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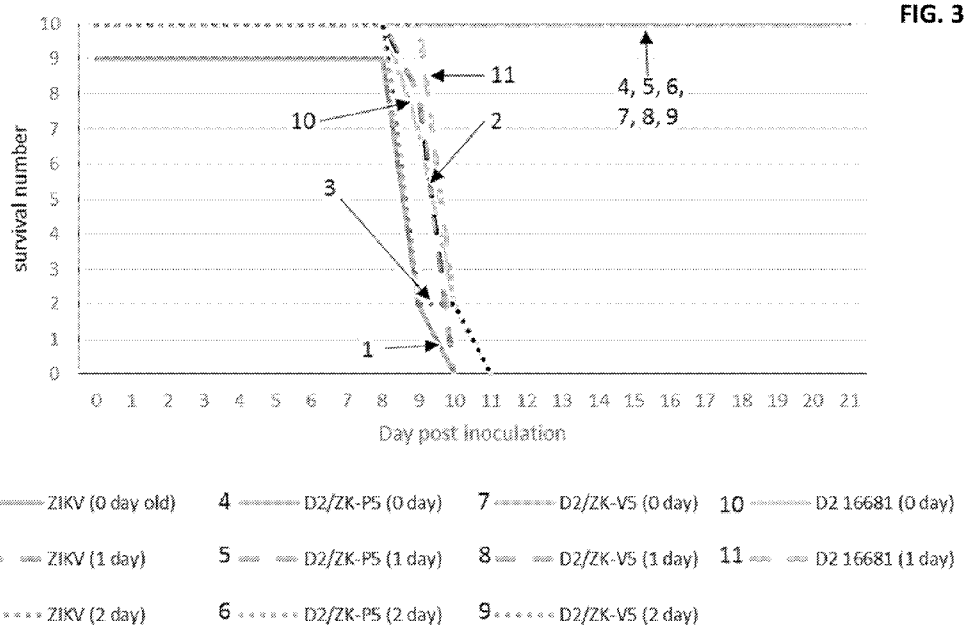
FIG. 3



(57) Abstract: Chimeric flaviviruses that include non-coding regions, non-structural proteins, a capsid (C) protein and a portion of a premembrane (prM) signal sequence from an attenuated or wild-type dengue serotype 2 virus (DENV-2), and a portion of a prM signal sequence, a prM protein and at least a portion of an envelope (E) protein from a Zika virus (ZIKV) are described. Also described are immunogenic compositions and methods for eliciting an immune response in a subject, such as an immune response directed against ZIKV.

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CHIMERIC DENGUE/ZIKA VIRUSES AS LIVE-ATTENUATED ZIKA VIRUS VACCINES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/359,812, filed July 8, 2016, which is herein incorporated by reference in its entirety.

FIELD

This disclosure concerns chimeric, attenuated flaviviruses having non-structural proteins from an attenuated or wild-type dengue virus and at least one structural protein from a Zika virus. This disclosure further concerns use of the chimeric flaviviruses in Zika virus vaccine compositions.

BACKGROUND

Zika virus, a flavivirus classified within the *Flaviviridae* with other important mosquito-borne viruses, including yellow fever, dengue, West Nile and Japanese encephalitis viruses, has spread rapidly in a hemispheric-wide epidemic since the virus was introduced to Brazil in 2015, reaching Central and North Americas, including territories of the United States and now threatening the continental U.S. Initially isolated in 1947 in Uganda, the virus was first linked to human disease in 1952 and has been recognized sporadically as a cause of mild, self-limited febrile illness in Africa and Southeast Asia (Weaver *et al.*, *Antiviral Res* 130:69-80, 2016; Faria *et al.*, *Science* 352(6283):345-349, 2016). However, in 2007, an outbreak appeared in the North Pacific island of Yap, transferred there presumably from Asia, and subsequently disseminated from island to island across the Pacific, leading to an extensive outbreak in 2013-2014 in French Polynesia, with subsequent spread to New Caledonia, the Cook Islands, and ultimately to Easter Island, far to the East. An Asian lineage virus subsequently was transferred to the Western Hemisphere by routes that remain undetermined (Faria *et al.*, *Science* 352(6283):345-349, 2016). The virus is transmitted anthropontically by *Aedes aegypti, A. albopictus* and possibly *A. hensilli* and *A. polynieseinsis* (Weaver *et al.*, *Antiviral Res* 130:69-80, 2016).

