.

Crill WD, Roehrig JT. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J Virol. 2001; 75:7769–7773. [PubMed: 11462053]

de Alwis R, Smith SA, Olivarez NP, Messer WB, Huynh JP, Wahala WM, White LJ, Diamond MS, Baric RS, Crowe JE Jr, de Silva AM. Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:7439–7444. [PubMed: 22499787]

Diallo M, Ba Y, Faye O, Soumare ML, Dia I, Sall AA. Vector competence of Aedes aegypti populations from Senegal for sylvatic and epidemic dengue 2 virus isolated in West Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2008; 102:493–498. [PubMed: 18378270]

Diallo M, Ba Y, Sall AA, Diop OM, Ndione JA, Mondo M, Girault L, Mathiot C. Amplification of the sylvatic cycle of dengue virus type 2, Senegal, 1999–2000: entomologic findings and epidemiologic considerations. Emerging infectious diseases. 2003; 9:362–367. [PubMed: 12643833]

Diallo M, Sall AA, Moncayo AC, Ba Y, Fernandez Z, Ortiz D, Coffey LL, Mathiot C, Tesh RB, Weaver SC. Potential role of sylvatic and domestic african mosquito species in dengue emergence. The American journal of tropical medicine and hygiene. 2005; 73:445–449. [PubMed: 16103619]

Durbin AP, Karron RA, Sun W, Vaughn DW, Reynolds MJ, Perreault JR, Thumar B, Men R, Lai CJ, Elkins WR, Chanock RM, Murphy BR, Whitehead SS. Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region. The American journal of tropical medicine and hygiene. 2001; 65:405–413. [PubMed: 11716091]

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

Durbin AP, McArthur J, Marron JA, Blaney JE Jr, Thumar B, Wanionek K, Murphy BR, Whitehead SS. The live attenuated dengue serotype 1 vaccine rDEN1Delta30 is safe and highly immunogenic in healthy adult volunteers. Human vaccines. 2006; 2:167–173. [PubMed: 17012875]

Endy TP, Nisalak A, Chunsuttitwat S, Vaughn DW, Green S, Ennis FA, Rothman AL, Libraty DH. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. The Journal of infectious diseases. 2004; 189:990–1000. [PubMed: 14999601]

Fagbami AH, Monath TP, Fabiyi A. Dengue virus infections in Nigeria: a survey for antibodies in monkeys and humans. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1977; 71:60–65. [PubMed: 404737]

Falconar AK, de Plata E, Romero-Vivas CM. Altered enzyme-linked immunosorbent assay immunoglobulin M (IgM)/IgG optical density ratios can correctly classify all primary or secondary dengue virus infections 1 day after the onset of symptoms, when all of the viruses can be isolated. Clin Vaccine Immunol. 2006; 13:1044–1051. [PubMed: 16960117]

Fox A, Le NM, Simmons CP, Wolbers M, Wertheim HF, Pham TK, Tran TH, Trinh TM, Nguyen TL, Nguyen VT, Nguyen DH, Farrar J, Horby P, Taylor WR, Nguyen VK. Immunological and viral determinants of dengue severity in hospitalized adults in Ha Noi, Viet Nam. PLoS neglected tropical diseases. 2011; 5:e967. [PubMed: 21390156]

Franco L, Palacios G, Martinez JA, Vazquez A, Savji N, Ory FD, Sanchez-Seco MP, Martin D, Lipkin WI, Tenorio A. First report of sylvatic DENV-2-associated dengue hemorrhagic fever in West Africa. PLoS neglected tropical diseases. 2011

Guilarde AO, Turchi MD Jr, JB, Feres VC, Rocha B, Levi JE, Souza VA, Boas LS, Pannuti CS, Martelli CM. Dengue and Dengue Hemorrhagic Fever among Adults: Clinical Outcomes Related to Viremia, Serotypes, and Antibody Response. The Journal of infectious diseases. 2008

Guirakhoo F, Kitchener S, Morrison D, Forrat R, McCarthy K, Nichols R, Yoksan S, Duan X, Ermak TH, Kanesa-Thasan N, Bedford P, Lang J, Quentin-Millet MJ, Monath TP. Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. Human vaccines. 2006; 2:60–67. [PubMed: 17012873]

