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## Development of DENVax: A chimeric dengue-2 PDK-53-based tetravalent vaccine for protection against dengue fever

Jorge E. Osorio<sup>a,b,\*</sup>, Claire Y.-H. Huang<sup>c</sup>, Richard M. Kinney<sup>c</sup>, and Dan T. Stinchcomb<sup>a</sup>

<sup>a</sup>Inviragen, Inc., Fort Collins, CO 80525, and Madison, WI 53719, USA

<sup>b</sup>Department of Pathobiological Sciences, University of Wisconsin-Madison, School of Veterinary Medicine, Madison, WI 53706, USA

<sup>c</sup>Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80521, USA

### Abstract

Dengue virus infection is the leading arboviral cause of disease worldwide. A vaccine is being developed based on the attenuated DEN-2 virus, DEN-2 PDK-53. In this review, we summarize the characteristics of the parent DEN-2 PDK-53 strain as well as the chimeric viruses containing the prM and E genes of DEN-1, DEN-3 or DEN-4 virus in the genetic backbone of the DEN-2 PDK-53 virus (termed DENVax). Tetravalent DENVax formulations containing cloned, fully sequenced isolates of the DEN-2 PDK-53 virus and the three chimeras have been evaluated for safety and efficacy in preclinical animal models. Based on the safety, immunogenicity and efficacy in preclinical studies, Phase 1 clinical testing of DENVax has been initiated.

### Keywords

Dengue fever; Vaccine development; Dengue virus; DEN-2 PDK-53 virus; Recombinant viral vaccine

## 1. Introduction

### 1.1. Dengue fever: a worldwide public health threat

Dengue fever is caused by infection with dengue viruses, enveloped RNA viruses that occur as four recognized serotypes: Dengue type-1, -2, -3, and -4 (DEN-1 to -4) [1]. These viruses are transmitted from human to human by mosquitoes (primarily *Aedes aegypti*) [2]. Infection with a dengue virus can cause subclinical disease or overt illness ranging from mild symptoms to dengue fever to severe dengue hemorrhagic fever (DHF) [3,4]. Dengue fever is characterized clinically as an acute febrile illness with two or more manifestations that can include headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, or leucopenia [1]. The most severe forms of dengue fever – DHF and dengue shock syndrome (DSS) – are life threatening. Dengue is the most rapidly spreading mosquito-

\*Corresponding author at: Department of Pathobiological Sciences, University of Wisconsin-Madison, School of Veterinary Medicine, Madison, WI 53706, USA. osorio@svm.vetmed.wisc.edu (J.E. Osorio).

borne viral disease in the world, and 3.6 billion people in tropical and subtropical areas are at risk of dengue infection [5,6]. An estimated 36 million cases of dengue fever occur each year, resulting in about 2.1 million cases of DHF/DSS and more than 20,000 deaths, primarily among children [5,7].

Infection with one DEN virus serotype results in life-long protection from re-infection by that serotype, but does not prevent secondary infection by one of the other three DEN virus serotypes [8]. Significantly, previous infection with one DEN serotype may lead to an increased risk of severe disease (DHF/DSS) upon secondary infection with a different serotype [9]. The specific mechanism causing DHF/DSS is controversial. The pathogenesis presented in DHF/DSS patients is thought to result from both viral virulence factors and the host immune response [10,11]. It is hypothesized that non-neutralizing antibodies reacting with a second DEN strain contribute to viral pathogenesis by antibody-dependent enhancement (ADE) of infection [12,13]. Antibody:virus complexes can be directly internalized by Fc $\gamma$  receptor-bearing cells in which dengue virus can replicate, increasing virus load (extrinsic ADE). In addition, ligation of Fc $\gamma$  receptors can inhibit innate immunity and increase production of certain cytokines that can increase viral infection and pathogenesis (intrinsic ADE) [14].

No dengue vaccine is currently available nor is there an antiviral therapy for dengue virus infection. All four dengue serotypes have co-circulated in most endemic countries at various times, resulting in concurrent infection by, and protection from, multiple serotypes [15]. There have been no reports of sequential disease with a fourth DEN serotype, suggesting that effective protection can be obtained by immunization against several serotypes [15,16]. Finally, there is the potential for ADE associated with non-neutralizing cross-reactive antibodies arising from immunization with monovalent DEN vaccines. Thus, vaccine development has focused on tetravalent vaccines that simultaneously provide protection against all four serotypes of DEN virus [17].

## 1.2. Dengue virus vaccines

Tetravalent vaccine candidates in development include mixtures of four different live-attenuated viruses, recombinant live-attenuated viruses, protein subunit vaccines or DNA vaccines [17]. Early efforts at developing a tetravalent vaccine comprising four live-attenuated viruses developed at Mahidol University were suspended when the DEN-3 vaccine was found to be insufficiently attenuated [18]. Co-development of live-attenuated tetravalent vaccines by the Walter Reed Army Institute of Research and Glaxo-SmithKline Biologicals has completed Phase 2 trials [19] but there are currently no plans for additional testing.

Three chimeric recombinant dengue vaccines are under active development. Chimeric viruses based on a yellow fever virus backbone are in Phase 2b clinical testing in Thailand and Phase 3 testing worldwide by Sanofi Pasteur ([20] and reviewed by Guy et al., this issue). Recombinant vaccine viruses based on the DEN-2 PDK-53 attenuated virus backbone (DENVax) are the subject of this review. Recombinants with a 30 nucleotide deletion in the 3'-untranslated region (UTR) of the dengue virus genome were optimized for safety and efficacy in preliminary monovalent Phase 1 testing [21]; different tetravalent

formulations of these “delta 30” constructs are in Phase 1 clinical testing (reviewed by Durbin et al. in this issue).

A recombinant protein vaccine consisting of adjuvanted recombinant envelope (E) proteins expressed in insect cells [22] has completed monovalent Phase 1 clinical testing and tetravalent formulations have been manufactured (reviewed by Collier et al., this issue). Several additional approaches are currently being evaluated in preclinical studies. They include the use of (1) adenoviral vectors that express combinations of two of the four dengue serotypes [23,24]; (2) DNA vaccines expressing the E protein [25]; and (3) the E protein domain III either alone [26], or as domain-based single recombinant envelope protein that can induce tetravalent neutralizing antibody responses [27].

### 1.3. DEN-2 PDK-53: a promising DEN-2 vaccine

The four monovalent DENVax vaccine strains are based on a common DEN virus backbone: an attenuated DEN-2 virus strain, termed PDK-53, generated by 53 serial passages in primary dog kidney cells [28]. The strain was derived at Mahidol University, Bangkok, Thailand from a wild-type DEN-2 virus originally recovered from the serum of a patient with DHF/DSS in Thailand (DEN-2 16681). The DEN-2 PDK-53 strain has been extensively studied *in vitro* and *in vivo*. The strain exhibits an attenuated replication phenotype in mammalian and insect cell cultures [28–30], attenuated neurovirulence in newborn ICR mice [29,30], and attenuated replication in non-human primates [31]. In addition, DEN-2 PDK-53 has been extensively tested in human clinical trials.

