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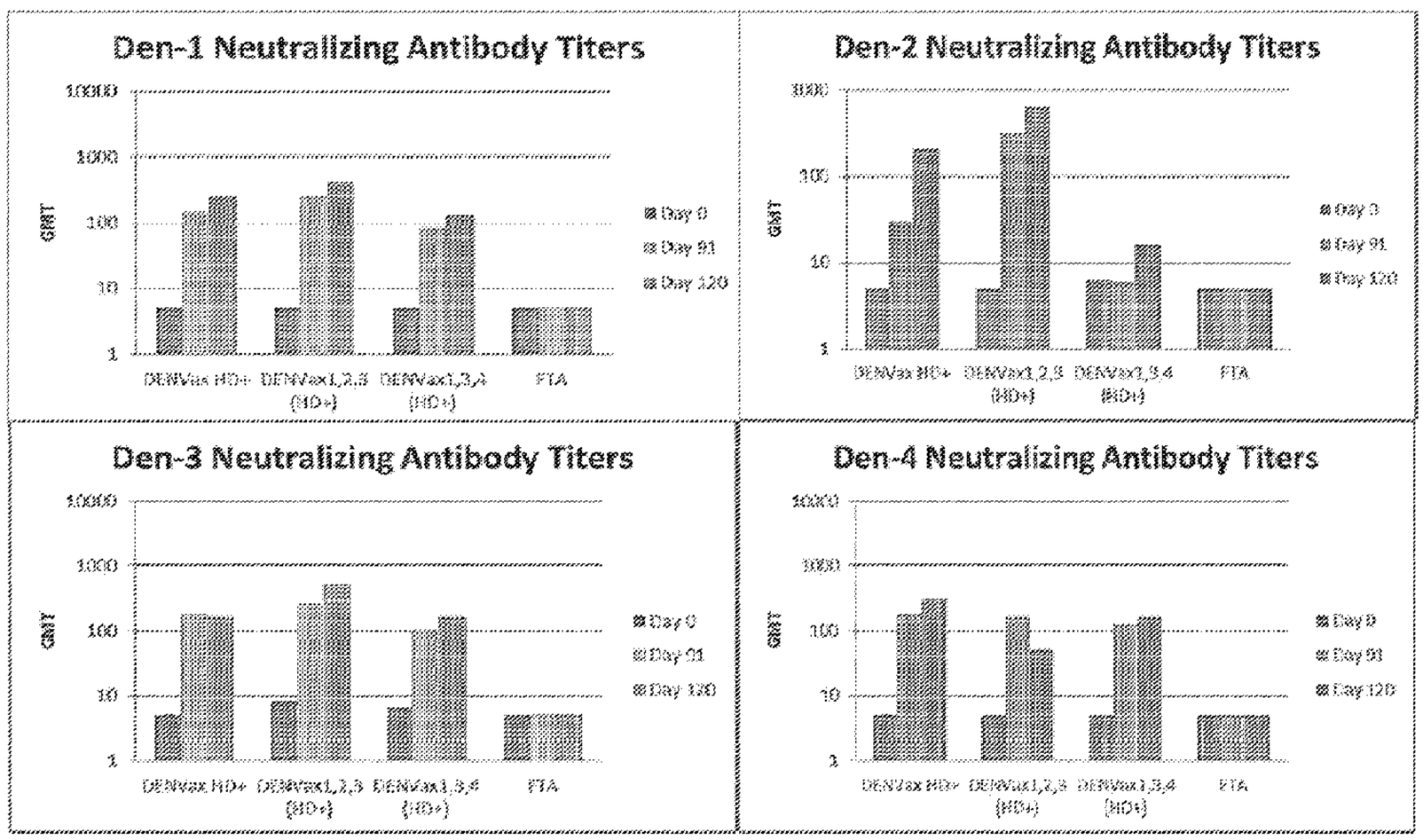
(54) **Titre : COMPOSITIONS, PROCEDES D'ADMINISTRATION ET UTILISATIONS DE FORMULATIONS TRIVALENTES CONTRE LE VIRUS DE LA DENGUE**
(54) **Title: COMPOSITIONS, METHODS OF ADMINISTRATION AND USES FOR TRIVALENT DENGUE VIRUS FORMULATIONS**

Fig. 1



(57) **Abrégé/Abstract:**
Embodiments of the present invention report compositions and methods for vaccinating a subject using trivalent dengue virus vaccine compositions. In some embodiments, more than one vaccine composition may be administered to a subject in different anatomical locations in order to induce a rapid response to at least three of four dengue virus serotypes. In certain embodiments, administration of a trivalent dengue virus vaccine composition can be combined with administration of a monovalent dengue virus vaccine composition.

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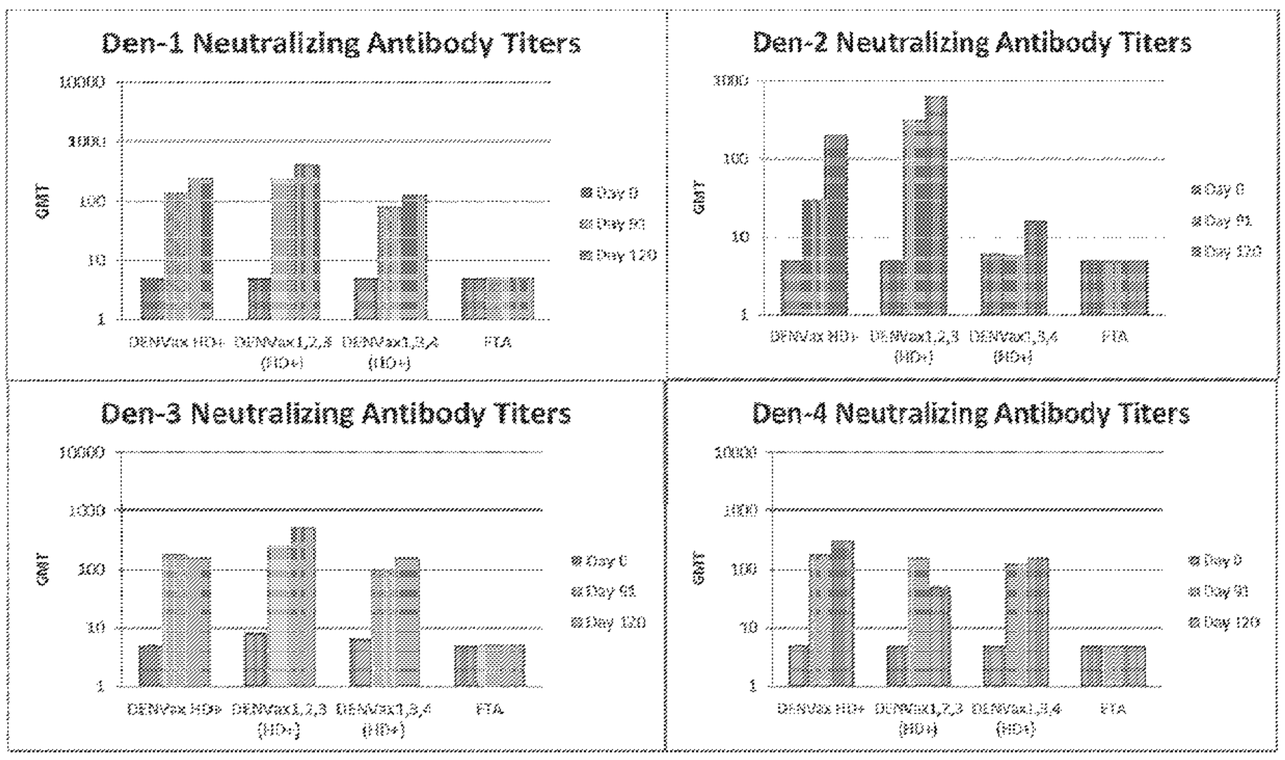
Fig. 1



(57) Abstract: Embodiments of the present invention report compositions and methods for vaccinating a subject using trivalent dengue virus vaccine compositions. In some embodiments, more than one vaccine composition may be administered to a subject in different anatomical locations in order to induce a rapid response to at least three of four dengue virus serotypes. In certain embodiments, administration of a trivalent dengue virus vaccine composition can be combined with administration of a monovalent dengue virus vaccine composition.

WO 2014/093182 A1

COMPOSITIONS, METHODS OF ADMINISTRATION AND USES FOR TRIVALENT DENGUE VIRUS FORMULATIONS

FIELD

[0001] Embodiments of the present invention report compositions and methods for administering a vaccine to a subject against dengue virus serotypes. In some embodiments, vaccine compositions disclosed herein may be administered by subcutaneous, intradermal, intramuscular or other injection or introduction methods against three dengue virus serotypes. In other embodiments, vaccine compositions disclosed herein may be administered to a subject against three dengue virus serotypes (trivalent formulation) followed by a monovalent formulation, bivalent or other trivalent dengue virus vaccine formulation. In certain embodiments, administration of a vaccine to a subject can include administration to two or more anatomical sites of one or more trivalent and optionally, monovalent vaccine compositions. Other embodiments include follow-on injections from within days of a first vaccination to up to 12 months after initial injection(s). In certain embodiments, compositions against dengue virus include trivalent formulations where the formulations comprise chimera constructs having a DEN-2 backbone and for example having combination compositions of chimeras of DEN 1, 3 or 4 on a DEN-2 backbone.

BACKGROUND

[0002] Vaccines for protection against viral infections have been effectively used to reduce the incidence of human disease. One of the most successful technologies for viral vaccines is to immunize animals or humans with a weakened or attenuated strain of the virus (a “live, attenuated virus”). Due to limited replication after immunization, the attenuated strain does not cause disease. However, the limited viral replication is sufficient to express the full repertoire of viral antigens and can generate potent and long-lasting immune responses to the virus. Thus, upon subsequent exposure to a pathogenic strain of the virus, the immunized individual is protected from disease. These live, attenuated viral vaccines are among the most successful vaccines used in public health.