In late 2015, a significant increase in fetal abnormalities (*e.g.* microcephaly) and Guillain-Barré syndrome (GBS) in areas of widespread Zika virus infection raised concerns that Zika virus might be much more virulent than originally thought and prompted the World Health Organization (WHO) to declare a Public Health Emergency of International Concern (PHEIC) (Heymann *et al.*, *Lancet* 387(10020):719-721, 2016).

SUMMARY

Disclosed herein are chimeric flaviviruses that include non-coding regions, non-structural proteins, a capsid (C) protein and a portion of a premembrane (prM) signal sequence from a dengue serotype 2 virus (DENV-2); and a portion of a prM signal sequence, a prM protein and at least a portion of an envelope (E) protein from a Zika virus (ZIKV). Also described are immunogenic compositions and methods for eliciting an immune response against ZIKV in a subject.

Provided herein are nucleic acid chimeras that include a first nucleic acid molecule comprising a 5' non-coding region, a nucleic acid encoding non-structural proteins and a C protein, and a 3' non-coding region, each from a DENV-2 strain genome, wherein the C protein comprises a portion of a prM signal sequence from the DENV-2 genome and a portion of a prM signal sequence from a ZIKV genome; and a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding a prM protein and at least a portion of an E protein from the ZIKV genome. In some embodiments, the DENV-2 is an attenuated DENV-2 strain, such as strain PDK-53, or the attenuated strain includes one or more attenuating mutations present in the PDK-53 genome. In other embodiments, the DENV-2 is a wild-type DENV-2, such as strain 16681.

Also provided are chimeric flaviviruses that include a nucleic acid chimera disclosed herein. Immunogenic compositions that include a chimeric DENV-2/ZIKV are further provided.

Further provided herein are methods of eliciting an immune response against ZIKV in a subject by administering to the subject a chimeric flavivirus disclosed herein, or immunogenic composition thereof.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of the genomic structure of chimeric DENV-2/Zika viruses (D2/ZKV). The C/prM and E/NS1 junction site sequences for DENV-2, ZIKV and D2/ZKV are also shown. For the chimeric viruses, ZIKV sequence is indicated by boxes. The C/prM junction sequences shown are from DENV-2 (nucleotides 388-447 of SEQ ID NO: 11; amino acids 98-117 of SEQ ID NO: 12), ZIKV (nucleotides 411-482 of SEQ ID NO: 7; amino acids 102-125 of SEQ ID NO: 8), D2/ZKV Strategy 1 (D2/ZKV-V nucleotides 388-459 of SEQ ID NO: 1 and amino acids 98-121 of SEQ ID NO: 2) and D2/ZKV Strategy 2 (nucleotides 388-459 of SEQ ID NO: 3;

amino acids 98-121 of SEQ ID NO: 4). The E/NS1 junction sequences shown are from DENV-2 (nucleotides 2356-2430 of SEQ ID NO: 11; amino acids 754-778 of SEQ ID NO: 12), ZIKV (nucleotides 2424-2498 of SEQ ID NO: 7 and amino acids 773-797 of SEQ ID NO: 8) and D2/ZKV (nucleotides 2401-2475 of SEQ ID NO: 1 and amino acids 769-793 of SEQ ID NO: 2). The introduced NgoMIV in the DENV-2 backbone for the E/NS1 junction site resulted in a Val to Ala substitution at the amino acid 754 (DENV-2 E-482 residue).

FIGS. 2A-2B are graphs showing growth kinetics of D2/ZK-P5 and D2/ZK-V5. (FIG. 2A) In Vero cells, both P5 and V5 chimeras reached peak titers of greater than 1 x 10^7 pfu/ml, but V5 virus grew slightly slower than the P5 virus. Both viruses replicated slightly less than the wt ZIKV pRVABC59 in Vero cells. (FIG. 2B) In C6/36 cells, P5 virus replicated significantly less than the wt ZIKV, and somewhat less than its backbone D2 16681 virus. However, P5 still replicated to greater than 1 x 10^8 pfu/ml. The V5 virus retained the same crippled growth in C6/36 cells as its D2 PDK-53 vaccine backbone virus.