### 1.4. Clinical studies with DEN-2 PDK-53 virus

The DEN-2 PDK-53 strain has been studied clinically both as a monovalent vaccine and in multivalent formulations with one or more attenuated dengue virus strains of other serotypes. An overview of previous human experience with the DEN-2 PDK-53 strain, based on literature reports of completed clinical studies, is provided here (Table 1).

An uncontrolled study of the safety and immunogenicity of DEN-2 PDK-53 was conducted in 10 healthy male Thai volunteers in 1984 [32]. All subjects were non-immune to dengue virus; however, five showed evidence of prior exposure to Japanese encephalitis virus. The subjects were vaccinated subcutaneously with 4.3–4.4 log<sub>10</sub> plaque forming units (PFU) of DEN-2 PDK-53 and observed for 21 days post-immunization. No serious adverse reactions or abnormal signs (identified as elevated temperature, bleeding, hypotension or organ involvement) were observed, and there were no reactions at the injection site. Transient mild headache or mild abdominal pain occurring between days 8 and 17 were reported by half of the subjects. Other symptoms, including myalgia and eye pain, occurred less frequently. No abnormal clinical chemistry findings were observed. Hematology parameters were within normal values. There was a slight reduction in total white blood cell counts observed on day 10 post-immunization that returned to near baseline levels by day 14. All 10 volunteers developed neutralizing antibodies to wild-type DEN-2 which persisted for at least 18 months. Viremia was detected in only one subject on day 10 post-vaccination by amplification in cell culture (but not by direct plaque isolation). The isolated virus was

identified as DEN-2 and was phenotypically attenuated *in vitro*. All subjects seroconverted, with serum neutralizing antibodies to DEN-2 that persisted for 1.5 years [32].

Vaughn et al. [33] reported on a second Phase 1 study of the safety of the monovalent DEN-2 PDK-53 vaccine. A single 3.9–4.3 log<sub>10</sub> PFU dose of the DEN-2 PDK-53 vaccine was administered subcutaneously in the deltoid region of 10 18–24-year-old American male ( $N = 7$ ) and female ( $N = 3$ ) volunteers who were non-immune to flavivirus (including dengue virus serotypes 1–4, Japanese encephalitis virus, St. Louis encephalitis virus, and yellow fever virus). In this uncontrolled study, subjects were observed for 21 days post-vaccination. Overall, there were no serious adverse reactions reported. There was no induration, redness or warmth at the injection site although one subject reported mild arm soreness for a few hours after injection. Two subjects did not report any symptoms during the observation period. The remaining eight subjects experienced symptoms including headache, myalgia and arthralgia, most of which were mild in intensity. One subject reported intermittent (5 min to 2 h) headaches on seven days beginning five days after vaccination. The headaches resolved without sequelae. Most laboratory results were within normal limits. Elevated aspartate amino transferase (AST) levels were observed in two patients; both correlated with exercise. As in the Thailand study, a slight reduction in total white blood cell counts was observed 8–12 days post-vaccination in seven of the 10 volunteers. Viremia was detected in eight of the 10 subjects between study days 4 and 12; all viruses had small plaque phenotype. All subjects developed neutralizing antibodies that persisted for at least one year, and out to two years for the five subjects who were available for evaluation at that time point.

### 1.5. Clinical studies with multivalent formulations containing DEN-2 PDK-53

DEN-2 PDK-53 virus has also been used in combination with up to three live, attenuated DEN vaccine strains representing other dengue serotypes, in an attempt to develop a live-attenuated multivalent vaccine. Bhamarapavati and co-workers [34,35] reported studies in healthy adult volunteers in which DEN-2 PDK-53 was evaluated in bivalent combination with an attenuated DEN-1 strain (seven volunteers), an attenuated DEN-4 strain (11 volunteers), in a trivalent mixture with both the DEN-1 and DEN-4 strains (11 volunteers), and finally in a tetravalent formulation with attenuated DEN-1, DEN-3 and DEN-4 virus strains (six volunteers). The authors state the vaccines were well-tolerated and did not produce clinically significant signs, symptoms or laboratory abnormalities [35]. All individuals vaccinated with a single dose of the bivalent vaccine formulations demonstrated serum neutralizing antibodies against DEN-2 and DEN-1 or DEN-4. Among individuals who received a single dose of the tetravalent formulation, five out of six developed DEN-2 neutralizing antibodies, six out of six developed neutralizing antibodies for DEN-1 and DEN-3, and four out of six developed neutralizing antibodies for DEN-4.

On the basis of the promising preliminary data cited above, several clinical trials were performed to evaluate and optimize tetravalent vaccine formulations containing DEN-2 PDK-53 and three other live-attenuated DEN vaccines developed at Mahidol University. The attenuated DEN-1, DEN-3 and DEN-4 viruses in these investigational vaccine

formulations were derived separately from DEN-2 PDK-53 by serial passage on primary dog kidney (PDK) cells or primary African green monkey kidney cells [28].

Kanesathasan et al. [36] described a study of the four monovalent vaccines ( $N = 5$  for each group) and the tetravalent formulation ( $N = 10$ ) compared to a vehicle control ( $N = 10$ ) in adult volunteers. The monovalent vaccines were given in single subcutaneous doses of 3.6–4.4  $\log_{10}$  PFU and were well-tolerated with few side effects. All vaccinated individuals seroconverted to the respective DEN virus. The tetravalent formulation showed significantly increased clinical effects compared to the placebo control. The clinical effects were associated with high levels of viremia by the DEN-3 vaccine component. Tetravalent vaccine recipients seroconverted predominantly to DEN-3 and showed variable seroconversion to the other DEN viruses.

Due to the predominance of DEN-3 virus replication, and increased clinical symptoms with formulations that included the DEN-3 vaccine, several formulations with higher concentrations of the DEN-1, DEN-2 and DEN-4 components and a lower dose of the DEN-3 component were tested in adults ( $N = 59$  total for seven formulations [37]) and children ( $N = 40$  and  $N = 42$  for each of two different formulations [38]). In these studies, the DEN-3 virus was still the dominant replicating vaccine virus and still dominated the immune responses. A study with the two preferred formulations was conducted in dengue-naïve adult volunteers in Australia. Subjects showed significant dengue-like clinical signs associated with DEN-3 replication [18] and further studies of this live, attenuated tetravalent vaccine were suspended.

Despite the clinical symptoms associated with the attenuated DEN-3 virus component of these tetravalent formulations, two follow-up studies of previously vaccinated children provided insight into the safety of the DEN-2 PDK-53 component. Using sera from Thai children that were given a single dose of the tetravalent vaccine 3–8 years prior, as well as samples from naturally infected age-matched controls, Guy et al. [39] studied the ability of these sera to stimulate antibody-dependent enhancement (ADE) of viral replication *in vitro*. At low serum dilutions that approximated the concentrations *in vivo*, assays with sera from the vaccinated children resulted either in no ADE or ADE at a similarly low level to that observed in naturally infected children. Three of the 16 individuals studied had seroconverted only to DEN-2. Two of these sera demonstrated low levels of ADE *in vitro* at levels similar to or much lower than those observed with corresponding age-matched control subjects in the endemic area. Within the limits of the *in vitro* test system used, the vaccinees had no apparent increased risk of ADE relative to naturally-infected, age-matched controls.