[0003] Dengue viruses are mosquito-borne pathogens of the genus Flavivirus (family Flaviviridae). Four serotypes of dengue virus (often abbreviated "DEN" or “DENV”) had been identified, including dengue-1, dengue-2, dengue-3 and dengue-4 (DEN-1 to DEN-4). The flavivirus genome is a single-stranded, positive-sense RNA approximately 11 kb in length, containing a 5'-noncoding region (5'NC); a coding region encoding the viral structural

proteins; five nonstructural proteins, designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5; and a 3'-noncoding region (3'NC). The viral structural proteins include the capsid, premembrane/membrane and envelope. The structural and nonstructural proteins are translated as a single polyprotein. The polyprotein is then processed by cellular and viral proteases.

[0004] Transmitted by Aedes aegypti mosquitoes to humans in tropical and subtropical regions of the world, dengue viruses cause millions of cases of disease every year, ranging from dengue fever to the often fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Secondary infection of humans with a heterologous serotype of DEN virus may induce an immunopathological response and is considered a possible risk factor for DHF/DSS. Therefore, the need exists for development of a vaccine that confers simultaneous protection against all dengue virus strains.

SUMMARY

[0005] Embodiments of the present invention generally relate to methods and compositions for inducing protection in a subject against multiple a dengue viruses by, for example, by administering a trivalent dengue virus vaccine composition to a subject. In accordance with these embodiments, a trivalent dengue virus vaccine composition includes immunogenic agents to at least three of four dengue virus serotypes or even five of the dengue virus serotypes.

[0006] Some embodiments can include introducing a trivalent dengue virus vaccine composition disclosed herein to a subject in one or more anatomical locations in the subject. In accordance with these embodiments, dengue virus vaccine composition can be introduced to a subject by any method known in the art to, for example, induce neutralizing antibodies against at least three dengue virus serotypes. Other embodiments include administering a trivalent dengue virus vaccine formulation to a subject and then following administration of the trivalent formulation with a monovalent formulation on the same or within 12 months after first administration. Other embodiments include treating a subject with a monovalent dengue virus vaccine composition first and then administering on the same or sometime later, but at least within 12 months of the monovalent dengue virus vaccine formulation at least one trivalent dengue virus vaccine composition.

[0007] Some embodiments concern administering a single trivalent dengue virus vaccine composition to a subject in need thereof to induce a response to three or four dengue virus serotypes.

[0008] In certain embodiments, a vaccine composition can include, but is not limited to, a single dose formulation of a trivalent dengue virus serotype vaccine. In accordance with these embodiments, a trivalent formulation can be a trivalent formulation of dengue-1 (DEN-1); dengue-2 (DEN-2), dengue-3 (DEN-3) and/or dengue-4 (DEN-4). Further, these formulations can be a predetermined ratio of dengue virus serotypes.

[0009] In other embodiments, a vaccine composition may include, but is not limited to; an initial dose of a trivalent dengue virus vaccine composition followed by one or more boosts of the same, or a different trivalent or one or more monovalent dengue-virus vaccine composition administered to a subject in need thereof.

[00010] Other aspects herein can concern inducing a humoral or cellular immune response in a subject by, for example, introducing a vaccine composition to a subject via an intradermal route wherein the vaccine composition includes, but is not limited to, a dengue virus vaccine. In accordance with these embodiments, compositions disclosed can be administered intradermally to a subject for modulating neutralizing antibody production in the subject against three or more dengue virus serotypes. Some aspects concern predetermined composition ratios of three of DEN-1:DEN-2:DEN:3:DEN:4 (*e.g.* 1:1:1, 1:2:1, 1:1:100,000 etc. or any ratio of three serotypes) of the various serotypes of dengue virus or fragments thereof or attenuated compositions thereof in a single vaccine composition in order to increase cross protection and levels of neutralizing antibodies in a subject against at least three dengue virus serotypes when the subject is administered the single vaccine composition. Other embodiments concern administering a trivalent dengue virus vaccine composition to a subject in need thereof via subcutaneously or by other mode known in the art. In addition, certain embodiments concern treating a subject with at least one additional injection(s) of a trivalent or monovalent dengue virus vaccine composition administered at a separate site from the first injection, for example, in close proximity to the initial injection or in a distant anatomical site on the subject. In addition, at least one additional intradermal injection(s) may be performed less than 30 days after the first administration to the subject while others are performed 30 days and up to 12 months after the first administration of the vaccine.

[00011] In some embodiments, a single dose trivalent vaccine against dengue virus can include constructs of three different dengue virus serotype antigens (e.g. structural proteins) in a single trivalent composition. In accordance with these embodiments, constructs contemplated herein include attenuated dengue-2 virus as a backbone for one or more structural dengue virus proteins capsid (C), premembrane/membrane (prM/M), or envelope (E). In addition, nonstructural proteins may also be included in a construct, including, nonstructural proteins NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, and 3' NCR. In certain embodiments, a trivalent dengue virus vaccine composition can include constructs having an attenuated dengue-2 virus (e.g. PDK-53) where structural proteins of dengue-2 are replace with one or more structural proteins of DEN-1, DEN-3 or DEN-4. Trivalent dengue virus vaccine compositions can be DENVax 1, 2, 3; DENVax 2, 3, 4; DENVax1, 2, 4 or DENVax1, 3, 4 on an attenuated DEN-2 backbone.

[00012] In certain embodiments, a trivalent dengue virus vaccine composition disclosed herein can include dengue virus constructs in any combination of three where pfus of the various compositions can range from 1 to about 100,000 fold difference depending on desired ratio and endemic conditions in a given location. Some embodiments include up to 100,000 fold pfu difference between DEN-2 and DEN-4 where DEN-4 can be represented up to 100,000 fold higher than DEN-2. Certain embodiments include compositions where dengue virus serotypes are 10 fold higher to 10 fold lower than for example when DENVax1 is 1.25×10^4; DENVax2 is $6.04 \times 10^{4;}$ DENVax3 is 1.30×10^5 and DENVax4 is $1.34 \times 10.^6$

[00013] Other embodiments disclosed herein relate to methods and compositions for inducing protection in a subject against all dengue virus serotypes by, for example, administering a trivalent vaccine to a subject against three dengue virus serotypes in two or more doses in one or more than one anatomical location consecutively within a short interval of time. Some embodiments can include introducing a vaccine composition to a subject via intradermal (ID), subcutaneous (SC), or intramuscular (IM) injection in one location and consecutively in another anatomical location by ID, SC, IM or by other introduction method at a second different anatomical location. Other embodiments include using any combination of modes of administration for introducing a dengue virus vaccine of all dengue virus serotypes to a subject where administration of the vaccine occurs at two or more anatomical sites or by two or more different routes consecutively on the same day to the subject.

[00014] In other embodiments, a subject may be administered dengue virus trivalent vaccinations consecutively at two or more anatomical locations, then the subject can be

administered at least a third vaccine within 30 days such as about 7, about 14, about 21 or about 28 days later with a composition comprising dengue virus serotypes which can include a monovalent formulation. Subsequent vaccinations may depend on personalized titers of antibodies post dual injection or other criteria such as results of test populations. In certain embodiments, a subsequent vaccination may only include a single dengue serotype (e.g. DEN-4).

[00015] In certain embodiments, the composition introduced to the subject comprises vaccines against three of four dengue virus serotypes, for example a trivalent formulation based on DENVax or another similar formulation. DENVax comprises a tetravalent dengue vaccine of predetermined ratio where the vaccine is made up of constructs on an attenuated DEN-2 backbone (see for example, PCT Application Number PCT/US01/05142 filed on February 16, 2001 incorporated herein by reference in its entirety for all purposes). In other compositions, all dengue vaccine virus serotypes are in equal proportions in the composition. In yet other compositions, each dengue vaccine virus serotype may be in a particular ratio to one another such that introduction of the composition induces sufficient levels of neutralizing antibodies which would provide the subject with sufficient protection against infection (*e.g.* DEN-1, DEN-2, DEN-3 and/or DEN-4). In accordance with these embodiments, samples from a subject may be analyzed for resistance to dengue infection using standard means known in the art in order to assess immunity to dengue virus serotypes.

[00016] In other embodiments, a vaccine composition can include attenuated dengue virus serotypes in combination with other anti-pathogenic compositions (*e.g.* Japanese encephalitis, yellow fever, West Nile, influenza, Chikungunya or other).

Brief Description of the Drawings

[00017] The following drawings form part of the present specification and are included to further demonstrate certain embodiments. Some embodiments may be better understood by reference to one or more of these drawings alone or in combination with the detailed description of specific embodiments presented.

[00018] **Fig. 1** represents neutralizing antibody titers produced against two different trivalent formulations over time compared to control compositions (e.g. FTA).

Definitions

[00019] As used herein, “a” or “an” may mean one or more than one of an item.

[00020] As used herein, vessel can include, but is not limited to, test tube, mini- or

micro-fuge tube, channel, vial, microtiter plate or container.

[00021] As used herein the specification, “subject” or “subjects” may include but are not limited mammals such as humans or mammals, domesticated or wild, for example dogs, cats, other household pets (*e.g.*, hamster, guinea pig, mouse, rat), ferrets, rabbits, pigs, horses, cattle, prairie dogs, or zoo animals.

[00022] As used herein, “about” or “approximately” can mean plus or minus ten percent.