Halstead SB. Etiologies of the experimental dengues of Siler and Simmons. The American journal of tropical medicine and hygiene. 1974; 23:974–982. [PubMed: 4615598]

Halstead SB, Rojanasuphot S, Sangkawibha N. Original antigenic sin in dengue. The American journal of tropical medicine and hygiene. 1983; 32:154–156. [PubMed: 6824120]

Hanafusa S, Chanyasanha C, Sujirarat D, Khuankhunsathid I, Yaguchi A, Suzuki T. Clinical features and differences between child and adult dengue infections in Rayong Province, southeast Thailand. The Southeast Asian journal of tropical medicine and public health. 2008; 39:252–259. [PubMed: 18564710]

Hanley KA, Manlucu LR, Manipon GG, Hanson CT, Whitehead SS, Murphy BR, Blaney JE Jr. Introduction of mutations into the non-structural genes or 3' untranslated region of an attenuated dengue virus type 4 vaccine candidate further decreases replication in rhesus monkeys while retaining protective immunity. Vaccine. 2004; 22:3440–3448. [PubMed: 15308370]

Hervy JP, Legros F, Roche JC, Monteny N, Diaco B. Circulation du Dengue 2 dans plusieurs milieux boises des savanes soudaniennes de la region de Bobo-Dioulasso (Burkina Faso). Considerations entomologiques et epidemiologiques. Cah. ORSTOM. ser Ent. Med. et Parasitol. 1984; 22:135–143.

Kaufman BM, Summers PL, Dubois DR, Cohen WH, Gentry MK, Timchak RL, Burke DS, Eckels KH. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. The American journal of tropical medicine and hygiene. 1989; 41:576–580. [PubMed: 2817214]

Kaufman BM, Summers PL, Dubois DR, Eckels KH. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. The American journal of tropical medicine and hygiene. 1987; 36:427–434. [PubMed: 3826503]

Koh BK, Ng LC, Kita Y, Tang CS, Ang LW, Wong KY, James L, Goh KT. The 2005 dengue epidemic in Singapore: epidemiology, prevention and control. Annals of the Academy of Medicine, Singapore. 2008; 37:538–545.

Kuno G, Gubler DJ, Oliver A. Use of 'original antigenic sin' theory to determine the serotypes of previous dengue infections. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1993; 87:103–105. [PubMed: 8465377]

Kyle JL, Harris E. Global spread and persistence of dengue. Annual review of microbiology. 2008; 62:71–92.

Lanata CF, Andrade T, Gil AI, Terrones C, Valladolil O, Zambrano B, Saville M, Crevat D. Immunogenicity and safety of tetravalent dengue vaccine in 2–11 year-olds previously vaccinated against yellow fever: Randomized, controlled, phase II study in Piura, Peru. Vaccine. 2012; 30:5935–5941. [PubMed: 22863660]

Low JG, Ooi EE, Tolfvenstam T, Leo YS, Hibberd ML, Ng LC, Lai YL, Yap GS, Li CS, Vasudevan SG, Ong A. Early Dengue infection and outcome study (EDEN) - study design and preliminary findings. Annals of the Academy of Medicine, Singapore. 2006; 35:783–789.

Mathers CD, Ezzati M, Lopez AD. Measuring the burden of neglected tropical diseases: the global burden of disease framework. PLoS neglected tropical diseases. 2007; 1:e114. [PubMed: 18060077]

McArthur JH, Durbin AP, Marron JA, Wanionek KA, Thumar B, Pierro DJ, Schmidt AC, Blaney JE Jr, Murphy BR, Whitehead SS. Phase I clinical evaluation of rDEN4Delta30-200,201: a live attenuated dengue 4 vaccine candidate designed for decreased hepatotoxicity. The American journal of tropical medicine and hygiene. 2008; 79:678–684. [PubMed: 18981503]

Monlun E, Zeller H, Traore-Lamizana M, Hervy JP, Adam F, Mondo M, Digoutte JP. Caracteres cliniques et epidemiologiques de la dengue 2 au Senegal. Med. Mal. Infect. 1992; 22:718–721.