Chanthavanich et al. [40] reported on the long-term follow-up of 113 Thai children given a single dose of the tetravalent vaccine 3–8 years prior, aged 4–15 years at the time of vaccination. These children were age- and address-matched with two controls per vaccinee in a retrospective study to evaluate the immune response and occurrence of dengue infection after vaccination. 75% of the individuals had seroconverted to DEN-2 virus 6–12 months post-vaccination while 82% had antibodies to DEN-2 virus 3–8 years (average 6.8 years) after vaccination. Similar trends were observed for DEN-1, DEN-3 and DEN-4 viruses. The increase in seroconversion is likely due to natural exposure of the children who lived in an

endemic area. Importantly, there were no excess hospitalizations for clinically suspected dengue fever (DF) or dengue hemorrhagic fever (DHF) in vaccinees (4 of 113, one with DF and three with DHF) compared to the unvaccinated control children (14 of 226 with DHF). While neither of these studies was sufficiently powered to preclude modest increases in severe dengue disease, it is encouraging that immunization with the tetravalent vaccine did not cause a high incidence of either ADE *in vitro* or an increase in the incidence of severe dengue illness after long-term follow-up.

### 1.6. DENVax: chimeric DEN-2 PDK-53-based tetravalent vaccine

As members of the *Flavivirus* genus of the virus family *Flaviviridae*, dengue viruses possess a single-stranded, positive-sense, approximately 11-kb RNA genome that contains a single long open reading frame bracketed by 5' and 3' non-coding regions. The gene order in the translated polypeptide is capsid–premembrane/membrane (prM/M)–envelope (E)–non-structural protein 1 (NS1)–NS2A–NS2B–NS3–NS4A–NS4B–NS5 (Fig. 1). Upon cDNA cloning and sequence analysis of virus sampled directly from a vial of the Mahidol vaccine, two major genetic variants, termed V and E, were identified in the DEN-2 PDK-53 candidate vaccine virus. Both DEN-2 PDK-53 variants share eight nucleotide mutations, relative to the wild-type DEN-2 16681 parent, but the DEN-2 PDK-53-V variant possesses a unique ninth mutation in the NS3 gene which modifies the wild-type glutamic acid at residue 250 (single letter code, E) to valine (single letter code, V) in the translated NS3 protein [30].

The DENVax vaccines are based on the genetic background of the DEN-2 PDK-53-V variant. The DENVax-2 virus was derived from an infectious cDNA clone of the DEN-2 PDK-53-V variant. Whole genome sequencing and molecular genetics techniques have demonstrated that the mutations that are necessary and sufficient for attenuation of DEN-2 PDK-53 lie outside the structural genes in the 5' non-coding region (5'NC), and in non-structural proteins 1 and 3 (NS1 and NS3) [29]. Attenuated vaccine strains for the DEN-1, -3 and -4 were engineered by replacing the DEN-2 PDK-53 structural genes, premembrane (prM) and envelope (E), with the prM and E genes of wild-type DEN-1, DEN-3 or DEN-4 virus, as shown in Fig. 1 [41]. The origins of the four wild-type DEN virus strains on which the DENVax vaccine is based are shown in Table 2. The chimeric viruses express the surface antigens of DEN-1, DEN-3 or DEN-4 and retain six of the nine PDK-53 virus-specific genetic alterations (mutation in the 5'NC, four amino acid mutations in NS1, NS2A, NS3, and NS4A, and a silent mutation at nt 5547), including the three major genetic determinants (in the 5'NC, NS1, and NS3) responsible for the attenuation of the DEN-2 PDK-53 strain (triangles in Fig. 1). Only the DEN-2 PDK-53-V component of DENVax retained the PDK-53 virus-specific amino acid mutation in prM and silent mutation at nt 2055 in the E gene. At the locus of the ninth, silent PDK-53 virus-specific nucleotide mutation, the DEN-2 16681 virus-specific C nucleotide was retained in all of the engineered vaccine strains. The research grade chimeric DEN viruses have been referred to as “ChiDEN-V”. The equivalent viruses, as well as the DEN-2 PDK-53-V virus, re-derived under Good Manufacturing Practices (GMP) for vaccine manufacture are referred to as “DENVax” (Huang et al., in preparation).

The ChiDEN-V viruses were shown to retain critical DEN serotype-specific neutralizing epitopes using reference neutralizing antibody reagents. In a plaque reduction neutralization test, the ChiDEN-V viruses were neutralized to the same extent as the homologous wild-type DEN virus. The mean plaque size of each of the three ChiDEN-V virus strains was significantly smaller than that of the corresponding homologous, wild-type strain in LLC-MK<sub>2</sub> monolayers grown in six-well plates (see fig. 1 in [41]). The ChiDEN-V viruses replicated to peak titers of about 6.4–6.9 log<sub>10</sub> PFU/mL in LLC-MK<sub>2</sub> cells versus the 7.0–7.6 log<sub>10</sub> PFU/mL peak titers of the homologous wild-type DEN viruses (see fig. 1 in [41]). The ChiDEN-V viruses also replicated efficiently in Vero cells, with peak titers ranging from 6.7 to 7.4 log<sub>10</sub> PFU/mL (see fig. 2 in [41]). The ChiDEN-V viruses all exhibited a temperature-sensitive phenotype ( 90% reduction in viral titer at 38.7 °C versus 37 °C) in LLC-MK<sub>2</sub> cells (data not shown; see fig. 1 in [41]).

The ChiDEN-V viruses all exhibited greatly reduced replication profiles and peak replication titers when grown in C6/36 (*Aedes albopictus* mosquito) cells (fig. 2 in [41]). The peak titers of these ChiDEN-V viruses were decreased by 3.7–5.8 log<sub>10</sub> PFU/mL, relative to the peak titers of the wild-type DEN viruses. The common DEN-2 PDK-53 genetic background shared by these ChiDEN-V viruses clearly controlled their crippled replication phenotype in C6/36 cells, as ChiDEN viruses with the wild-type DEN-2 16681 genetic background exhibited only modest reductions of 0.6–1.8 log<sub>10</sub> PFU/mL relative to the peak titers of the respective wild-type DEN viruses (see fig. 2 in [41]).