FIG. 3 is a graph showing neurovirulence of wild-type and D2/ZK chimeric viruses in mice. Litters of newborn CD-1 (ICR) mice 0-2 days after birth were placed into groups of 10 according to their age, and inoculated with 10^4 pfu/30 µl of ZIKV, D2/ZK-P5, D2/ZK-V5 or D2 16681 through intracranial inoculation. All 29 mice (0-, 1-, or 2-days old) challenged with wild-type ZIKV became severely ill or died between 9 and 11 days post-inoculation. The 20 mice (0- or 1-day old) that received wild-type D2 16681 virus also succumbed to virus infection between about days 10 and 11. All 30 mice inoculated with either D2/ZK-P5 or D2/ZK-V5 survived virus inoculation.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on July 5, 2017, 562 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOs: 1 and 2 are D2/ZK-V nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 3 and 4 are D2/ZK-5V nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 5 and 6 are D2/ZK-V2A nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 7 and 8 are nucleotide and amino acid sequences of ZIKV strain R103451.

SEQ ID NOs: 9 and 10 are nucleotide and amino acid sequences of DENV-2 16681.

SEQ ID NOs: 11 and 12 are nucleotide and amino acid sequences of DENV-2 PDK-53.

SEQ ID NOs: 13 and 14 are nucleotide and amino acid sequences of ZIKV strain PRVABC59.

SEQ ID NOs: 15 and 16 are D2/ZK-P-RFNN nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 17 and 18 are D2/ZK-V-RFNN nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 19 and 20 are D2/ZK-P4 nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 21 and 22 are D2/ZK-V4 nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 23 and 24 are D2/ZK-P5 nucleotide and amino acid sequences, respectively.

SEQ ID NOs: 25 and 26 are D2/ZK-V5 nucleotide and amino acid sequences, respectively.

DETAILED DESCRIPTION

I. Abbreviations

ASD	average survival days
C	capsid protein
D2/ZKV	chimeric dengue-2/Zika virus
DENV	dengue virus
E	envelope glycoprotein
GBS	Guillain-Barré syndrome
MOI	multiplicity of infection
NS	non-structural
pfu	plaque forming unit
p.i.	post-infection
prM	premembrane protein
PHEIC	Public Health Emergency of International Concern
WHO	World Health Organization
ZIKV	Zika virus

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-

02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Adjuvant: A substance or vehicle that non-specifically enhances the immune response to an antigen. Adjuvants can include a suspension of minerals (alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in mineral oil (for example, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity. Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants (for example, see U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological molecules, such as costimulatory molecules. Exemplary biological adjuvants include IL-2, RANTES, GM-CSF, TNF-α, IFN-γ, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL.

Administer: As used herein, administering a composition (*e.g.* an immunogenic composition, such as a chimeric virus) to a subject means to give, apply or bring the composition into contact with the subject. Administration can be accomplished by any of a number of routes, such as, for example, topical, oral, subcutaneous, intramuscular, intraperitoneal, intravenous, intrathecal and intramuscular.

Antibody: A protein (or protein complex) that includes one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad of immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is generally a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" (about 50-70 kDa) chain. The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms "variable light chain" (V_L) and "variable heavy chain" (V_H) refer, respectively, to these light and heavy chains.

As used herein, the term "antibodies" includes intact immunoglobulins as well as a number of well-characterized fragments. For instance, Fabs, Fvs, and single-chain Fvs (scFvs) that bind to

target protein (or epitope within a protein or fusion protein) would also be specific binding agents for that protein (or epitope). These antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) $(Fab')_2$, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; (4) $F(ab')_2$, a dimer of two Fab' fragments held together by two disulfide bonds; (5) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (6) single chain antibody, a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Methods of making these fragments are routine (see, for example, Harlow and Lane, *Using Antibodies: A Laboratory Manual*, CSHL, New York, 1999).

Antibodies for use in the methods and devices of this disclosure can be monoclonal or polyclonal. Merely by way of example, monoclonal antibodies can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495-97, 1975) or derivative methods thereof. Detailed procedures for monoclonal antibody production are described in Harlow and Lane, *Using Antibodies: A Laboratory Manual*, CSHL, New York, 1999.