[00023] As used herein, “Dengue viruses" or "DENs" or “DENVs” are positive, single-stranded RNA viruses belonging to the Flavivirus genus of the flaviviridae family. The genomic RNA contains a type I cap at the 5' end but lacks a poly-A tail at the 3' end. The genomic organization consists of the following elements: 5' noncoding region (NCR), structural proteins (capsid (C), premembrane/membrane (prM/M), envelope (E)) and nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5), and 3' NCR. The genomic viral RNA is associated with the capsid proteins so as to form a nucleocapsid. As for the other flaviviruses, the DEN viral genome encodes an uninterrupted coding region which is translated into a single polyprotein.

[00024] As used herein, “attenuated virus” can mean a virus that demonstrates reduced or no clinical signs of disease when administered to a subject such as a mammal (*e.g.*, human or an animal).

[00025] As used herein, “consecutively” can mean in close temporal proximity, usually within a single patient visit and within 24 hours.

[00026] As used herein, “administration” can mean delivery of a vaccine or therapy to an individual animal or human by any one of many methods such as intradermal, subcutaneous, intramuscular, intranasal, inhalation, vaginal, intravenous, oral, buccal, by inhalation, intranasally, or any others known in the art.

DESCRIPTION

[00027] In the following sections, various exemplary compositions and methods are described in order to detail various embodiments. It will be obvious to one skilled in the art that practicing the various embodiments does not require the employment of all or even some of the details outlined herein, but rather that concentrations, times and other details may be modified through routine experimentation. In some cases, well-known methods or components have not been included in the description.

[00028] Certain aspects of the present invention include, but are not limited to, administration of vaccine compositions against dengue virus.

[00029] Embodiments of the present invention generally relate to methods and compositions for inducing protective neutralizing antibodies in a subject against three or more dengue virus serotypes. Other embodiments can include introducing a vaccine composition to a subject via any method known in the art including, but not limited to, intradermal, subcutaneous, intramuscular, intranasal, inhalation, orally, intranasally, vaginal, intravenous, ingested, and any other method wherein the vaccine composition so introduced induces neutralizing antibodies against three or more dengue virus serotypes. In certain embodiments, the vaccine composition comprises a dose of a vaccine against three (trivalent) dengue virus serotypes administered to a subject. In other embodiments, the vaccine composition comprises an initial dose against three dengue serotypes then, one or more of the same other vaccine compositions administered to a subject.

[00030] Vaccines against dengue virus may include a composition comprising predetermined ratios of three of the four live, attenuated dengue vaccine viruses, recombinant dengue vaccine viruses, chimeric viruses or mutants thereof.. The ratios of various dengue serotypes may be equivalent or nearly equal in representation or certain serotypes may be represented at higher concentrations than others depending on need or ability to induce a balanced neutralizing antibody response in the subject. In accordance with these embodiments, ratios of different dengue vaccines may differ by 2 to 100,000 fold (e.g. plaque forming units (pfus)) between any two serotypes. This can depend on, for example, number of serotypes represented in the formulation, predetermined response and desired effect. It is contemplated that any dengue vaccine virus serotype formulation may be used to generate a vaccine (*e.g.* attenuated virus etc.) of use in consecutive administration to a subject in need thereof where the composition includes, but is not limited to, three or more dengue virus serotypes.

[00031] In other embodiments, compositions of dengue virus vaccine formulations may be introduced to a subject prior to, during or after exposure to dengue virus by the subject. In accordance with these embodiments, a subject may receive more than one administration consecutively or more than one administration comprising a dengue virus formulation, optionally, followed by one or more additional administrations at a later time. Intradermal, subcutaneous, intramuscular, intranasal, inhalation, vaginal, intravenous, oral, and any other method of applications of formulations described herein may be combined with any other anti-viral treatment. In some embodiments, it is contemplated that intradermal, subcutaneous, intramuscular introduction of a formulation contemplated herein

may be administered to any appropriate region of a subject's body (*e.g.* arm, shoulder, hip, intranasally etc). In addition, parenteral administration of vaccine formulations may be combined with other modes of administration such as intranasal, pulmonary, oral, buccal, or vaginal in consecutive administrations. In some embodiments, it is contemplated that, after consecutive administrations as described herein primary or booster administrations may occur consecutively on the same day, consecutive days, weekly, monthly, bi-monthly or other appropriate treatment regimen.

[00032] Dengue is endemic in Asia, Central and South America including Colombia, the Caribbean, the Pacific Islands, and parts of Africa and Australia. It is estimated that 3.6 billion people (55% of the world's population) live in areas at risk of dengue virus transmission (DVI). Infection with a dengue virus can result in a range of symptoms, from subclinical disease to debilitating but transient dengue fever to life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Currently, there is no therapeutic treatment or prophylactic vaccine for dengue fever. Given the impact of dengue on populations in endemic countries and on travelers to those regions, a vaccine to prevent dengue is needed.

[00033] Dengue is a mosquito borne viral disease, transmitted from human to human primarily by the mosquito, *Aedes aegypti*. Dengue viruses (DEN) contain a single-stranded, positive-sense RNA genome of approximately 11 kb. The genome consists of three structural proteins, capsid (C), premembrane (prM), and envelope (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. There are four different serotypes of dengue viruses, DEN-1, DEN-2, DEN-3 and DEN-4. Primary infection with a given serotype induces lifelong serotype specific immunity. However, there is no long-term cross-protective immunity against the other three dengue virus serotypes, and subsequent infection with an alternate serotype leads to increased probability of more severe disease, such as DHF or DSS.

[00034] Due to the disease enhancement associated with secondary DENV infections, a multivalent (e.g. trivalent) vaccine that stimulates immunity against at least three dengue virus serotypes is contemplated. Several DENV vaccine candidates attenuated by classical serial passage in cell culture have proven unsafe or poorly immunogenic. Chimeric live-attenuated, recombinant DENV vaccines candidates, including viruses based on the attenuated genetic background of yellow fever 17D (YF-17D) vaccine virus, DENV-2 PDK-53 vaccine virus, or DENV-4 containing a 30-nucleotide 3' non-coding region (NCR) deletion are known in the art.

[00035] Live, attenuated dengue viruses of all four serotypes have been developed at Mahidol University in Thailand by passaging the wild-type viruses in cell culture. These are currently the most promising live, attenuated vaccine candidates for immunization against dengue virus infection and/or disease. These vaccine candidates have been designated by a combination of their dengue serotype, the cell line through which they were passaged and the number of times they were passaged.

[00036] Preliminary human clinical trials with these attenuated viruses have indicated that DEN-2 PDK-53 has the lowest infectious dose (50% minimal infectious dose of 5 plaque forming units or PFU) in humans, is strongly immunogenic, and produces no unacceptable clinical symptoms. The DEN-1 PDK-13, DEN-3 PGMK-30/FRhL-3 and DEN-4 PDK-48 vaccine virus candidates have higher 50% minimal infectious doses of 10,000, 3500, and 150 PFU, respectively, in humans.

[00037] The DEN-2 PDK-53 virus vaccine candidate, henceforth abbreviated PDK-53, has several measurable biological markers associated with attenuation, including temperature sensitivity, small plaque size, decreased replication in mosquito C6136 cell culture, decreased replication in intact mosquitoes, loss of neurovirulence for suckling mice and decreased incidence of viremia in monkeys. Clinical trials of the candidate PDK-53 vaccine have demonstrated its safety and immunogenicity in humans. Furthermore, the PDK-53 vaccine induces dengue virus-specific T-cell memory responses in human vaccine recipients. In certain embodiments herein, a chimeric dengue virus construct can include a dengue-2 backbone where PDK-53 has been selected to differ from the known PDK-53 backbone and contain additional mutations that further attenuate the virus.

[00038] Accordingly, there is a need for avirulent, yet immunogenic, dengue viruses to be used in the development of dengue virus vaccines to confer protection against all dengue virus serotypes.

[00039] A challenging issue in the development of an effective live-attenuated dengue virus (DENV) vaccine is the interference between the four dengue vaccine viruses when administered as a tetravalent formulation. Interference is manifest when one or more components of a multivalent mixture will induce lower immune responses than those elicited by each individual monovalent vaccine. Interference has been observed with vaccines for diseases with multiple pathogenic serotypes, such as polio, dengue or others. Due in part to this interference, it was previously discovered that three dose regimen of oral polio vaccine is required to induce adequate immune responses to the three key serotypes. Historically studies with live attenuated tetravalent dengue vaccines have shown that the DENV serotype

that elicits the strongest neutralizing antibody response when administered alone tends to dominate immune responses when administered in the context of a multivalent formulation containing other serotypes. As an example, tetravalent mixtures of four different live, attenuated dengue vaccines showed dominant responses to the DEN-3 component and reduced immune responses to DEN-1, -2 and -4 (see for example, Sabchareon, et al., 2002, Kitchener, et al. 2006). As a result of this dominance, clinical development of the tetravalent mixtures was suspended. Interference has been seen with recombinant, live attenuated viruses as well. Interference was documented in tetravalent mixtures of dengue/yellow fever chimeras (Guy, et al. 2009. Evaluation of Interferences between Dengue Vaccine Serotypes in a Monkey Model. Am. J. Trop Med. Hyg. 80: 3012-311). In these studies, two serotypes were found to dominate the responses in tetravalent formulations of ChimeriVax vaccine strains. Interference could be overcome by administering two bivalent vaccine formulations, either in separate anatomical locations or sequentially in time, or by a third administration of the tetravalent formulation after one year. Similarly, it was demonstrated that improved multivalent responses with tetravalent recombinant vaccine strains (in this case, formulations containing DENV or chimeric DENV with deletions in the 3' non-coding region) could be obtained only with a prolonged four month internal between the first and second administration. (Blaney, et al., 2005. Recombinant, Live-Attenuated Tetravalent Dengue Virus Vaccine Formulations Induce a Balanced, Broad, and Protective Neutralizing Antibody Response against Each of the Four Serotypes in Rhesus Monkeys. J. Virology 79: 5516-5528).