Okuno Y, Fukunaga T, Tadano M, Fukai K, Ikeda T, Sekii K, Ariyoshi H. Serological studies on volunteers inoculated experimentally with a dengue virus strain in 1943. Biken J. 1983; 26:161–163. [PubMed: 6678151]

Papaevangelou G, Halstead SB. Infections with two dengue viruses in Greece in the 20th century. Did dengue hemorrhagic fever occur in the 1928 epidemic? J Trop Med Hyg. 1977; 80:46–51. [PubMed: 327086]

Pengsaa K, Luxemburger C, Sabchareon A, Limkittikul K, Yoksan S, Chambonneau L, Chaovarind U, Sirivichayakul C, Lapphra K, Chanthavanich P, Lang J. Dengue virus infections in the first 2 years of life and the kinetics of transplacentally transferred dengue neutralizing antibodies in thai children. The Journal of infectious diseases. 2006; 194:1570–1576. [PubMed: 17083042]

Rico-Hesse R. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology. 1990; 174:479–493. [PubMed: 2129562]

Robin Y, Cornet M, Heme G, Le Gonidec G. Isolement du virus dela dengue au Senegal. Ann. Virol (Institut Pasteur). 1980; 131:149–154.

Roche JC, Cordellier R, Hervy JP, Digoutte JP, Monteny N. Isolement de 96 souches de virus Dengue 2 a partir de moustiques captures en Cote d'Ivoire et en Haute Volta. Ann. Virol (Institut Pasteur). 1983; 134E:233–244.

Roehrig JT, Bolin RA, Kelly RG. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology. 1998; 246:317–328. [PubMed: 9657950]

Rosen L. The pathogenesis of dengue haemorrhagic fever. A critical appraisal of current hypotheses. S Afr Med J Suppl. 1986:40–42.

Rudnick A. Studies of the ecology of dengue in Malaysia: a preliminary report. Journal of medical entomology. 1965; 2:203–208. [PubMed: 5827577]

Rudnick A. Ecology of dengue virus. Asian. J. Infect. Dis. 1978; 2:156–160.

Rudnick, A. The ecology of the dengue virus complex in peninsular Malaysia. In: Pang, T.; Pathmanatan, R., editors. Proceedings of the International Conference on dengue/DHF; University of Malaysia Press; Kuala Lampur, Malaysia. 1984.

Rudnick A. Dengue virus ecology in Malaysia. Inst. Med. Res. Malays. Bull. 1986; 23:51–152.

Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S, Jiwariyavej V, Dulyachai W, Pengsaa K, AnhWartel T, Moureau A, Saville M, Bouckenooghe A, Viviani S, Tornieporth NG, Lang J. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet. 2012:1–9.

Sabin AB. Research on dengue during World War II. The American journal of tropical medicine and hygiene. 1952; 1:30–50. [PubMed: 14903434]

Saluzzo JF, Cornet M, Castagnet P, Rey C, Digoutte JP. Isolation of dengue 2 and dengue 4 viruses from patients in Senegal. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1986; 80:5. [PubMed: 3726996]

Schlesinger JJ, Brandriss MW, Cropp CB, Monath TP. Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. J Virol. 1986; 60:1153–1155. [PubMed: 3783816]

Siqueira JB Jr, Martelli CM, Coelho GE, Simplicio AC, Hatch DL. Dengue and dengue hemorrhagic fever, Brazil, 1981–2002. Emerging infectious diseases. 2005; 11:48–53. [PubMed: 15705322]

Smith CE. The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. J Trop Med Hyg. 1956; 59:243–251. [PubMed: 13368255]

Smith CE. The distribution of antibodies to Japanese Encephalitis, dengue, and yellow fever viruses in five rural communities in Malaya. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1958; 52:237–252. [PubMed: 13556868]

Tadano M, Okuno Y, Fukunaga T, Fukai K. Retrospective serological studies on dengue epidemics in Osaka and Okinawa. Biken J. 1983; 26:165–167. [PubMed: 6378175]