Antibody binding affinity: The strength of binding between a single antibody binding site and a ligand (*e.g.*, an antigen or epitope). The affinity of an antibody binding site X for a ligand Y is represented by the dissociation constant (K_d), which is the concentration of Y that is required to occupy half of the binding sites of X present in a solution. A smaller K_d indicates a stronger or higher-affinity interaction between X and Y and a lower concentration of ligand is needed to occupy the sites. In general, antibody binding affinity can be affected by the alteration, modification and/or substitution of one or more amino acids in the epitope recognized by the antibody paratope. In one example, antibody binding affinity is measured by end-point titration in an Ag-ELISA assay.

Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. In one embodiment, an antigen is a virus antigen, such as a flavivirus E protein.

Attenuated: In the context of a live virus, the virus is attenuated if its ability to infect a cell or subject and/or its ability to produce disease is reduced (for example, eliminated) compared to a wild-type virus. Typically, an attenuated virus retains at least some capacity to elicit an immune response following administration to an immunocompetent subject. In some cases, an attenuated virus is capable of eliciting a protective immune response without causing any signs or symptoms of infection. In some embodiments, the ability of an attenuated virus to cause disease in a subject is reduced at least about 10%, at least about 25%, at least about 50%, at least about 75% or at least about 90% relative to wild-type virus. Accordingly, an "**attenuating mutation**" is a mutation in the viral genome and/or an encoded polypeptide that results in an attenuated virus.

Biological sample: A sample obtained from a subject (such as a human or veterinary subject). Biological samples, include, for example, fluid, cell and/or tissue samples. In some embodiments herein, the biological sample is a fluid sample. Fluid sample include, but are not limited to, serum, blood, plasma, urine, feces, saliva, cerebral spinal fluid (CSF) and bronchoalveolar lavage (BAL) fluid.

Capsid protein (C protein): A flavivirus structural protein that functions to package viral RNA into the nucleocapsid core during virus assembly. The C-terminal portion of the C protein includes an internal signal sequence (referred to herein as either C(ss) or prM signal sequence) for translocation of the prM protein into the endoplasmic reticulum, where cleavage of the C and prM proteins occurs. This signal sequence varies in length among different flaviviruses. For example, the C(ss) of both WNV and ZIKV is 18 amino acids, while the C(ss) of DEN viruses is 14 amino acids.

Chimera: A molecule (*e.g.*, nucleic acid or protein) composed of parts that are of different origin (such as at least two nucleic acids or polypeptides) that, while typically unjoined in their native state, are joined or linked to form a single continuous molecule. A chimera may include nucleic acids or polypeptides that are joined end-to-end (for example, the amino-terminus of one sequence is joined to the carboxyl-terminus of a second sequence) or may include a sequence from one molecule that is embedded within that of another molecule (for example, the amino-terminus and carboxyl-terminus of the chimera are from one molecule, while an intervening sequence comes from another molecule).

A chimera may include a chimeric protein, for example a protein that is composed of amino acids from more than one protein. A chimera may also include a chimeric nucleic acid composed of nucleic acid sequences from more than one source, such as a chimeric nucleic acid which encodes a chimeric protein. In other examples, a chimera may include a chimeric genome, such as a flavivirus genome, which is composed of sequences from two or more flaviviruses. For example,

a chimeric flavivirus genome may comprise nucleic acid sequences from more than one flavivirus genome, such as a dengue virus and a Zika virus. In some examples, a chimeric flavivirus includes nucleic acids encoding one or more proteins from a first flavivirus and nucleic acids encoding one or more proteins from a second flavivirus. In particular examples, a chimeric flavivirus is composed of a nucleic acid encoding the non-structural proteins and a C protein or a portion thereof from a dengue virus genome linked to a nucleic acid encoding a prM protein and at least a portion of an E protein (and optionally a portion of a C protein) from a Zika virus genome.

Conservative substitution: A substitution of one amino acid residue in a protein sequence for a different amino acid residue having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting polypeptide. For example, ideally, a flavivirus protein (such as a prM, E, or non-structural protein) including one or more conservative substitutions (for example 1-10, 2-5, or 10-20, or no more than 2, 5, 10, 20, 30, 40, or 50 substitutions) retains the structure and function of the wild-type protein. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR. In one example, such variants can be readily selected for additional testing by infecting cells with a virus containing a variant protein and determining its ability to replicate, by producing virus containing a variant protein and determining its neurovirulence or neuroinvasion properties, and/or by testing antibody cross-reactivity.