[00040] Certain embodiments disclosed herein concern derivatives of DENVax (a tetravalent vaccine composition). DENVax is a dengue vaccine that consists of a mixture of four recombinant dengue virus strains designed to generate immune responses to the four dengue serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). Not to be bound by any limitations to a particular tetravalent formulation, DENVax™, the dengue serotype 2 vaccine component (DENVax-2) corresponds to an attenuated DEN-2 PDK-53 strain. This construct has already been investigated in many clinical studies. The other dengue vaccine strains (DENVax-1, DENVax-3 and DENVax-4) are chimeras consisting of the DEN-1, DEN-3 or DEN-4 structural pre-membrane (prM) and envelope (E) protein genes cloned into a DEN-2 PDK-53 non-structural gene backbone. These recombinant viruses express the surface antigens of DEN-1, DEN-3 or DEN-4 and retain the genetic alterations responsible for the attenuation of the DEN-2 PDK-53 strain. In certain embodiments, DENVax can be used as an example of a trivalent live, attenuated dengue vaccine having three of four dengue virus serotypes

represented in one vaccine composition at various ratios. Other embodiments relate to optimizing tetravalent vaccine administrations. Yet other embodiments relate to DENVax™ immunization methods.

[00041] In some embodiments, a mixture of three or more attenuated dengue viruses comprises one or more attenuated dengue-2 viruses and one or more dengue-dengue chimeric viruses further comprising capsid and non-structural proteins of the attenuated dengue-2 virus and pre-membrane and envelope proteins of at least a second dengue virus.

[00042] In certain embodiments, a chimeric construct of the instant application can include the pre-membrane (PM) and envelope (E) proteins of at least a second dengue virus are dengue-2, dengue-3 or dengue-4 when the attenuated dengue virus is dengue-1; or dengue-1, dengue-3 or dengue-4 when the attenuated dengue virus is dengue-2; or dengue-1, dengue-2 or dengue-4 when the attenuated dengue virus is dengue-3; or dengue-1, dengue-2 or dengue-3 when the attenuated dengue virus is dengue-4.

[00043] In other embodiments, the composition introduced to the subject comprises vaccines against three of dengue virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4). In other embodiments, a composition contemplated herein can include a modified formula of DENVax where three of four dengue virus serotypes are represented or other similar formulation. In some compositions, vaccine viruses against all dengue serotypes are in equal proportions in the composition. In yet other compositions, each dengue vaccine virus serotype may be in a particular ratio to one another such that introduction of the composition provides the subject with sufficient levels of neutralizing antibodies against all dengue viruses (*e.g.* DEN-1, DEN-2, DEN-3, DEN-4).

[00044] Certain embodiments disclosed herein relate to methods and compositions for a rapid induction of protection in a subject against all dengue virus serotypes by, for example, administering a vaccine to a subject against three of four dengue virus serotypes in more than one anatomical location consecutively on the same day. Some embodiments can include introducing a vaccine composition to a subject via intradermal (ID) or subcutaneous (SC) injection or other administration mode in one anatomical location then introducing at least a second vaccine composition at another anatomical location by ID, SC or other administration mode. Some embodiments include using any combination of modes of administration for introducing a dengue virus vaccine of all dengue virus serotypes using a trivalent formulation to a subject where administration of the vaccine occurs at two or more anatomical sites or by two or more different routes on day 0 to the subject. Some embodiments include using the

same mode of administration but at different anatomical locations.

[00045] Some dengue virus vaccine compositions described herein range in dosage from from 10^2 to 5 x 10^6 PFU for each serotype in a single trivalent composition. Other compositions (e.g. follow-on vaccinations) contemplated herein include compositions that have dosages less than or more than this range based on immune response in the subject after primary immunization. In certain embodiments, ratios can vary for the various Dengue vaccine virus serotypes depending on need and immune response in a subject.

[00046] In certain embodiments, compositions introduced on the first vaccination or in any follow-on vaccination contemplated herein may include one trivalent dengue virus composition. In accordance with these embodiments, the composition can include three of four dengue virus serotypes of a DENVax composition or other similar trivalent formulation of equal or equivalent ratios or at predetermined serotype ratios. Other embodiments, can include using different formulations (*e.g.* serotype ratios) for each of the vaccine compositions administered at the primary vaccination or any follow-on vaccinations (*e.g.* less than 30 days later).

[00047] Some embodiments herein include treating a subject in need of such a vaccine, on day 0 at two or more anatomical locations then administering at least a second vaccine within 30 days such as about 7, about 14, about 21 or about 28 days later with a composition comprising dengue virus serotypes which may or may not have all serotypes. In certain embodiments, each vaccination has all dengue virus serotypes represented in the vaccine formulation. Vaccine compositions of follow-on administration disclosed herein may include two or more dengue virus serotypes at a predetermined ratio for the subsequent administration(s).

[00048] In certain vaccine compositions, the ratio of three of four of DEN-1:DEN-2:DEN-3:DEN-4 or even DEN-5 can be 3:3:3, 4:3:4, 5:4:5,:4:5:5, 5:5:5, 1:10:100 or other ratio where the ratio between 2 serotypes can be about 2 to about 100,000 fold difference in a single composition. In certain embodiments a dengue serotype ratio can be DEN-1 at 2 x10^4: DEN-2 at 5 x10^4: DEN-3 at 1 x10^5: DEN-4 at 3 x10^5 PFUs; DEN-1 at 8 x10^3: DEN-2 at 5 x10^3: DEN-3 at 1 x10^4: DEN-4 at 2 x10^5 PFUs and in any combination of three of the four dengue virus serotypes. In certain embodiments, a trivalent formulation can contain a ratio that includes a higher ratio for dengue-4 than for other serotypes. In some compositions, all dengue vaccine virus serotypes are in equal proportions in the composition. In yet other

compositions, each dengue vaccine virus serotype may be in a particular ratio to another serotype such that introduction of the composition provides the subject with adequate or more than adequate levels of neutralizing antibodies which confer protection against all dengue viruses (*e.g.* any combination of three of the four of Dengue 1, 2, 3 and 4). For example, if after receiving two or more consecutive vaccinations on day 0 at two or more anatomical locations, the subject has lower protection to one or more particular dengue virus serotypes, then a booster for that subject can contain an increased concentration of the one or more dengue vaccine virus serotype (that demonstrated lower neutralizing antibodies) to provide better protection against all dengue virus types. In accordance with these embodiments, samples from a subject may be analyzed for an immune response to dengue serotype infection (e.g. DEN-1, -2, -3, -4) using standard means known in the art.

[00049] In certain embodiments, the vaccine composition can be simultaneously or consecutively introduced to a subject intradermally in multiple anatomical locations to, for example, protect against all dengue serotypes (*e.g.* cross protection). In certain embodiments, a vaccine composition can include, but is not limited to, a single formulation of three of four dengue vaccine virus serotypes administered to a subject capable of providing full protection against infection by all dengue virus serotypes. In other embodiments, a vaccine composition can include attenuated dengue virus serotypes in combination with other anti-pathogenic compositions (*e.g.* Japanese encephalitis, West Nile, influenza etc.). Compositions contemplated herein can be administered by any method known in the art including, but not limited to, intradermal, subcutaneous, intramuscular, intranasal, inhalation, vaginal, intravenous, ingested, and any other method. Introduction in two or more anatomical sites can include any combination administration including by the same mode in two or more anatomical sites or by two different modes that include two separate anatomical sites. In accordance with these embodiments, two or more anatomical sites can include different limbs.

[00050] For example, if a subject, after receiving two or more consecutive vaccinations on day 0 at two or more anatomical locations and the subject does not induce poor levels of neutralizing antibodies to one or more particular dengue virus serotypes, then a booster vaccination for that subject can contain an increased concentration of the one or more dengue vaccine virus serotype (that demonstrated lower levels of neutralizing antibodies) to provide complete protection against infection by all dengue virus types. In accordance with these embodiments, samples from a subject may be analyzed for resistance to dengue infection

using standard means known in the art.

[00051] In certain embodiments, vaccine compositions disclosed herein can be chimeric constructs that can include a mixture of constructs that make up three dengue serotypes in a vaccine composition for administration to a subject. In other embodiments, dengue virus vaccines can include constructs having an attenuated flavivirus backbone with various dengue serotype substitutions representing each of the four serotypes where the constructs can be mixed in a composition for administration as a vaccine.

[00052] Trivalent formulations, e.g. using a variation of DENVax, can be prepared by mixing predetermined amounts of each monovalent vaccine component. Based on input titer of each vaccine component, a defined volume of monovalent vaccines can be added to a final volume of either 0.1mL (e.g. for intradermal) or 0.5mL (e.g. for subcutaneous) vaccine formulation. The remaining volume of the tetravalent DENVax™ vaccine can be composed of diluent containing Trehalose (15%) F127 (1%) and human serum albumin (0.1%) in a saline buffer to stabilize the live, attenuated vaccine formulation. FTA (F127: Trehalose: Albumin)

Methods

Construction of Flavivirus Chimeras

[00053] The flavivirus chimeras described herein can be produced by splicing one or more of the structural protein genes of the flavivirus against which immunity is desired into a PDK-53 dengue virus genome backbone, or the equivalent thereof as described above, using recombinant engineering techniques well known to those skilled in the art to remove the corresponding PDK-53 gene and replace it with the desired gene.

[00054] Alternatively, using the sequences, nucleic acid molecules encoding the flavivirus proteins may be synthesized using known nucleic acid synthesis techniques and inserted into an appropriate vector. Avirulent, immunogenic virus is therefore produced using recombinant engineering techniques known to those skilled in the art.