Twiddy SS, Holmes EC, Rambaut A. Inferring the rate and time-scale of dengue virus evolution. Molecular biology and evolution. 2003; 20:122–129. [PubMed: 12519914]

Vasilakis N, Durbin A, Travassos da Rosa APA, Munoz-Jordan JL, Tesh RB, Weaver SC. Antigenic relationships between sylvatic and endemic dengue viruses. The American journal of tropical medicine and hygiene. 2008a; 79:128–132. [PubMed: 18606776]

Vasilakis N, Fokam EB, Hanson CT, Weinberg E, Sall AA, Whitehead SS, Hanley KA, Weaver SC. Genetic and phenotypic characterization of sylvatic dengue virus type 2 strains. Virology. 2008b; 377:296–307. [PubMed: 18570968]

Vasilakis N, Shell EJ, Fokam EB, Mason PW, Hanley KA, Estes DM, Weaver SC. Potential of ancestral sylvatic dengue-2 viruses to re-emerge. Virology. 2007; 358:402–412. [PubMed: 17014880]

Vasilakis N, Tesh RB, Weaver SC. Sylvatic dengue virus type 2 activity in humans, Nigeria, 1966. Emerging infectious diseases. 2008c; 14:502–504. [PubMed: 18325274]

Wahala WM, Donaldson EF, de Alwis R, Accavitti-Loper MA, Baric RS, de Silva AM. Natural strain variation and antibody neutralization of dengue serotype 3 viruses. PLoS pathogens. 2010; 6:e1000821. [PubMed: 20333252]

Wang E, Ni H, Xu R, Barrett AD, Watowich SJ, Gubler DJ, Weaver SC. Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. J Virol. 2000; 74:3227–3234. [PubMed: 10708439]

Wichmann O, Hongsiriwon S, Bowonwatanuwong C, Chotivanich K, Sukthana Y, Pukrittayakamee S. Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi, Thailand. Trop Med Int Health. 2004; 9:1022–1029. [PubMed: 15361117]

Witayathawornwong P. DHF in infants, late infants and older children: a comparative study. The Southeast Asian journal of tropical medicine and public health. 2005; 36:896–900. [PubMed: 16295542]

Zulueta A, Martin J, Hermida L, Alvarez M, Valdes I, Prado I, Chinea G, Rosario D, Guillen G, Guzman MG. Amino acid changes in the recombinant Dengue 3 Envelope domain III determine its antigenicity and immunogenicity in mice. Virus research. 2006; 121:65–73. [PubMed: 16781791]

- Sylvatic dengue viruses are both evolutionary and ecologically distinct from human DENV
- Low to nonexistent adaptive barrier for emergence into human transmission cycle
- Sylvatic DENV emergence is constrained by natural infection to DENV strains from human transmission cycle
- Sylvatic DENV emergence is constrained by vaccination

Table 1

Sylvatic and Human DENV Strains Used in this Study

Isolate[a]	Serotype	Host[b]	Passage History[c]	Epidemiological Type[d]	Location	Year	GenBank Accession No.[e]
P72-1244	1	*Ae. niveus*	?, C6/36-3	Sylvatic?	Malaysia	1975	EF457905
OBS7690	1	Human	C6/36-3	Human	Bolivia	1999	N/A[f]
16681	2	Human	BSC-1 –X, MK2 x– 6, Rh. Macaque –1,Tx. Amboinensis – 2, C6/36 – 4, MK2 – 1, C6/36 – 1	Human	Thailand	1964	U87411
DKD811	2	Human	C6/36-4	Sylvatic	Malaysia	2008	FJ467493
P8-1407	2	Sentinel monkey	SM – 3, C6/36 – 2	Sylvatic	Malaysia	1970	EF105379
JKT85-934	3	Human	?, NHP-1, C6/36-2	Human	Indonesia	1985	N/A[f]
814669[g]	4	Human	MK2-1, C6/36-4	Human	Dominica	1981	AF326573
H241	4	Human	SM-17, C6/36-4	Human	The Philippines	1956	AY947539
India G11337	4	Human	?, C6/36-2	Human	India	1961	JF262783
INH6412	4	Human	C6/36-2	Human	Venezuela	1995	JF262781
Haiti73	4	Human	C6/36-3	Human Haiti	1994	JF262782	
P75-215	4	Sentinel onkey	*Ae. niveus,* C6/36-3	Sylvatic	Malaysia	1975	EF457906
P75-514	4	Sentinel monkey	*Presbytis ristata-2,* 6/36-2	Sylvatic	Malaysia	1975	JF262780
P73-1120	4	Sentinel monkey	*Presbytis cristata-1,* C6/36-2	Sylvatic	Malaysia	1973	JF262779