### 1.7. Genetic stability of DEN-2 PDK-53-based chimeras

The genotype leading to attenuation of the DEN-2 PDK-53 strain has been investigated in detail using recombinant viruses with varying combinations of the nine nucleotide differences between the DEN-2 PDK-53-V strain and the wild-type parent, 16681. Mutations at three loci were found to contribute individually, and synergistically, to PDK-53 attenuation, specifically, a C to T transition at nucleotide 57 in the 5' non-coding region (5'NC-57), a Gly-to-Asp substitution in non-structural protein 1, amino acid 53 (NS1-53) and a Glu-to-Val substitution in non-structural protein 3, amino acid 250 (NS3-250) [29]. It is possible that the PDK-53 virus-specific mutations in prM (DEN-2 PDK-53-V virus only), NS2A, and/or NS4A might further modulate attenuation to an undefined extent. Additionally, the juxtaposition of heterologous genes in the chimeric DEN viruses, particularly in ChiDEN-3-V and -4-V, may also contribute to an attenuated phenotype, relative to wild-type virus [41].

The genetic stabilities of the ChiDEN-V viruses at the three loci associated with PDK-53 viral attenuation were evaluated by serial passage of the viral stocks in Vero cell cultures (table 2 in [42]). No evidence of reversion at the NS1-53 or NS3-250 locus was observed by sequence analyses of the ChiDEN-V viruses at the Vero passage 10. However, the 5'NC-57-T mutation did show a propensity to revert during passage in Vero cells. A sensitive, quantitative single nucleotide polymorphism assay was developed to permit finer assessment of the level of reversion at this locus. This TaqMan-based mismatch amplification mutation assay (TaqMAMA) permitted detection of less than 1% reversion at the 5'NC-57 locus in the viral population [42]. In eight separate ChiDEN-V viral stocks, up to 5% reversion at the

5'NC-57 locus was observed at passage level 1. The level of reversion increased with passage in all stocks, reaching >50% of the population by passage level 9 in two of the eight stocks. This suggests that during passage of the virus in Vero cells, the virus with reversion at the 5'NC-57 locus replicates more efficiently than viruses with the attenuating 5' mutation. Refinements in the TaqMAMA currently permit detection of <0.05% reversion at the 5'NC-57 locus, and enable the establishment of release criteria to verify an upper limit of reversion at this locus in vaccine lots.

Given the propensity of the 5'NC-57 locus to revert, detailed knowledge regarding the contribution of each of the three genetic loci of attenuation (individually and in various combinations with the other two attenuation loci) to the overall attenuated phenotype of the virus is important. Such data have been generated for the DEN-2 PDK-53 virus ([29]; summarized in [43]). The NS1-53 attenuation locus (highly stable during passage of the ChiDEN-V viruses in Vero cells), when engineered alone in the genetic background of wild-type DEN-2 16681 virus, encoded nearly complete attenuation of neurovirulence for newborn ICR mice, and decreased plaque size in LLC-MK<sub>2</sub> cells and decreased replicative ability in C6/36 cells to an extent equivalent to that of the single 5'NC-57 attenuating mutation engineered in the 16681 background. Only the highly stable NS1-53 and NS3-250 mutations were identified as genetic loci that significantly contributed to the temperature-sensitive phenotype of DEN-2 PDK-53 virus [29]. Furthermore, experimental biological reversion of any of these indicated phenotypic markers of attenuation to a corresponding phenotype approaching that of the wild-type DEN-2 16681 virus required engineered reversions in at least two of the three loci, one necessarily at the NS1-53 locus, in the PDK-53 genetic background [29]. Thus, in the event of significant reversion at the 5'NC-57 locus, the current evidence suggests that the partially reverted vaccine virus would retain a significantly attenuated phenotype as a result of the remaining, stable attenuating mutations at NS1-53 and NS3-250.

## 2. *In vivo* properties of DEN-2-PDK-53 chimeric viruses

### 2.1. Neurovirulence in newborn ICR mice

The neurovirulence of three ChiDEN-V viruses expressing the prM/E genes of DEN serotypes 2, 3 and 4 has been examined in newborn ICR mice. Because the wild-type DEN-1 16007 strain lacked neurovirulence in newborn ICR mice [44], the ChiDEN-2/1 virus was not tested for neurovirulence in this model. However, an earlier study demonstrated that ChiDEN-2/1 viruses expressing the capsid gene, as well as prM/E genes, of DEN-1 16007 virus in the PDK-53 background also lacked neurovirulence for newborn ICR mice [44].

The DEN-2 PDK-53 virus and the two ChiDEN-V viruses expressing the prM/E genes of DEN serotypes 3 and 4 exhibited attenuation of neurovirulence in groups of newborn ICR mice inoculated intracranially with 10<sup>4</sup> PFU of virus (see Fig. 2, modified from fig. 3 in [41]). No mouse mortality, or evidence of illness requiring euthanasia, occurred in mice injected with these three viruses. These results contrast with the 87.5%, 100%, and 100% morbidity/mortality observed following intracranial challenge with 10<sup>4</sup> PFU of the corresponding wild-type DEN-2 16681, DEN-3 16562 or DEN-4 1036 virus, respectively.

## 2.2. Immunogenicity of DEN-2-PDK-53 chimeric viruses in mice

Wild-type DEN-2, DEN-3, and DEN-4 viruses fail to induce robust anti-viral neutralizing antibody responses after administration to ICR mice [41,44]. Therefore, the immunogenicity of each chimeric virus was tested in transgenic, AG129 knockout mice. This mouse strain lacks receptors for interferon- $\alpha/\beta$  and interferon- $\gamma$ , and has been shown to be more permissive for DEN-2 replication following infection [45]. Immunization of AG129 mice with all four serotypes of DEN virus consistently results in production of neutralizing antibodies, as measured by neutralization of 50% of DEN virus in a plaque-reduction neutralization test (PRNT<sub>50</sub>). Intraperitoneal immunization of adult AG129 mice with 10<sup>4</sup> PFU of ChiDEN-2/1-V virus elicited a pooled reciprocal PRNT<sub>50</sub> titer of 640 against DEN-1 virus, and protected the immunized mice against a severe challenge with the AG129 mouse-virulent DEN-1 Mochizuki virus (see Table 3, modified from table 5 in [41]). In a separate experiment, ChiDEN-2/3V and ChiDEN-2/4-V viruses, each at a dose of 10<sup>5</sup> PFU, elicited neutralizing antibodies in these mice. The magnitude of the neutralizing response in the mice immunized with the ChiDEN-2/4-V virus (pooled reciprocal primary and boosted titers of 40 and 320, respectively) was less robust than in mice that received ChiDEN-3-V (160 and 640, respectively) (Table 3, modified from table 6 in [41]).

A tetravalent formulation of the ChiDEN-V viruses, containing 10<sup>5</sup> PFU of each virus, elicited high neutralizing antibody titers against wild-type DEN-1 (pooled reciprocal primary and boosted titers of 640 and 2560, respectively), DEN-2 (1280 and 2560), DEN-3 (160 and 1280), and DEN-4 (80 and 320) viruses in adult AG129 mice (Table 4; see table 7 in [41]). The neutralizing antibody titers were similar to the homologous titers elicited by each monovalent virus, indicating no significant interference among the vaccine components in tetravalent formulations in the AG129 mouse model.