[00055] As recited above, a target gene to be inserted into the backbone encodes a flavivirus structural protein. In accordance with these embodiments, a flavivirus gene to be inserted is a gene encoding a C protein, a PrM protein and/or an E protein. The sequence inserted into the dengue-2 backbone can encode both the PrM and E structural proteins. The sequence inserted into the dengue-2 backbone can encode the C, prM and E structural proteins. The dengue virus backbone is the PDK-53 dengue-2 virus genome and includes

either the spliced genes that encode the C, PrM and/or E structural proteins of dengue-1 (DEN-2/1), the spliced genes that encode the PrM and/or E structural proteins of dengue-3 (DEN-2/3), or the spliced genes encode the PrM and/or E structural proteins of dengue-4 (DEN-2/4). In a particular embodiment of this invention, the spliced gene that encodes the structural protein of dengue-3 virus directs the synthesis of an E protein that contains a leucine at amino acid position 345.

[00056] In a particular embodiment, the chimera of this invention encodes the C structural protein of dengue-2 virus and directs the synthesis of a C protein that contains a serine at amino acid position 100 and comprises a spliced gene encoding the structural proteins of dengue-4 which directs the synthesis of an E protein that contains a leucine at amino acid position 447.

[00057] In a further embodiment, the chimera of this invention encodes the C structural protein of dengue-2 virus and directs the synthesis of a C protein that contains a serine at amino acid position 100 and comprises a spliced gene encoding the structural proteins of dengue-4 which directs the synthesis of an E protein that contains a leucine at amino acid position 447 and a valine at amino acid position 364. The structural proteins described herein can be present as the only flavivirus structural protein or in any combination of flavivirus structural proteins in a viral chimera of this invention.

[00058] Certain chimeras contemplated herein are engineered by recombination of full genome-length cDNA clones derived from both DEN-2 16681 wild type virus and either of the PDK-53 dengue-2 virus variants (-E or -V(SEQ ID NO: 15)). The uncloned PDK-53 vaccine contains a mixture of two genotypic variants, designated herein as PDK-53-E and PDK-53-V. The PDK-53-V variant contains all nine PDK-53 vaccine-specific nucleotide mutations, including the Glu-to-Val mutation at amino acid position NS3-250. The PDK-53-E variant contains eight of the nine mutations of the PDK-53 vaccine and the NS3-250-Glu of the parental 16681 virus. Infectious cDNA clones are constructed for both variants, and viruses derived from both clones are attenuated in mice. The phenotypic markers of attenuation of DEN-2 PDK-53 virus include small plaque size, temperature sensitivity (particularly in LLC-MK.sub.2 cells), limited replication (particularly in C6/36 cells), attenuation for newborn mice (specifically loss of neurovirulence for suckling mice) and decreased incidence of viremia in monkeys. The chimeras that are useful as vaccine candidates are constructed in the genetic backgrounds of the two DEN-2 PDK-53 variants which all contain mutations in nonstructural regions of the genome, including 5'NC-57 C-to-

T (16681-to-PDK-53) in the 5' noncoding region, as well as mutations in the amino acid sequence of the nonstructural proteins, such as, for example, NS1-53 Gly-to-Asp and NS3-250 Glu-to-Val.

[00059] In certain embodiments, an immunogenic composition that includes chimeric dengue constructs of the present invention can be a combination of three or more of DEN-1, DEN-2, DEN-3 or DEN-4 to confer simultaneous protection against all four dengue virus serotypes in a single vaccine administration. In other embodiments, an immunogenic composition including combinations of three DEN-1, DEN-2, DEN-3 and DENV-4 constructs of embodiments disclosed herein can be administered to a subject to induce improved immunogenic responses against each dengue virus serotype and where immune response interference to the dengue virus constructs is reduced.

[00060] In certain embodiments, dengue virus constructs can include a dengue-dengue chimeric construct having adaptive mutations in the structural or non-structural regions of the various dengue virus serotypes. In other embodiments, a chimeric construct can include a DEN-2 backbone where structural or non-structural regions of DENV-1, -3, -4 are substituted for DEN-2 structural or non-structural regions. In accordance with these embodiments, a DEN-2 backbone can include any live attenuated DEN-2 virus. In other embodiments, a DEN-2 backbone can include live attenuated DEN-2 PDK-53 virus as a backbone where the live attenuated DEN-2 PDK virus further includes structural proteins of one or more of prM (premembrane) and E (envelope) structural proteins of DEN-1, DEN-3 or DEN-4. In addition, a DEN-2 PDK-53 backbone can include additional mutations or reversions of mutations of DEN-2 PDK-53 generating a novel construct in order to enhance *in vitro* growth, or *in vivo* the immune response to DEN-1, DEN-3 or DEN-4 in a subject upon administration.

[00061] In some embodiments, a current dengue chimeric construct denoted as DENVax-4 strain was modified to contain a capsid/PrM junction of the DEN-2 backbone to be more genetically similar to that of DENV-4 instead of DEN-2 in order to improve replication efficiency of the virus both in vitro for production and in vivo as a construct of use for inducing an immune response to DENV-4. The current strain of DEN-4, DENVax-4, has a capsid/PrM sequence that is identical to DEN-2 instead of DEN-4, possibly creating an inefficient transcription and translation from the genomic RNA, which is different than that of wild type DENV-4. It is contemplated that these DENV-4 constructs can be used in any

trivalent composition contemplated herein, in combination with DEN-1, DEN-2 and DEN-3 as live, attenuated and/or chimeric dengue-dengue constructs.

[00062] In some embodiments, structural protein genes can include prM and E genes of DENV-4 on another dengue virus backbone (e.g. dengue-2, DEN-2 PDK-53), making a dengue-dengue chimera. For example, a DEN-4 construct, in certain embodiments can include those construct termed DENVax-4e (Capsid 107 Cysteine to Tyrosine; DenVax-4b backbone, modifications at Capsid/prM junction), DENVax-4f (where the PDK-53 backbone NS2A and NS4A mutations are reverted to that of 16681) or DENVax-4h (Envelope 417 Glu to Lys) where for certain constructs the DEN-2 PDK-53 backbone has one or more reversions to wild-type DEN-2 (e.g. in the non-coding region (NCR) or a non-structural region (NS2 etc.)) and one or more mutations in the DENV-4 structural region (e.g. prM or E), while encoding one or more structural proteins of DENV-4 (e.g. strain 1036). A modified DENV-4 construct disclosed herein can include a modified attenuated DEN-2 PDK-53 backbone, having one or more modified structural proteins of DENV-4 strain 1036. In some embodiments, one or more mutations present in live, attenuated DEN-2 PDK-53 virus can be reverted back to a wildtype nucleic acid (which may be a silent mutation) or another nucleic acid to produce constructs herein that generate a modified DEN-2/DENV-4 construct having increased replication ability and immunogenicity without affecting its attenuation or safety but may affect growth and/or replication of the DEN-4 virus. In certain embodiment, the reversions may lead to increased growth and/or replication.

[00063] In other embodiments, trivalent compositions disclosed herein can include a modified DENV-4 (or DENV-1 or DENV-3) construct can incorporate mutations introduced to one or more structural regions and/or non-structural regions of the dengue serotype in order to generate constructs inducing an improved immunological response while maintaining safety and viral attenuation. For example, a modified or mutated dengue-dengue chimera of DEN-2/DENV-4 may contain mutations at one or more non-structural regions of a DEN-2 PDK-53 backbone, such as NS2A, and NS4A, and/or mutations at 5' non-coding region (5'NCR). In another embodiment, a modified DENV-4 chimera construct can include NS2A and NS4A of DEN-2 16681 by reverting mutations at NS2A and NS4A of PDK-53 (*e.g.* an M-L substitution at NS4A). Some embodiments include a modified DENV-4 chimera construct having 5'NCR, NS2A and NS4A of DEN-2 16681 by reverting corresponding mutations in the DEN-2 PDK-53 backbone of a target construct. Other embodiments can include a modified DENV-4 chimera construct having 5'NCR of DEN-2 16681 by reverting corresponding mutations in the DEN-2 PDK-53 backbone. A modified

DEN-4 chimera construct can also include DEN-2 PDK-53 backbone, and encode one or more structural proteins of DEN-4 strain H241. It is contemplated that, to induce an immune response, any DEN-4 structural protein can be substituted for structural regions of a chimeric virus containing a dengue -2 serotype backbone (e.g. PDK-53 or modified PDK-53). In some embodiments, a modified DEN-4 construct contains live attenuated DEN-2 PDK-53 as a backbone, and DEN-4, DEN-2 or DEN-3 structural proteins where mutations can be introduced to modify structural regions of a DEN-4 (e.g. strain 1036) or DEN-1 or DEN-3 strain.

[00064] In other embodiments, trivalent compositions can include constructs with mutations introduced to capsid/prM junction amino acid sequences of a DENV virus in order to increase immunogenicity of a construct containing such a mutation. For example, a mutation in DEN-4 can be a Cys-Tyr mutation at capsid position 107 of the DEN-4. In other embodiments, it is contemplated that the cysteine in position 107 can be mutated to any other aromatic amino acid with a hydrophobic side chain (see for example DEN-4e). Other DEN-2 PDK-53 reversion of a chimeric construct can be found in NS2A or NS4A. Yet other embodiments include a DEN-4 construct where a DEN-2 backbone comprises PDK-53 (MVS, SEQ ID NO:21) where amino acid positions 102-107 of the capsid region of PDK-53 are converted to a homologous DEN-4 counterpart amino acid to generate DENV-4b. These backbone constructs can then further comprise a cysteine in the capsid region to aromatic amino acid in position (e.g. tyrosine, tryptophan etc). In certain embodiments, this construct is represented by SEQ ID NO:22 or SEQ ID NO:23.