Contacting: Placement in direct physical association; includes both in solid and liquid form. "Contacting" is often used interchangeably with "exposed." In some cases, "contacting" includes transfecting, such as transfecting a nucleic acid molecule into a cell. In other examples, "contacting" refers to incubating a molecule (such as an antibody) with a biological sample.

Control: A reference standard, for example a positive control or negative control. A positive control is known to provide a positive test result. A negative control is known to provide a negative test result. However, the reference standard can be a theoretical or computed result, for example a result obtained in a population.

Dengue virus (DENV): An RNA virus of the family *Flaviviridae*, genus *Flavivirus*. The dengue virus genome encodes the three structural proteins (C, prM and E) that form the virus particle and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) that are only found in infected host cells, but are required for replication of the virus. There are four serotypes of dengue virus, referred to as DENV-1, DENV-2, DENV-3 and DENV-4. All four serotypes can cause the full spectrum of dengue disease. Infection with one serotype can produce lifelong immunity to that serotype. However, severe complications can occur upon subsequent

infection by a different serotype. Dengue virus is primarily transmitted by *Aedes* mosquitoes, particularly *A. aegypti*. Symptoms of dengue virus infection include fever, headache, muscle and joint pain and a skin rash similar to measles. In a small percentage of cases, the infection develops into a life-threatening dengue hemorrhagic fever, typically resulting in bleeding, low platelet levels and blood plasma leakage, or into dengue shock syndrome characterized by dangerously low blood pressure.

Envelope glycoprotein (E protein): A flavivirus structural protein that mediates binding of flavivirus virions to cellular receptors on host cells. The flavivirus E protein is required for membrane fusion, and is the primary antigen inducing protective immunity to flavivirus infection. Flavivirus E protein affects host range, tissue tropism and viral virulence. The flavivirus E protein contains three structural and functional domains, DI-DIII. In mature virus particles the E protein forms head to tail homodimers lying flat and forming a dense lattice on the viral surface.

Flavivirus non-structural protein: There are seven non-structural (NS) proteins of a flavivirus, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, which are encoded by the portion of the flavivirus genome that is 3' to the structural proteins. NS1 has been implicated in RNA replication and has been shown to be secreted from infected mammalian cells (Post *et al., Virus Res.* 18:291-302, 1991; Mackenzie *et al., Virology* 220:232-240, 1996; Muylaert *et al., Virology* 222:159-168, 1996). NS1 can elicit strong humoral immune responses and is a potential vaccine candidate (Shlesinger *et al., J. Virol.* 60:1153-1155, 1986; Qu *et al., J. Gen. Virol.* 74:89-97, 1993). NS2 is cleaved into NS2A and NS2B. NS2A is involved in RNA replication and virus particle assembly and secretion and NS2B forms a complex with NS3 and functions as a cofactor for the NS3 protease, which cleaves portions of the virus polyprotein. NS3 also functions as an RNA helicase and is used to unwind viral RNA during replication (Li *et al., J. Virol.* 73:3108-3116, 1999). While the exact functions of NS4A and NS4B remain to be elucidated, they are thought to be involved in RNA replication and RNA trafficking (Lindenbach and Rice, In: *Fields Virology*, Knipe and Howley, eds., Lippincott, Williams, and Wilkins, 991-1041, 2001). Finally, the NS5 protein is an RNA-dependent RNA polymerase involved in genome replication (Rice *et al., Science* 229:726-733, 1985). NS5 also shows methyltransferase activity commonly found in RNA capping enzymes (Koonin, *J. Gen. Virol.* 74:733-740, 1993).