## 2.3. Preparation for clinical trials: a summary of preclinical development of DENVax

The extensive clinical data with DEN-2 PDK-53 and the *in vitro* and *in vivo* preclinical characterization of the DEN-2 PDK-53-based recombinant chimeras reviewed above support the testing of tetravalent DENVax formulations in human clinical trials. To complete preclinical development of the DENVax vaccine, manufacturing quality DENVax strains were derived by transfection of clinical grade Vero cells with RNA transcribed from cDNA clones of each of the four DENVax viruses. These strains were plaque purified and expanded to make the master seed viruses, working seed viruses and bulk vaccine preparations. The master seed strains were completely sequenced and characterized *in vitro* and *in vivo* to verify retention of all attenuation markers (Huang, et al., in preparation). Tetravalent formulations were manufactured by combining the bulk vaccines with excipients that promote the thermal stability of dengue viruses (Wiggan et al., submitted). Tetravalent DENVax formulations were tested in preclinical models for safety (Huang et al., in preparation). The DENVax formulations also were tested for efficacy in non-human primates [46].

## 2.4. Safety, immunogenicity and efficacy of tetravalent DENVax vaccines in monkeys

Monkeys infected with DEN virus do not develop clinical symptoms. However, viremia and the development of DEN virus-specific neutralizing antibodies have been regarded as

markers of safety and immunogenicity for candidate DEN vaccines. Early immunogenicity studies in monkeys with the ChiDEN-V viruses were conducted at Mahidol University [47]. More recently, we evaluated the safety and efficacy of three tetravalent formulations containing different ratios of DENVax viruses in cynomolgus macaques (*Macaca fascicularis*) [46]. A formulation termed 3:3:3:3 contained  $10^3$  PFU per dose of each of the four DENVax viruses. The 3:3:5:5 formulation contained  $10^3$  PFU of DENVax-1 and DENVax-2 and  $10^5$  PFU of DENVax-3 and DENVax-4 per dose. A 5:5:5:5 formulation contained  $10^5$  PFU of each of the four viruses. Subcutaneous injection of the three DENVax formulations was well-tolerated. Transient, low level viremia of only the DENVax-2 component was detected after the primary immunization (  $2.4 \log$  PFU/mL for no more than five consecutive days), yet virus neutralizing antibody titers were induced against all four dengue virus serotypes after one or two administrations of vaccine (see Fig. 3, from table 1 in [46]). Significant neutralizing antibody titers were induced to DEN-1, DEN-2 and DEN-3 by one or two administrations of the tetravalent formulations. The neutralizing antibody responses to DEN-4 were significantly lower. The 3:3:5:5 formulation, containing increased titers of DENVax-3 and DENVax-4, provided the most balanced neutralizing antibody responses. Many animals seroconverted to multiple DEN serotypes (as defined as a PRNT<sub>50</sub> titer of >10 and a four-fold increase over pre-immunization titer). Significant seroconversion to each DEN serotype (greater than 87.5% seroconversion) was observed after two immunizations with either the 3:3:5:5 or 5:5:5:5 formulation (Table 5). After immunization, animals were segregated into four cohorts for challenge with each of the four wild-type DEN serotypes. While non-human primates do not show clinical signs after DEN infection, there is demonstrable viremia. All animals immunized with the high dose formulation (5:5:5:5) were protected from viremia after challenge with each of the four DENV. Immunization with the lower dose formulations (3:3:3:3 and 3:3:5:5) completely protected animals from viremia caused by DEN-3 virus or DEN-4 virus; immunized animals were partially protected from viremia caused by DEN-1 and DEN-2. These data guided the choice of tetravalent DENVax formulations to be used in subsequent human clinical trials.

The manufacturing and characterization of the DENVax vaccines and data from the preclinical safety, immunogenicity and efficacy studies were submitted to the Food and Drug Administration (FDA) as an Investigational New Drug application (IND). In addition, the data were submitted to the Instituto Nacional de Vigilancia de Medicamentos y Alimentos (INVIMA) the regulatory authorities in Colombia, in support of Phase 1 clinical trials.

## 2.5. Initial Phase 1 study in healthy U.S. volunteers

The first clinical trial to investigate the safety and preliminary immunogenicity of the tetravalent DENVax vaccine is being conducted in healthy adult volunteers in the United States and is sponsored by the Division of Microbiology and Infectious Disease (DMID) of the National Institute of Allergy and Infectious Disease (NIAID). DMID is conducting the clinical trial at its Vaccine and Treatment Evaluation Unit (VTEU) at St. Louis University, St. Louis, MO (see [www.clinicaltrials.gov](http://www.clinicaltrials.gov)). The primary objective of the DMID-sponsored study is to assess the safety and tolerability of the vaccine following administration of two doses of vaccine on days 0 and 90. The study is enrolling a total of 72 subjects, randomized

to one of four groups. Subjects within each group are randomized to receive vaccine or placebo (Phosphate Buffered Saline; PBS) in a 12:6 ratio. The first two groups receive a low dose formulation of DENVax (approximately  $10^4$  PFU of DENVax-1,  $10^3$  PFU of DENVax-2,  $10^4$  PFU of DENVax-3 and  $10^5$  PFU of DENVax-4) (or placebo) administered by either subcutaneous (SC) or intradermal (ID) injection in the deltoid region. The subsequent two groups are receiving a high dose formulation of DENVax (approximately  $10^5$  PFU of DENVax-1,  $10^4$  PFU of DENVax-2,  $10^5$  PFU of DENVax-3 and  $10^5$  PFU of DENVax-4 per dose) or placebo, again administered by either SC or ID injection in the deltoid region. Subjects are vaccinated on day 0 and boosted with the same dose on day 90. Primary outcome measures include the incidence and severity of vaccine-associated Adverse Events (AEs) from the time of the first dose of vaccine through the end of the study, as well as an assessment of vaccine reactogenicity based on the incidence and severity of solicited AEs recorded during the first 14 days after each dose administration. The secondary objective of the study is to evaluate the immune response to the vaccine based on the levels of neutralizing antibodies to each of the four dengue serotypes and the proportion of subjects who seroconvert to each of the four dengue serotypes. The level of viremia associated with each vaccine virus serotype is being assessed based on the quantity of viral RNA in the blood.

## 2.6. Ongoing Phase 1 study in a dengue non-endemic region of Colombia

To assess the safety and efficacy of the vaccine in a population reflective of those at most risk of disease, a second Phase 1 study is being conducted in healthy, flavivirus-naïve, adult volunteers in a high altitude region of Colombia. At high altitude, there is no *Aedes aegypti* and no endemic dengue disease. The objectives of this Inviragen-sponsored study are to evaluate the safety, tolerability and immunogenicity of the DENVax vaccine in healthy adults following subcutaneous or intradermal administration at two different dose levels compared to a saline placebo. The study is evaluating the same dose levels and dosing schedule as the DMID-sponsored study being conducted in the United States, but will enroll a larger number of subjects per group from a population more representative of those in most need of the vaccine.