[a] DENV isolates were obtained from the UTMB World Reference Center for Emerging Viruses and Arboviruses (WRCEVA)

[b] Source of virus isolation.

[c] SM – suckling mouse; C6/36 – *Ae. albopictus* cell line; MK2 – Rhesus monkey kidney cells; BSC-1 – African green monkey kidney cells; AP61 – Aedes pseudoscutellaris cell line; ? – unknown number of passages prior been deposited to WRCEVA

[d] Endemic indicates human or *Ae. aegypti* isolates or those associated with peridomestic transmission; sylvatic indicates sentinel monkey or canopy-dwelling mosquito isolate.

[e] Although DENV-1 P72-1244 strain had been identified initially by Rudnick (Rudnick, 1986) as sylvatic, subsequent analyses suggest that this strain may represent a spill-back from the human transmission cycle (Vasilakis et al., 2011)

[f] N/A – not available.

[g] Kindly provided by Dr Anna Durbin

Table 2

Homotypic and Heterotypic Neutralization[a] by DENV--4 Sera from Persons Inoculated with Candidate rDEN4Δ30-200,201 DENV-- 4 Vaccine at day 42 post vaccination

DENV-4 Immune sera	Virus													
	Endemic DENV				Sylvatic DENV--4					DENV--		DENV--3	DENV	
	814669	H241	Ind G11337	NH6412	Haiti73	P75-215	P75-514	P73-1120	16681	DKD811[b]	P8-1407[b]	JKT85-934	OBS7690	P72-1244[c]
214.01.01	NT	40	40	80	40	40	20	20	<20	<20	<20	<20	<20	<20
214.01.06	NT	20	20	40	40	20	40	20	<20	<20	<20	<20	<20	<20
214.01.08	NT	80	40	40	20	40	40	40	<20	<20	<20	<20	<20	<20
214.01.09	NT	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
214.01.11	NT	40	40	40	80	80	40	20	<20	<20	<20	<20	<20	<20
214.01.12	NT	20	20	80	40	20	40	20	<20	<20	<20	<20	<20	<20
214.01.13	NT	40	20	80	40	20	20	20	<20	<20	<20	<20	<20	<20
214.01.14	NT	<20	<20	40	20	20	20	20	<20	<20	<20	<20	<20	<20
214.01.16	NT	40	<20	40	20	20	20	20	NT	NT	<20	<20	<20	<20
214.01.18	NT	20	<20	20	20	20	20	20	<20	<20	<20	<20	<20	<20
214.01.19	NT	160	40	160	80	80	80	40	<20	NT	<20	<20	<20	<20
214.01.20	NT	20	20	20	40	20	40	20	<20	<20	<20	<20	<20	<20
214.01.21	NT	80	80	80	40	80	80	40	<20	<20	<20	<20	<20	<20
214.01.22	NT	NT	80	80	40	40	20	20	NT	NT	NT	<20	NT	NT
214.01.23	NT	80	80	80	40	40	40	20	<20	<20	<20	<20	<20	<20
214.01.25	NT	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
214.01.26	NT	80	20	80	40	80	40	20	<20	<20	<20	<20	<20	<20
214.01.27	NT	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
214.02.12	NT	40	20	80	160	80	40	20	<20	<20	<20	<20	<20	<20

[a] 60% reduction measured by Plaque Reduction Neutralization Test ($PRNT_{60}$).

[b] Sylvatic DENV-2 strains.

[c] Sylvatic DENV-1 strain.