Flavivirus structural protein: The capsid (C), premembrane (prM), and envelope (E) proteins of a flavivirus are the viral structural proteins. Flavivirus genomes consist of positive-sense RNAs that are roughly 11 kb in length. The genome has a 5' cap, but lacks a 3' polyadenylated tail (Wengler *et al., Virology* 89:423-437, 1978) and is translated into one polyprotein. The structural proteins (C, prM, and E) are at the amino-terminal end of the

polyprotein followed by the non-structural proteins (NS1-5). The polyprotein is cleaved by virus and host derived proteases into individual proteins. The C protein forms the viral capsid while the prM and E proteins are embedded in the surrounding envelope (Russell *et al., The Togaviruses: Biology, Structure, and Replication*, Schlesinger, ed., Academic Press, 1980). The E protein functions in binding to host cell receptors resulting in receptor-mediated endocytosis. In the low pH of the endosome, the E protein undergoes a conformational change causing fusion between the viral envelope and the endosomal membranes. The prM protein is believed to stabilize the E protein until the virus exits the infected cell, at which time prM is cleaved to the mature M protein (Reviewed in Lindenbach and Rice, In: *Fields Virology*, Knipe and Howley, eds., Lippincott, Williams, and Wilkins, 991-1041, 2001).

Heterologous: Originating from a different genetic sources or species.

Immune response: A response of a cell of the immune system, such as a B-cell, T-cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigen. An immune response can include any cell of the body involved in a host defense response for example, an epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate immune response or inflammation.

Immunize: To render a subject protected from an infectious disease, such as by vaccination.

Isolated: An "isolated" or "purified" biological component (such as a nucleic acid, peptide, protein, protein complex, or particle) has been substantially separated, produced apart from, or purified away from other components in a preparation or other biological components in the cell of the organism in which the component occurs, that is, other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins that have been "isolated" or "purified" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell, as well as chemically synthesized nucleic acids or proteins. The term "isolated" or "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, an isolated biological component is one in which the biological component is more enriched than the biological component is in its natural environment within a cell, or other production vessel. Preferably, a preparation is purified such that the biological component represents at least 50%, such as at least 70%, at least 90%, at least 95%, or greater, of the total biological component content of the preparation.

Nucleic acid molecule: A polymeric form of nucleotides, which may include both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of

the above. A nucleotide refers to a ribonucleotide, deoxynucleotide or a modified form of either type of nucleotide. The term "nucleic acid molecule" as used herein is synonymous with "nucleic acid" and "polynucleotide." A nucleic acid molecule is usually at least 10 bases in length, unless otherwise specified. The term includes single- and double-stranded forms of DNA. A polynucleotide may include either or both naturally occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages.

Operably linked: A first nucleic acid is operably linked to a second nucleic acid when the first nucleic acid is placed in a functional relationship with the second nucleic acid. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame. Operably linked nucleic acids include a first nucleic acid contiguous with the 5′ or 3′ end of a second nucleic acid. In other examples, a second nucleic acid is operably linked to a first nucleic acid when it is embedded within the first nucleic acid, for example, where the nucleic acid construct includes (in order) a portion of the first nucleic acid, the second nucleic acid, and the remainder of the first nucleic acid.

Pharmaceutically acceptable carrier: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington: The Science and Practice of Pharmacy*, The University of the Sciences in Philadelphia, Editor, Lippincott, Williams, & Wilkins, Philadelphia, PA, 21st Edition (2005), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compositions, such as a chimeric virus, and additional pharmaceutical agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Premembrane protein (prM protein): A flavivirus structural protein. The prM protein is an approximately 25 kDa protein that is the intracellular precursor for the membrane (M) protein. prM is believed to stabilize the E protein during transport of the immature virion to the cell surface. When the virus exits the infected cell, the prM protein is cleaved to the mature M protein, which is

part of the viral envelope (Reviewed in Lindenbach and Rice, In: *Fields Virology*, Knipe and Howley, eds., Lippincott, Williams, and Wilkins, 991-1041, 2001).

Preventing, treating or ameliorating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. "Ameliorating" refers to the reduction in the number or severity of one or more signs or symptoms of a disease.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nucleic acid preparation is one in which the nucleic acid is more enriched than the nucleic acid is in its natural environment (such as within a cell) or in a preparation or production vessel. In other examples, a purified virus preparation is one in which the virus is more enriched than in a cell or organism, a preparation, or a production vessel. A purified nucleic acid or virus also includes one that is substantially free of undesired components, such as an inactivating agent. Preferably, a preparation is purified such that the nucleic acid or virus represents at least 50% of the total content of the preparation. In some embodiments, a purified preparation contains at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or more of the nucleic acid or virus.