## 3. Discussion

There are numerous advantages to a tetravalent, chimeric dengue vaccine based on the well-characterized DEN-2 PDK-53 backbone. The mutations in the viral non-structural genes which are necessary and sufficient to express the attenuated phenotype of DEN-2 PDK-53 have been genetically identified [29,41]. The three recombinant virus strains for DEN-1, DEN-3, and DEN-4 contain the same attenuating mutations as the parental DEN-2 PDK-53 strain. Since all four DENVax components share the identical attenuating mutations, recombination between vaccine strains cannot generate more pathogenic viruses.

Direct knowledge of the attenuating mutations in these vaccine candidates permits the design of specific genetic quality control tests to ensure that the vaccine used in clinical studies maintains the attenuated genotype. We have sequenced the genomes of the master seed DENVax viruses to assure the presence of the attenuating mutations in the 5'NCR, NS-1 and NS-3. In addition, we use spot-sequencing of the attenuation loci as a release test

for GMP preparations of each of the four DENVax viruses. The presence of the attenuating mutations assures that the vaccine viruses do not replicate as efficiently as wild-type dengue viruses in mammalian and mosquito cells [41]. Animal model testing demonstrates that the pathogenicity of the vaccine viruses is significantly attenuated compared to wild-type dengue viruses [41].

Attenuation of the viruses through the three mutations in the DEN-2 PDK-53 backbone is achieved without compromising the ability of each of the four viruses to elicit an appropriate serotype-specific neutralizing antibody response. The individual monovalent vaccines and mixtures of all four DENVax viruses induce neutralizing antibody responses to all four DEN viruses in mice [41] and in monkeys [46]. Although there currently is no DEN-3 or DEN-4 challenge model in adult mice, AG-129 mice immunized with tetravalent DENVax formulations are protected from infection by wild-type DEN-1 or DEN-2 viruses ([41] and Huang et al., in preparation). Monkeys immunized with tetravalent DENVax formulations are protected from challenge with wild-type DEN-1, DEN-2, DEN-3 or DEN-4 virus [46]. In this study, animals were challenged 30 days post second vaccination and the short timeframe from vaccination to challenge would be considered by some to not be optimal when testing vaccine protective efficacy.

Mice or monkeys immunized with a priming dose of tetravalent DENVax show no, or significantly reduced, viremia and no signs of disease when a second dose of DENVax is administered. Similarly, monkeys immunized with two doses of DENVax show no signs of disease and no, or low, viremia upon subsequent challenge with wild-type DEN viruses [46]. Thus, DENVax administration does not enhance the infectivity of subsequent doses of live-attenuated or wild-type dengue viruses.

Phase 1 studies of the monovalent DEN-2 PDK-53 candidate vaccine showed that the strain is safe, well-tolerated and generates long-lasting neutralizing antibody and cell-mediated immune responses to DEN-2 [32,33,40]. Vaccination of healthy adult subjects and children with the live-attenuated DEN-2 PDK-53 virus has been well tolerated and immunogenic in both monovalent and multivalent vaccine formulations. The vaccine development studies described in this review have culminated in the testing of tetravalent DENVax formulations in human clinical trials. The Phase 1 studies in progress at the time of this review are designed to determine if DENVax is safe in healthy adults who are flavivirus negative. The safety profile of DENVax is being determined not only in U.S. adults but also in adults in Colombia, in a high altitude, non-endemic region. In addition, preliminary immunogenicity results from these trials will assess whether two different formulations of DENVax can elicit neutralizing antibodies to all four dengue vaccines. Finally, DENVax is being delivered by two different routes of administration (SC and ID) in these early Phase 1 studies.

The DENVax vaccine is considerably different from previously tested tetravalent vaccines in that all four strains contain the same attenuating mutations as the DEN-2 PDK-53 strain, a strain that has been shown to be both safe and immunogenic in humans. Clinical data from the ongoing studies will shed light on whether these advantages translate to a safe dengue vaccine that can rapidly induce neutralizing antibodies against all four DEN serotypes. Such

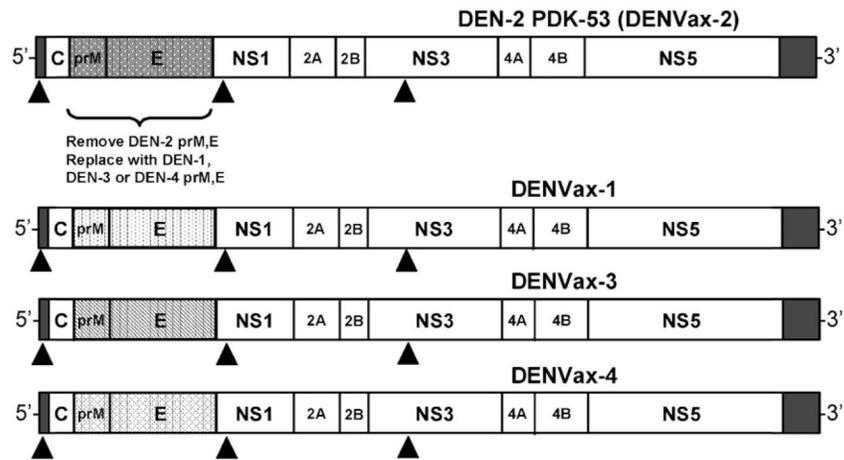
a vaccine is critically needed to protect people from the threat of dengue infection and improve public health worldwide.

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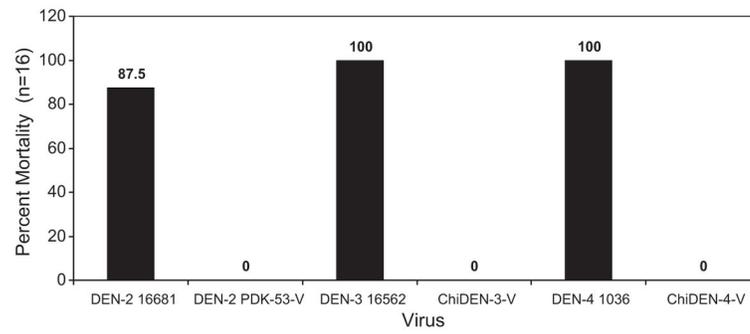
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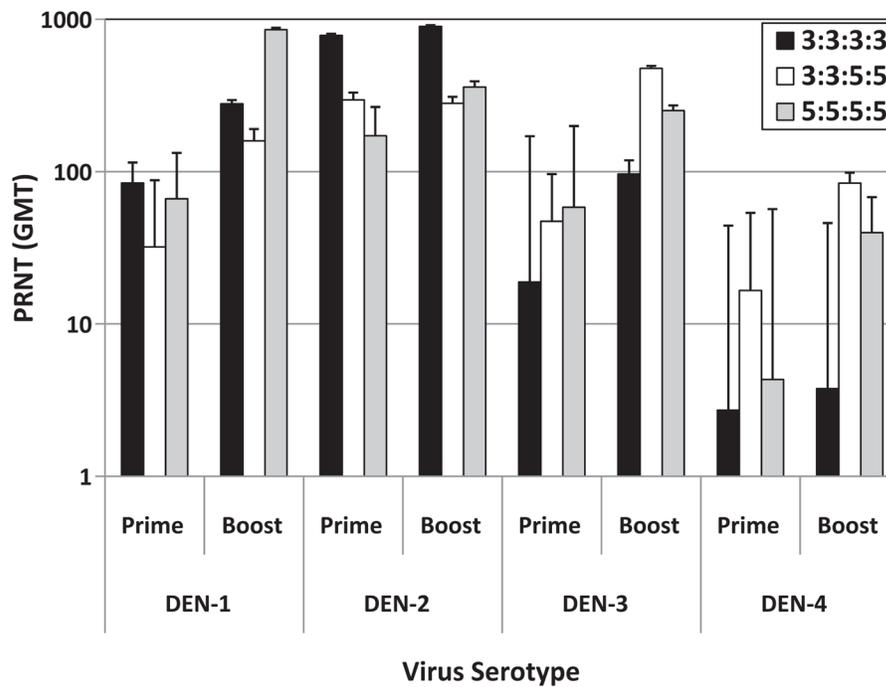