NT – not tested

Table 3

Homotypic and Heterotypic Neutralization[a] by DENV-4 Sera from Persons Inoculated with Candidate rDEN4Δ30 DENV-4 Vaccine at day 42 post vaccination

DENV-4 Immune sera	Virus													
	Endemic DEN			Sylvatic DENV--4					DE-2		DENV--3		DENV--1	
	814669	H241	India G11337	INH6412	Haiti73	P75-215	P75-514	P73-1120	16681	DKD811[b]	P8-1407[b]	JKT85934	OBS7690	P72-1244[c]
JHU 01	>640	160	>640	>640	320	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 02	20	40	80	80	20	20	80	160	<20	>640	<20	<20	<20	<20
JHU 03	320	<20	<20	320	20	20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 04	160	<20	20	>640	<20	40	20	<20	<20	<20	<20	<20	<20	<20
JHU 05	<20	<20	<20	20	20	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 06	160	40	320	>640	160	320	160	320	<20	<20	<20	<20	<20	<20
JHU 07	80	80	160	>640	>640	80	40	320	<20	<20	<20	<20	<20	<20
JHU 08	20	<20	20	20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 09	320	40	80	>640	40	80	40	80	<20	<20	<20	<20	<20	<20
JHU 10	40	<20	<20	>640	40	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 11	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 12	160	20	20	320	80	20	20	20	<20	20	<20	<20	<20	<20
JHU 13	320	<20	40	>640	80	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 14	80	20	20	160	40	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 15	>640	20	20	>640	40	80	40	160	<20	<20	<20	<20	<20	<20
JHU 16	320	<20	20	320	40	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 17	80	<20	<20	80	20	<20	<20	<20	NT	NT	<20	<20	<20	<20
JHU 18	40	<20	20	80	40	<20	<20	40	<20	<20	<20	<20	<20	<20
JHU 19	>640	<20	20	>640	20	20	<20	<20	NT	<NT	<20	<20	<20	<20
JHU 20	80	40	20	160	320	160	<20	<20	<20	<20	<20	<20	<20	<20
JHU 21	80	<20	80	320	80	<20	<20	<20	<20	<20	<20	<20	<20	<20
JHU 22	160	80	320	160	160	40	80	80	>640	>640	80	80	320	40

[a] 60% reduction measured by Plaque Reduction Neutralization Test ($PRNT_{60}$).

[b] Sylvatic DENV-2 strains.

[c] Sylvatic DENV-1 strain.

NT – not tested

Table 4

Epidemiologic and demographic history of human primary convalescent DENV-4 used in this study

Origin	Ethnic background	Age	Sex	Onset[a, c]	Collection[b, c]	Diagnosis
Puerto Rico (PR	Hispanic	39	M	12/07/1994	12/16/1994	DF
Puerto Rico (PR	Hispanic	43	F	09/12/1995	09/19/1995	DHF
Puerto Rico (PR	Hispanic	10	F	01/15/1997	01/21/1997	DF
Puerto Rico (PR	Hispanic			09/09/1988		DF[d]
		48	M		09/16/1988	
Puerto Rico (PR	Hispanic	29	M	06/11/1996	06/17/1996	DF
Puerto Rico (PR	Hispanic	35	F	03/06/1996	03/12/1996	DHF
Puerto Rico (PR	Hispanic	17	F	12/01/1991	12/18/1991	DF[d]
Puerto Rico (PR	Hispanic	15	M	09/01/1993	09/09/1993	DF
Puerto Rico (PR	Hispanic	75	M	10/10/1993	10/22/1993	DF
Puerto Rico (PR	Hispanic	29	M	10/13/1993	10/20/1993	DHF
Puerto Rico (PR	Hispanic	21	M	11/10/1993	11/27/1993	DF[d]
Puerto Rico (PR	Hispanic	10	M	12/01/1991	12/13/1991	DF[d]
Mexico	Hispanic	40	M	08/30/2008	02/04/2009	DF
Mexico	Hispanic	20	F	06/25/2008	08/04/2009	DF
Singapore	Chinese	25	M	07/17/2006	08/10/2006	DF
United States	Caucasian	26	F	2005[e]	05/01/2009	DF
United States	Caucasian	52	M	2007[f]	05/08/2009	DF
Thailand[g]	Unknown	Unknown	Unknown	Unknown	Late conv.[i]	Unknown
Sri Lanka[h]	Unknown	Unknown	Unknown	Unknown	Late conv.[k]	Unknown

[a] Indicates date when onset of symptoms occurred

[b] Indicates date when collection of serum occurred

[c] Date is represented as: month/day/year

[d] Cases of presumptive DF based on the case investigation form only. No additional information was received after the sample was obtained and consequently we do not know how the clinical presentation progressed.