[00065] Other DENV-4 constructs of use in trivalent compositions disclosed herein can include a virus construct with an amino acid substitution at Envelope position 417. For example, DEN-4 strain 1036 strain sequence or equivalent strain position thereof where a PDK-53 (MVS DEN2/4, SEQ ID NO:21) backbone of Dengue-2 with DEN-4 structural proteins is provided. Embodiments include further mutating Envelope position 417 from a negative to a positively charged side-chain amino acid (*e.g.* lysine). It is contemplated that any charged side chain will provide increased immunogenicity of the DEN-4 construct without affecting its safety or attenuation. In certain embodiments, this construct is represented by SEQ ID NO:24 or SEQ ID NO:25.

[00066] In certain embodiments, DEN-2 PDK-53 reversions of a chimeric DENV construct have the 5' NC, NS1 and NS3 mutations found in DEN-2 PDK-53 MVS while having other reversions or mutations that differ from DEN-2 PDK-53. It has been demonstrated that these three mutations can be important for attenuation (e.g. small plaque

size, reduced growth rate, lower titer, increased temperature sensitivity and decreased neurovirulence compared to a control).

[00067] In other embodiments, DEN-2 PDK-53 genome backbones can be used to generate chimeric constructs of DEN-1 and DEN-3, where one or more structural protein genes of DEN-2 PDK-53 genome can be replaced by one or more structural protein genes of DEN-1 and DEN-3. These constructs can include a combination of both DEN-1 and DEN-3 in a single chimera having a DEN-2 PDK-53 backbone. In some embodiments, a structural protein can be the C, prM or E protein of DEN-1 and/or DEN-3. In certain embodiments, structural protein genes include the prM and E genes of DEN-1 or DEN-3. These hybrid/chimeric viruses express the surface antigens of DEN-1, DEN-3 or DENV-4 while retaining the attenuation phenotypes of the parent DEN-2. In certain embodiments, these constructs can be represented by SEQ ID NO:15, DEN-2/DEN-1 and SEQ ID NO: 19, DEN-2/DEN-3 where these constructs can be used in di-, tri or tetravalent compositions disclosed herein.

[00068] In some embodiment, constructs disclosed herein can include chimeric constructs of DENV-4, DEN-2, DEN-1, and DEN-3 expressing surface antigens of DEN-1, DEN-3 and DENV-4 using attenuated DEN-2 PDK-53 virus as a backbone.

[00069] Suitable chimeric viruses or nucleic acid chimeras containing nucleotide sequences encoding structural proteins of other flaviviruses or dengue virus serotypes can be evaluated for usefulness as vaccines by screening them for the phenotypic markers of attenuation that indicate avirulence and by screening them for immunogenicity. Antigenicity and immunogenicity can be evaluated using in vitro or in vivo reactivity with flavivirus antibodies or immunoreactive serum using routine screening procedures known to those skilled in the art.

Flavivirus Vaccines

[00070] Chimeric viruses and nucleic acid chimeras provide live, attenuated viruses useful as immunogens or vaccines. In a preferred embodiment, chimeras exhibit high immunogenicity while at the same time producing no dangerous pathogenic or lethal effects. Chimeric viruses or nucleic acid chimeras of this invention can comprise the structural genes of either wild-type or attenuated virus in a virulent or an attenuated DEN-2 virus backbone. For example, the chimera may express the structural protein genes of wild-type DEN-1 16007 virus or its candidate PDK-13 vaccine derivative in either of the DEN-2 PDK-53 backgrounds.

[00071] All of the chimeric DEN-2/1 viruses containing the C, prM and B proteins of either DEN-1 16007 virus (DEN-2/1-EP and -VP chimeras) or PDK-13 virus (DEN-2/1-EV and -VV (SEQ ID NO:7) chimeras) in the backbones of DEN-2 PDK-53 retain all of the phenotypic attenuation markers of the DEN-2 PDK-53 virus. The chimeric DEN-2/1-EP and -VP(SEQ ID NO:5) viruses, which contain the C, prM and E proteins of DEN-1 16007 virus are more genetically stable after passing in cell culture than the DEN-2/1-EV and -VV viruses. The immunogenicity of the chimeric viruses expressing the structural proteins of DEN-1 16007 virus was higher as compared with the neutralizing antibody titers elicited by the PDK-13 vaccine virus and the chimeras expressing the structural proteins of the PDK-13 virus. Thus, the chimeric DEN-2/1-EP and -VP viruses, which express the structural genes of wild-type DEN-1 16007 virus within the genetic background of the two DEN-2 PDK-53 variants, are potential DEN-1 vaccine candidates that are superior to the candidate PDK-13 vaccine. These two chimeras replicate well in LLC-MK.sub.2 cells and retain the attenuation markers associated with DEN-2 PDK-53 virus, including small plaque size, temperature sensitivity, restricted replication in mosquito cells and attenuation for mice. They are at least as immunogenic as wild-type DEN-1 16007 virus in mice.

[00072] Other examples, such as DEN-2/3 and DEN-2/4 chimeras, are chimeric viruses containing structural protein genes from wild-type DEN-3 or DEN-4 virus within the DEN-2 PDK-53 backbones, are suitable vaccine candidates which retain all of the attenuated phenotypic markers of the DEN-2 PDK-53 viruses, while providing immunogenicity against DEN-3 or DEN-4 virus. The strategy described herein of using a genetic background that contains the determinants of attenuation in nonstructural regions of the genome to express the structural protein genes of heterologous viruses has lead to development of live, attenuated flavivirus vaccine candidates that express wild-type structural protein genes of optimal immunogenicity. Thus, vaccine candidates for immunogenic variants of multiple flaviviral pathogens can be designed.

[00073] Viruses used in the chimeras described herein are typically grown using techniques known in the art. Virus plaque titrations are then performed and plaques counted in order to assess the viability and phenotypic characteristics of the growing cultures. Wild type viruses are passaged through cultured cell lines to derive attenuated candidate starting materials.

[00074] Chimeric infectious clones are constructed from the various dengue serotype clones available. The cloning of virus-specific cDNA fragments can also be accomplished, if

desired. The cDNA fragments containing the structural protein or nonstructural protein genes are amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from dengue virus RNA with various primers. Amplified fragments are cloned into the cleavage sites of other intermediate clones. Intermediate, chimeric dengue virus clones are then sequenced to verify the accuracy of the inserted dengue virus-specific cDNA.

[00075] Full genome-length chimeric plasmids constructed by inserting the structural protein or nonstructural protein gene region of dengue serotype viruses into vectors are obtainable sing recombinant techniques well known to those skilled in the art.

Nucleotide and Amino Acid Analysis

[00076] A comparison of the critical nucleotide and amino acid substitutions that have been discovered between the parent strain and the attenuated virus are incorporated herein by reference. The sequence of the DEN-2 cDNA amplicons was amplified from DEN-2 viral genomic RNA by reverse transcriptase-polymerase chain reaction (RT-PCR).

[00077] Unlike PDK-53, which contains no amino acid mutations in the E protein relative to wild type dengue-2 virus, DEN-1, DEN-3 and DEN-4 attenuated viruses all have amino acid mutations in the E protein. The wild-type DEN-3 16562 was shown to include traces of a variant comprising a T at nucleotide position 1521 which directs incorporation of a leucine at polyprotein position 476, amino acid residue position 476 of the E protein.

[00078] Each of the latter three viruses possesses a Glu-to-Lys (parent-to-vaccine) mutation in the E protein, although the mutation is located at a different amino acid residue in the E protein. This substitution causes a shift from a negatively charged amino acid to a positively charged one. The Glu-to-Lys substitution in the E protein of DEN-4 vaccine virus was the only mutation present in the E protein, while the E proteins of DEN-1 and DEN-3 vaccine viruses had five and three amino acid mutations, respectively.

[00079] The NS1-53 mutation in the DEN-2 PDK-53 vaccine virus is significant for the attenuated phenotype of this virus, because the NS1-53-Gly of the DEN-2 16681 virus is conserved in nearly all flaviviruses, including the tick-borne viruses, sequenced to date. The mutations that occurred in the NS2A, NS2B, NS4A, and NS4B proteins of the DEN-1, -2, -3 and -4 attenuated strains were all conservative in nature. The NS4A-75 and NS4A-95 mutations of DEN-2 and DEN-4 vaccine viruses, respectively, occurred at sites of amino acid conservation among dengue viruses, but not among flaviviruses in general.

[00080] The flaviviral NS3 protein possesses at least two recognized functions: the

viral proteinase and RNA helicase/NTPase. The 698-aa long (DEN-2 virus) NS3 protein contains an amino-terminal serine protease domain (NS3-51-His, -75-Asp, -135-Ser catalytic triad) that is followed by sequence motifs for RNA helicase/NTPase functions NS3-196-GAGKT, -284-DEAH, -459-GRIGR. None of the mutations in the NS3 proteins of DEN-1, DEN-2, or DEN-3 virus occurred within a recognized motif. The NS3-510 Tyr-to-Phe mutation in DEN-1 PDK-13 virus was conservative. Since the wild-type DEN-2, -3 and -4 viruses contain Phe at this position, it is unlikely that the Tyr-to-Phe mutation plays a role in the attenuation of DEN-1 virus. The NS3-182 Glu-to-Lys mutation in DEN-1 PDK-13 virus occurred at a position that is conserved as Asp or Glu in most mosquito-borne flaviviruses and it may play some role in attenuation. This mutation was located 15 amino acid residues upstream of the GAGKT (helicase motif. As noted in previous reports, the NS3-250-Glu in DEN-2 16681 virus is conserved in all mosquito-borne flaviviruses except for yellow fever virus.

Nucleic Acid Amplification

[00081] Nucleic acids may be used in any formulation or used to generate any formulation contemplated herein. Nucleic acid sequences used as a template for amplification can be isolated viruses (*e.g.* dengue viruses), according to standard methodologies. A nucleic acid sequence may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary cDNA. In some embodiments, the RNA is whole cell RNA and is used directly as the template for amplification. Any method known in the art for amplifying nucleic acid molecules is contemplated (*e.g.*, PCR, LCR, Qbeta Replicase, etc).