**Fig. 1.** Genetic structure of DENVax vaccine strains. The diagram shows the gene arrangements for the parental DEN-2 PDK-53 as well as the three chimeras, DENVax-1, -3 and -4. Arrows indicate the three pivotal mutations in DEN-2 PDK-53 that determine the attenuating phenotype of the viruses (see text). Shading indicates the origin of the prM and E genes in the chimeras.

See Huang et al. [41] for details.



**Fig. 2.** Neurovirulence of ChiDEN-V viruses in newborn ICR mice. Newborn ICR mice were challenged intracranially with  $10^4$  PFU of each virus. Percent mortality = dead mice and mice euthanized at signs of morbidity. Data are from fig. 2 in Huang et al. [41].



**Fig. 3.** Neutralizing antibody titers after DENVax administration to non-human primates. Groups of eight cynomolgus macaques were immunized with the three indicated tetravalent DENVax formulations (see text) on days 0 and 60. Sera were collected at various time points after immunization. The figure indicates geometric mean PRNT<sub>50</sub> 30 days after the primary (“Prime”) and 30 days after the secondary (“Boost”) immunizations for each of the four DEN serotypes.

Table 1

Summary of literature pertaining to human clinical experience with DEN-2 PDK-53.

Author (year of publication) [reference]	Study design	Observations
Bhamarapravati (1987) [32]	Single subcutaneous (SC) dose of <i>monovalent</i> DEN-2 PDK-53 to 10 healthy adult dengue negative volunteers.	<i>Safety</i> : No reactions at the injection site. Headache and abdominal pain reported in 5 of 10 subjects. Myalgia, anorexia and nausea occurred less frequently. <i>Viremia</i> : Detected in one volunteer on day 10. <i>Immunogenicity</i> : Seroconversion in all subjects to DEN-2 that persisted for 1.5 years
Vaughn (1996) [33]	Single SC dose of <i>monovalent</i> DEN-2 PDK-53 to 10 healthy adult dengue negative volunteers.	<i>Safety</i> : No reactions at the injection site. Transient headache, myalgia and arthralgia reported in several subjects. <i>Viremia</i> : Detected in 8 of 10 subjects between days 4 and 12. <i>Immunogenicity</i> : Seroconversion to DEN-2 detected in all subjects. Neutralizing antibody response persisted.
Bhamarapravati (2000) [35]	Single SC dose of <i>bivalent</i> DEN-2 PDK-53 with DEN-4 or DEN-1 or <i>trivalent</i> with DEN-1 and DEN-4. Single SC dose of <i>tetravalent</i> formulation.	<i>Safety</i> : No details of observed side effects; authors stated that the vaccines were well-tolerated. <i>Viremia</i> : No data provided. <i>Immunogenicity</i> : Seroconversion in all subjects to two or three serotypes given bivalent or trivalent vaccines, respectively. All six subjects given tetravalent vaccine seroconverted to DEN-1 and DEN-3, five of six converted to DEN-2 and four of six seroconverted to DEN-4.
Kanesathasan (2001) [36]	Single SC dose of <i>monovalent</i> DEN-2 PDK-53 to five healthy flavivirus seronegative adult volunteers.	<i>Safety</i> : No reactions at the injection site. Mild symptoms of headache (4), myalgia, malaise, pruritus or transient fever reported. Slight leukopenia in two subjects around day 10. <i>Viremia</i> : Virus isolation by polymerase chain reaction (PCR) in 20% of subjects days 7–10 post dosing. <i>Immunogenicity</i> : All seroconverted.
Kanesathasan (2001) [36]	Single SC dose of <i>tetravalent</i> vaccine: DEN-2 PDK-53 combined with live-attenuated DEN-1, DEN-3 and DEN-4 <sup>a</sup> , to 10 healthy adult volunteers.	<i>Safety</i> : Erythema at the injection site in 2 of 10 volunteers. Moderate headache, malaise and myalgia, eye pain and pruritus were common. Four subjects had mild fever and all experienced maculopapular rashes over the trunk and extremities. Increased liver enzymes in a majority of subjects. <i>Viremia</i> : Detected in all subjects between days 5 and 12, identified as DEN-3. One volunteer had concurrent DEN-3 and DEN-4 viremia on day 11. <i>Immunogenicity</i> : Highest antibody titers were to DEN-3 but only one subject seroconverted to all four DEN serotypes.
Sabchareon (2002) [37]	Two doses of <i>tetravalent</i> vaccine given 6 months apart to 59 adults. Dosed with placebo ( $N = 10$ ) or various tetravalent formulations <sup>a</sup> combinations of dose. <i>Safety</i> : Majority of subjects with mild to headache, rash, eye pain, fever and myalgia. Five subjects were hospitalized for dengue-like fever with concomitant severe neutropenia and thrombocytopenia. Best safety profile observed in groups with lowest dose of DEN-3 (1 log <sub>10</sub> PFU) suggesting the DEN-3 virus was associated with adverse events. <i>Viremia</i> : DEN-3 virus detected in 47 of 49 subjects after first dose. DEN-2 or DEN-4 viremia detected in 21 of 47 after second dose. <i>Immunogenicity</i> : 100% seroconversion to DEN-3, 85%, 78% and 71% seroconversion to DEN-1, DEN-2 and DEN-4 after the second dose	
Sabchareon (2004) [38]	Three doses of <i>tetravalent</i> vaccine <sup>a</sup> ; 0, 3–5 months and 8–12 months. Subjects were 103 healthy children aged 5–12 years. Two tetravalent formulations tested.	<i>Safety</i> : After first dose, mild to moderate symptoms fever, rash, headache and myalgia observed in 79%–90% of subjects. Five subjects had severe reactions including dengue-like fever in one subject. Mild and moderate increases in ALT and neutropenia observed after first dose. <i>Viremia</i> : DEN-3 viremia observed in >75% of subjects after first dose but not seen thereafter. Viremia for DEN-1, DEN-2 and DEN-4 seen after first, second and third doses in a small percentage of subjects. <i>Immunogenicity</i> : 100% seroconversion for DEN-1, DEN-2 and DEN-3; 89–100% seroconversion to DEN-4 after three doses.