[e] Infection acquired in the French West Indies

[f] Infection acquired in Honduras

[g] WHO primary Thai DENV-4 reference sera (3 samples)

[h] Sera screened from Colombo, Sri Lanka, blood donors

[i] Sera were collected greater than 2 years after documented DENV infection

[k] Length of convalescence for the Sri Lankan blood bank donor is unknown

Abbreviations: M – male; F – female.

Table 5

Homotypic and Heterotypic DENV-4 Neutralization[a] by Convalescent Patient Sera[b]

DENV-4 Immune sera[b]	Virus													
	Endemic DENV			Sylvatic DENV						DENV-N-2V--3			ENV	
	814669	H241	India G11337	INH6412	Haiti73	P75-215	P75-51	P73-1120	16681	DKD811[c]	P8-1407[c]	JKT85-934	OBS7690	P72-1244[d]
PR-A01	NT	>1280	640	>1280	>1280	>1280	>1280	>1280	<20	NT	80	>1280	160	160
PR-A02	NT	160	<20	320	160	160	80	40	20	NT	<20	80	<20	<20
PR-A03	NT	640	80	640	320	640	320	80	20	NT	80	320	160	80
PR-A04	NT	320	80	320	160	160	320	160	NT	NT	160	640	NT	>1280
PR-A05	NT	40	80	160	320	80	160	80	<20	40	40	160	<20	40
PR-A06	NT	640	160	320	320	80	320	160	<20	<20	<20	40	<20	40
PR-A07	NT	1280	320	640	320	320	640	320	<20	NT	80	320	160	320
PR-A08	NT	320	80	320	320	160	320	160	<20	<20	80	320	<20	80
PR-A09	NT	320	320	160	320	320	320	1280	<20	80	80	40	160	80
PR-A10	NT	160	160	160	320	320	80	80	<20	<20	80	40	160	40
PR-A11	NT	160	160	160	160	160	160	160	<20	40	80	40	80	40
PR-A12	NT	320	160	320	320	160	160	40	<20	NT	40	40	40	40
MX-01	NT	20	40	20	20	40	20	40	NT	NT	<20	<20	NT	160
MX-02	NT	40	20	40	40	20	40	20	<20	<20	<20	<20	<20	<20
SG-01	NT	640	640	>1280	160	320	160	160	NT	NT	80	40	160	160
DT100[e]	160	NT	NT	160	320	160	NT	80	NT	NT	NT	NT	NT	NT
DT102[e]	160	NT	NT	320	160	40	NT	640	NT	NT	NT	NT	NT	NT
DV002[e]	40	NT	NT	80	80	40	NT	80	NT	NT	NT	NT	NT	NT
GS58e	20	NT	NT	40	40	20	NT	20	NT	NT	NT	NT	NT	NT
SS06/105[e]	320	NT	NT	640	320	80	NT	40	NT	NT	NT	NT	NT	NT
SS06/302[e]	>1280	NT	NT	>1280	320	80	NT	40	NT	NT	NT	NT	NT	NT
SS07/333[e]	>1280	NT	NT	>1280	>1280	160	NT	80	NT	NT	NT	NT	NT	NT

[a] 60% reduction measured by Plaque Reduction Neutralization Test ($PRNT_{60}$).

[b] Sera were obtained from confirmed primary DENV-4 infections from patients in Puerto Rico (PR), Mexico (MX) and Singapore (SG).

[c] Sylvatic DENV-2 strains.

[d] Sylvatic DENV-1 strain.

[e] Monotypic profile confirmed against WHO reference strains (DENV-1 West Pac 74, DENV-2 S16803, DENV-3 CH53498)