Expressed Proteins or Peptides

[00082] Genes can be expressed in any number of different recombinant DNA expression systems to generate large amounts of the polypeptide product, which can then be purified and used in methods and compositions reported herein. Any method known in the art for generating and using constructs is contemplated. In certain embodiments, genes or gene fragments encoding one or more polypeptide may be inserted into an expression vector by standard cloning or subcloning techniques known in the art.

[00083] Proteins, peptides and/or antibodies or fragments thereof may be detected or analyzed by any means known in the art. In certain embodiments, methods for separating and analyzing molecules may be used such as gel electrophoresis or column chromatography methods.

Electrophoresis

[00084] Electrophoresis may be used to separate molecules (*e.g.*, large molecules such as proteins or nucleic acids) based on their size and electrical charge. There are many variations of electrophoresis known in the art. A solution through which the molecules move may be free, usually in capillary tubes or it may be embedded in a matrix or other material known in the art. Common matrices can include, but are not limited to, polyacrylamide gels, agarose gels, mass spec, blotting and filter paper.

[00085] Some embodiments, using a gene or gene fragment encoding a polypeptide may be inserted into an expression vector by standard subcloning techniques. An expression vector may be used which produces the recombinant polypeptide as a fusion protein, allowing rapid affinity purification of a peptide or protein. Examples of such fusion protein expression systems are the glutathione S-transferase system (Pharmacia, Piscataway, NJ), the maltose binding protein system (NEB, Beverley, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA).

Pharmaceutical Formulations

[00086] Any pharmaceutical formulation known in the art for a vaccine is contemplated herein. In certain embodiments, a formulation disclosed herein contains chimeric or live, attenuated virus that represent three dengue virus serotypes in various ratios in a single vaccine or one to all serotypes for follow-on compositions. It is contemplated that formulations can contain other agents of use in vaccination of a subject including, but not limited to other active or inactive ingredients or compositions known to one skilled in the art.

[00087] All contemplated vaccinal viruses herein can be administered in the form of vaccinal compositions which can be prepared by any method known to one skilled in the art. In certain embodiments, the virus compositions are lyophilized and are mixed with a pharmaceutically acceptable excipient (e.g. water, phosphate buffered saline (PBS), wetting agents etc.) In other embodiments, vaccine compositions can include stabilizers that are known to reduce degradation of the formulation and prolong shelf-life of the compositions.

[00088] In other embodiments, an adjuvant may be added to the composition to induce, increase, stimulate or strengthen a cellular or humoral immune response to administration of a vaccination described herein. Any adjuvant known in the art that is compatible with compositions disclosed herein is contemplated.

[00089] Some embodiments herein concern amounts or doses or volumes of administration of a trivalent dengue virus composition and the amount or dose can depend

on route of administration and other specifications such as the subject getting the vaccine (e.g. age, health condition, weight etc.).

[00090] It is contemplated herein that compositions described can be administered to a subject living in an area having dengue virus, a subject traveling to an area having dengue virus or other subject such as any human or animal capable of getting dengue fever or other dengue virus condition. In certain embodiments, it may be recommended that a subject traveling to an area having dengue virus is administered one or more vaccine compositions (*e.g.* or two or more on Day 0) about 1 to about 3 months prior to dengue virus exposure. Vaccines herein can be administered as a prophylactic treatment to prevent infection in adults and children. A subject can be naïve or non-naïve subject with respect to exposure to dengue virus and vaccine regimens disclosed herein.

Kits

[00091] Other embodiments concern kits of use with the methods (*e.g.* methods of application or administration of a vaccine) and compositions described herein. Some embodiments concern kits having vaccine trivalent compositions of use to prevent or reduce the incidence of a subject having been exposed or suspected of being exposed to one or more dengue viruses to get an infection from the virus. In certain embodiments, a kit may contain one or more than one formulation of dengue virus serotype(s) (*e.g.* live, attenuated viruses or chimeric constructs to make a trivalent formulation, DENVax) at predetermined ratios of the dengue virus serotypes. Kits can be portable, for example, able to be transported and used in remote areas such as military installations or remote villages in dengue endemic areas. Other kits may be of use in a health facility to treat a subject having been exposed to one or more dengue viruses or suspected of being at risk of exposure to dengue virus.

[00092] Kits can also include a suitable container, for example, a vessel, vials, tubes, mini- or microfuge tubes, test tube, flask, bottle, syringe or other container. Where an additional component or agent is provided, the kit can contain one or more additional containers into which this agent or component may be placed. Kits herein will also typically include a means for containing the agent (e.g. a vessel), composition and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained. Optionally, one or more additional agents such as immunogenic agents or other anti-viral agents, anti-fungal or anti-bacterial agents may be needed for compositions described, for example, for compositions of use as a vaccine against one or more additional

microorganisms.

[00093] In other embodiments, kits can include devices for administering one or more vaccination to a subject such as an ID, SQ, IM, an inhaler, intranasal applicator or other device for administering a vaccine composition disclosed herein.

[00094] The following examples are included to demonstrate certain embodiments presented herein. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered to function well in the practices disclosed herein. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the certain embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope herein.

EXAMPLES

Example 1

[00095] In one exemplary method, dengue virus trivalent vaccine compositions studies were performed using various trivalent dengue virus formulations alone, in multiple doses or in combination with follow-on monovalent dengue virus formulations. In certain exemplary methods, the following concentrations of dengue virus serotypes were used: DENVax-1: $1x10^5$PFU, DENVax-2; $1x10^5$PFU, DENVax3: $1x10^5$PFU, and DENVax4: $1x10^5$PFU. See for example Table 1 and 2. In certain embodiments various dengue-4 serotypes can be combined with live, attenuated dengue-2 and a chimeric construct of dengue-1 in order to induce immunity in the subject against all four serotypes.

[00096] Animals: Cynomolgus macaques

Route of Immunization & Challenge: Subcutaneous (0.5mL)

Vaccination Schedule & Dose: Days 0, 91 (DENVax-HD+)

Challenge: Day 120

Dose: DENV-2 (105pfu), DENV-4 (106pfu)

Sampling

Viremia: qRT-PCR using primer for E-gene

Pre & Post primary vaccination: 0, 5, 8, 11, 14, 17, 20, 23, 25

Pre & Post Boost: 91, 94, 98

Pre & Post Challenge:120, 122, 124, 126, 128, 130, 132, 134

Serology: 0, 30, 60, 91, 105, 120, 134, 148

Post-vaccination viremia (vRNA) is dominated by DENVax-2. DENVax-4 vRNA detected in one animal from DENVax-HD+ on day 8 post-vaccination.The trivalent DENVax-1,-2,-3

is more immunogenic and elicits a more balanced response than the DENVax-1,-3,-4 formulation. It is contemplated that the modified DEN-4 strains disclosed herein will improve this outcome. (See for example, Fig. 1).

[00097] Table 1 Preclinical evaluation of trivalent DENVax in non-human primates

Route of Immunization & Challenge: Subcutaneous (0.5mL)

DENVax Formulation					
DENVax HD+:	DENVax1 - 1.25×10^4	Trivalent (DENVax1,2,3)	DENVax1 - 1.25×10^4	Trivalent (DENVax1,3,4)	DENVax1 - 1.25×10^4
	DENVax2 - 6.04×10^4		DENVax2 - 6.04×10^4		
	DENVax3 - 1.30×10^5		DENVax3 - 1.30×10^5		DENVax3 - 1.30×10^5
	DENVax4 - 1.34×10^6				DENVax4 - 1.34×10^6

Table 2: Design:

Group	Treatment on Day 0 and 90	Challenge on day 120	Aim
1	DENVax HD+	Challenge with DENV-2 and DENV-4	Clinical dosing
2	Trivalent (DENVax1,2,3 – same titers as HD+)	Challenge with DENV-4	Assess trivalent vaccine protection against dengue virus serotypes (e.g. DEN-4)
3	Trivalent (DENVax1,3,4 – same titers as HD+)	Challenge wit DENV-2	Assess trivalent followed by monovalent administration and assess protection against dengue virus serotypes (e.g. What is the neutralizing antibody level against DEN-2 using only a DEN-2 backbone for immunization)
4	FTA	Challenge with DENV-2 and DENV-4	Challenge control group
5	Trivalent formulation on Day 0 and a monovalent formulation on the same day or within 90 days of the Trivalent	Challenge with DENV-2 and DENV-4	Challenge control group

	formulation (or reverse: monovalent first) DENVax 1, 2, 3; DENVax 2, 3, 4; DENVax1, 2, 4 or DENVax1, 3, 4 on a DEN-2 backbone		
6	Trivalent formulation on Day 0 and a monovalent formulation on the same day in a different anatomical site; or within 90 days of the Trivalent formulation (or reverse: monovalent first) DENVax 1, 2, 3; DENVax 2, 3, 4; DENVax1, 2, 4 or DENVax1, 3, 4 on a DEN-2 backbone	Challenge with DENV-2 and DENV-4	Challenge control group

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All of the COMPOSITIONS and METHODS disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods have been described in terms of preferred embodiments, it is apparent to those of skill in the art that variations maybe applied to the COMPOSITIONS and METHODS and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope herein. More specifically, certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept as defined by the appended claims.

What is claimed is:

1. A method for induction of neutralizing antibodies in a subject against three or more dengue virus serotypes, comprising, administering a single trivalent dengue virus vaccine composition of live, attenuated dengue virus, the single trivalent dengue virus vaccine composition comprises dengue virus constructs having one or more structural elements of dengue-1, dengue-2, dengue-3 or dengue-4 wherein three of four dengue virus serotype structural elements are present in the single trivalent dengue virus vaccine composition.