Author (year of publication) [reference]	Study design	Observations
Kitchener (2006) [18]	One dose of two formulations of <i>tetavalent</i> vaccine <sup>a</sup> administered adult volunteers. All subjects seronegative for dengue.	<i>Safety</i> : All 10 subjects developed mild to moderate reactions consistent with mild dengue-like syndrome, including fever, headache, malaise, nausea or vomiting, arthralgia, myalgia, eye pain and skin rash. Study was discontinued by the sponsor prior to administration of intended second dose. <i>Viremia</i> : DEN-3 detected in seven of 10 subjects. DEN-4 was detected in two subjects. DEN-1 and DEN-2 were not detected. <i>Immunogenicity</i> : Seroconversion in all subjects to DEN-3. Six of 10 seroconverted to DEN-1 and DEN-4 and four of 10 seroconverted to DEN-2.
Chanthavanich (2006) [40]	Single dose of various formulations of <i>tetavalent</i> vaccine <sup>a</sup> administered to 113 volunteers aged 4–15 years. Age and address matched with 226 controls. Follow-up 3–8 years after vaccination.	<i>Safety</i> : No excess hospitalizations for clinically suspected dengue fever or DHF in vaccinees (4 of 113: one with dengue fever and three with hemorrhagic fever) compared to the unvaccinated control children (14 of 226 with dengue hemorrhagic fever). <i>Immunogenicity</i> : 75% of the individuals had seroconverted to DEN-2 virus 6–12 months post-vaccination while 82% had seroconverted 3–8 years after vaccination. Similar trends were observed for DEN-1, DEN-3 and DEN-4. The increase in seroconversion is likely due to natural exposure of the children in the endemic area.

<sup>a</sup> Attenuated DEN-1, DEN-3 and DEN-4 strains in the tetavalent vaccines were independently derived by serial passage in cell culture and were *not* based on DEN-2 PDK-53.

**Table 2**

Sources of prM/E gene regions in DENVax vaccine viruses.

<b>Virus</b>	<b>Strain</b>	<b>Origin</b>	<b>Source</b>	<b>Reference</b>
Viral origins of the prM/E gene region expressed in ChiDEN and DENVax vaccine viruses <sup>a</sup>				
DEN-1	16007	Thailand, 1964	DHF/DSS patient	Halstead and Simasthien [48]
DEN-2	16681	Thailand, 1964	DHF/DSS patient	Halstead and Simasthien [48]
DEN-3	16562	Philippines, 1964	DHF patient	Halstead and Simasthien [48]
DEN-4	1036	Indonesia, 1976	DF patient	Gubler et al. [49]

<sup>a</sup>See table 1 in [43].

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Table 3

Immunogenicity of Monovalent ChIDEN-V viruses in adult AG129 mice.

Virus	Experiment	Immunization dose <sup>a</sup>	Antibody titer (PRNT <sub>50</sub> )	Survivors (A)	Neutralizing antibody titer at day 68 (B)
PBS	A <sup>b</sup>	n.a. <sup>c</sup>	<10 <sup>d</sup>	0/1 <sup>e</sup>	n.a.
DEN-1 16007	A	10 <sup>4</sup> PFU	2560	6/6	n.a.
ChIDEN-2/1	A	10 <sup>4</sup> PFU	640	6/6	n.a.
DEN-3 16562	B <sup>f</sup>	10 <sup>5</sup> PFU	320	n.a.	640
ChIDEN-2/3	B	10 <sup>5</sup> PFU	160	n.a.	640
DEN-4 1036	B	10 <sup>5</sup> PFU	640	n.a.	1280
ChIDEN-2/4	B	10 <sup>5</sup> PFU	40	n.a.	320

<sup>a</sup> Mice immunized, challenged, and boosted intraperitoneally.

<sup>b</sup> Experiment A: 3–5-week-old AG129 mice immunized intraperitoneally, bled at day 27, then challenged intraperitoneally at day 28 with 10<sup>7</sup> PFU of wild-type DEN-1 Mochizuki virus. Mice were bled again at 30 days after the DEN-1 virus challenge. See table 5 in [41].

<sup>c</sup> Not applicable.

<sup>d</sup> Serum-dilution plaque-reduction (PRNT) antibody titer for pooled sera. PRNT<sub>50</sub> titers represent reciprocal antibody titers at which 50% or more of the homologous wild-type input virus was neutralized.

<sup>e</sup> Number of survivors/total AG129 mice challenged intraperitoneally with 10 PFU of DEN-1 Mochizuki virus.

<sup>f</sup> Experiment B: 6–8-week-old AG129 mice were immunized intraperitoneally, bled on day 40, then boosted intraperitoneally with the same dose of virus on day 42. Mice were bled again at 26 days after the boost. See table 6 in [41].

**Table 4**

Immunogenicity of tetravalent ChiDEN viruses in adult AG129 mice.

Tetravalent formulation	a-DEN-1 PRNT <sub>50</sub>		a-DEN-2 PRNT <sub>50</sub>		a-DEN-3 PRNT <sub>50</sub>		a-DEN-4 PRNT <sub>50</sub>	
	Day 40	Day 68						
ChiDEN-Tetra-V <sup>a</sup>	640 <sup>b</sup>	2560	1280	2560	160	1280	80	320

Source of data: table 7 in Huang et al. [41].

<sup>a</sup> 6–8-week-old AG129 mice were immunized intraperitoneally with mixture of 10<sup>5</sup> PFU of each virus at days 0 and 42. Mice were bled on days 40 and 68.

<sup>b</sup> Reciprocal PRNT<sub>50</sub> antibody titer of pooled sera ( $n = 6$ ) against wild-type DEN-1 16007, DEN-2 16681, DEN-3 16562, and DEN-4 1036 viruses.

**Table 5**

Percentage of non-human primates that seroconverted\* following primary and secondary immunizations with DENVax formulations.

Formulation	DENV-1		DENV-2		DENV-3		DENV-4	
	Day 30	Day 91						
3:3:3:3	87.5%	100%	100%	100%	75%	100%	37.5%	37.5%
3:3:5:5	62.5%	87.5%	87.5%	100%	100%	100%	87.5%	100%
5:5:5:5	87.5%	100%	87.5%	100%	87.5%	100%	50%	87.5%
Control	0%	0%	0%	0%	0%	0%	0%	0%

Source of data: Osorio et al. [46].

\*: Seroconversion: defined as PRINT 10 and 4-fold increase in PRNT<sub>50</sub> over day 0 baseline titer. All animals received DENVax formulations or vaccine diluent subcutaneously in 0.5 mL on the upper back on days 0 and 60.