2. The method of claim 1, further comprising administering at least one booster of live, attenuated dengue virus vaccine composition comprising a monovalent live, attenuated dengue vaccine on the same day or up to 180 days after administration of the trivalent vaccine composition.

3. The method of claim 1, wherein the single trivalent dengue virus vaccine composition comprises constructs comprising a predetermined ratio of dengue virus serotype constructs comprising dengue-1: dengue-2, and dengue-3; dengue-1: dengue-3, and dengue-4; dengue-1: dengue-2, and dengue-4; or dengue-2, dengue-3 and dengue-4.

4. The method of claim 3, wherein the constructs comprise dengue virus structural proteins on an attenuated dengue-2 virus backbone.

5. The method of claim 4, wherein the attenuated dengue-2 virus comprises dengue-2 vaccine strain PDK-53.

6. The method of claim 1, further comprising administering at least a second trivalent dengue virus vaccine composition of the same or different trivalent dengue virus composition to the subject at the same time as the single trivalent dengue virus vaccine composition of claim 1 or up to 180 days after.

7. The method of claim 3, wherein concentration (plaque forming units (pfus)) of at least one dengue virus serotype is higher in concentration than at least one other dengue virus serotype in the single trivalent dengue virus vaccine composition.

8. The method of claim 7, wherein the higher concentration is 2 to 100,000 fold higher concentration.

9. The method of claim 2 or 5, wherein administration of at least a second vaccine on the same day comprises administration at a second anatomical site using the same mode of administration.

10. The method of claim 2 or 6, wherein administration of at least a second vaccine on the same day comprises administration at a second anatomical site using different modes of administration.

11. The method of claim 1, wherein modes of administration of the vaccines comprise subcutaneous (SC), intradermal (ID), or intramuscular (IM).

12. The method of claim 2, wherein at least one additional booster is administered to the subject less than 180 days after the same day vaccinations.

13. The method of claim 2, wherein at least one additional booster is administered to the subject 90 days or less after the same day vaccinations.

14. The method of claim 1, further comprising administering at least one immunogenic agent to the subject.

15. A method for inhibiting dengue virus infection in a subject, the method comprising:

administering to the subject a first vaccine of a trivalent composition of live, attenuated dengue virus comprising three dengue virus serotypes dengue-1: dengue-2, and dengue-3; dengue-1: dengue-3, and dengue-4; dengue-1: dengue-2, and dengue-4; or dengue-2, dengue-3 and dengue-4 and administering consecutively to the subject at least a second vaccine of a composition of live, attenuated dengue virus comprising a single dengue virus serotype (monovalent), the first and second vaccines are administered to the subject in different anatomical sites on the subject.

16. The method of claim 15, wherein the vaccines comprise a predetermined ratio of dengue virus serotypes.

17. The method of claim 15, wherein the vaccines comprise trivalent dengue virus vaccines having unequivalent distribution of the three dengue virus serotypes in the vaccine where concentration of one serotype can vary from 2 to 100,000 fold compared to another.

18. The method of claim 15, wherein the single dengue virus serotype comprises a dengue virus serotype not having a structural protein in the trivalent dengue virus vaccine composition.

19. A method of inhibiting a condition caused by dengue virus infection, the method comprising: a) administering a first trivalent chimeric vaccine composition to a subject having an effective amount of a first, second and third dengue virus serotype; and b) administering a second monovalent vaccine composition to the subject having an effective amount of a single dengue virus serotype wherein i) the first, second, and third dengue virus serotypes are each different from one another; ii) the second administration occurs on the same day or within 180 days after the first administration; iii) each dengue virus serotypes is administered in an amount that is sufficient to induce an immune response in the subject; iv) each of the dengue virus serotypes is a live, attenuated virus.

20. The method of claim 19, wherein the first, second and third dengue virus serotype are in a predetermined ratio.

21. The method according to claim 20, wherein trivalent vaccine composition comprises dengue-4 (DEN-4) at 2 to 100,000 times higher than the other two dengue virus serotypes.

22. The method of claim 19, wherein the trivalent chimeric vaccine composition comprises constructs having attenuated dengue-2 virus backbones.

23. The method of claim 19, wherein the attenuated dengue-2 virus strain is PDK-53.

24. The method according to claim 1, wherein the dosage forms comprise the vaccinal dengue viruses serotypes in a range of pfus from 10^3 to 10^7.

25. The method of claim 20, wherein the trivalent chimeric vaccine composition comprises constructs comprising a predetermined ratio of dengue virus serotype constructs comprising dengue-1: dengue-2, and dengue-3; dengue-1: dengue-3, and dengue-4; dengue-1: dengue-2, and dengue-4; or dengue-2, dengue-3 and dengue-4.

26. A vaccine kit comprising;

at least one vaccine composition against dengue virus consisting of a trivalent dengue virus vaccine;

at least a second monovalent dengue virus vaccine composition; and

at least two containers for the vaccine compositions.

27. The kit of claim 26, wherein the at least one vaccine composition comprises approximately equivalent PFUs of the three dengue virus serotypes.

28. The kit of claim 26, wherein the at least one vaccine composition comprises a dengue virus vaccine composition of predetermined ratio of the three dengue virus serotypes.

29. A trivalent dengue virus vaccine composition comprising, dengue-dengue chimeras and live, attenuated dengue viruses in a combination wherein three dengue virus serotypes of dengue-1, dengue-2, dengue-3 and dengue-4 are represented in the composition and a pharmaceutically acceptable excipient.

30. The composition of claim 29, wherein the trivalent composition includes a modified dengue-4 chimera that includes a dengue-2 backbone.

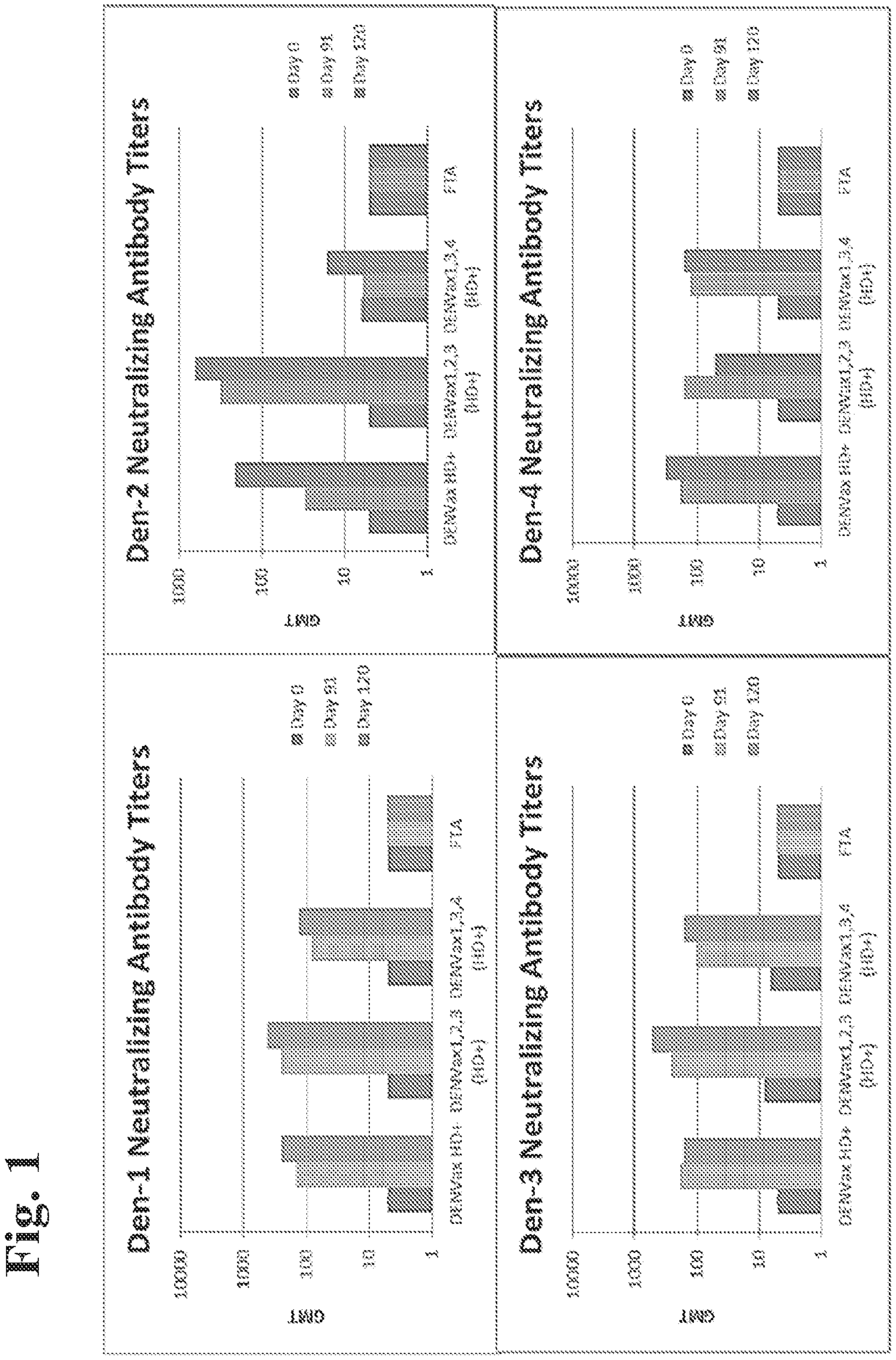
Den-2 Neutralizing Antibody Titers
Den-4 Neutralizing Antibody Titers
Den-1 Neutralizing Antibody Titers
Den-3 Neutralizing Antibody Titers
GMT
1000
100
10
1
Day 0
Day 51
Day 120
DENVax HDx
DENVax1,2,3 (HDx)
DENVax1,3,4 (HDx)
FDA

Fig. 1