Recombinant nucleic acid: A nucleic acid molecule (or protein or virus) that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques such as those described in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The term recombinant includes nucleic acids and proteins that have been altered solely by addition, substitution, or deletion of a portion of a natural nucleic acid molecule or protein.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences, is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman (*Adv. Appl. Math.*, 2:482, 1981); Needleman and Wunsch (*J. Mol. Biol.*, 48:443, 1970); Pearson and Lipman (*Proc. Natl. Acad. Sci.*, 85:2444, 1988); Higgins and Sharp (*Gene*, 73:237-44, 1988); Higgins and Sharp

(*CABIOS*, 5:151-53, 1989); Corpet *et al.* (*Nuc. Acids Res.*, 16:10881-90, 1988); Huang *et al.* (*Comp. Appls. Biosci.*, 8:155-65, 1992); and Pearson *et al.* (*Meth. Mol. Biol.*, 24:307-31, 1994). Altschul *et al.* (*Nature Genet.*, 6:119-29, 1994) presents a detailed consideration of sequence alignment methods and homology calculations.

The alignment tools ALIGN (Myers and Miller, *CABIOS* 4:11-17, 1989) or LFASTA (Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444-2448, 1988) may be used to perform sequence comparisons (Internet Program © 1996, W. R. Pearson and the University of Virginia, "fasta20u63" version 2.0u63, release date December 1996). ALIGN compares entire sequences against one another, while LFASTA compares regions of local similarity. These alignment tools and their respective tutorials are available on the Internet at the NCSA website. Alternatively, for comparisons of amino acid sequences of greater than about 30 amino acids, the "Blast 2 sequences" function can be employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment should be performed using the "Blast 2 sequences" function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). The BLAST sequence comparison system is available, for instance, from the NCBI web site; see also Altschul *et al.*, *J. Mol. Biol.*, 215:403-10, 1990; Gish and States, *Nature Genet.*, 3:266-72, 1993; Madden *et al.*, *Meth. Enzymol.*, 266:131-41, 1996; Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402, 1997; and Zhang and Madden, *Genome Res.*, 7:649-56, 1997.

Serum: The fluid portion of the blood that separates out from clotted blood. Serum contains many proteins, including antibodies, but does not contain clotting factors.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals (such as mice, rats, rabbits, sheep, horses, cows, and non-human primates).

Therapeutically effective amount: A quantity of a specified agent (such as a chimeric virus) sufficient to achieve a desired effect in a subject being treated with that agent. For example, this may be the amount of a virus vaccine useful for eliciting an immune response in a subject and/or for preventing infection by the virus. In the context of the present disclosure, a therapeutically effective amount of a Zika virus vaccine, for example, is an amount sufficient to increase resistance to, prevent, ameliorate, and/or treat infection caused by Zika virus in a subject without causing a substantial cytotoxic effect in the subject. The effective amount of a Zika virus vaccine (or Zika virus immunogenic composition) useful for increasing resistance to, preventing, ameliorating, and/or treating infection in a subject will be dependent on, for example, the subject being treated, the manner of administration of the therapeutic composition and other factors.

Transformed: A "transformed" cell is a cell into which has been introduced a nucleic acid molecule (such as a heterologous nucleic acid) by molecular biology techniques. The term encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Vaccine: A preparation of immunogenic material capable of stimulating an immune response, administered for the prevention, inhibition, amelioration, or treatment of infectious or other types of disease. The immunogenic material may include attenuated or inactivated (killed) microorganisms (such as bacteria or viruses), or antigenic proteins, peptides or DNA derived from them. An attenuated virus is a virulent organism that has been modified to produce a less virulent form, but nevertheless retains the ability to elicit antibodies and cell-mediated immunity against the virulent form. An inactivated (killed) virus is a previously virulent organism that has been inactivated with chemicals, heat, or other treatment, but elicits antibodies against the organism. Vaccines may elicit both prophylactic (preventative or protective) and therapeutic responses. Methods of administration vary according to the vaccine, but may include inoculation, ingestion, inhalation or other forms of administration. Vaccines may be administered with an adjuvant to boost the immune response.

Vector: A vector is a nucleic acid molecule allowing insertion of foreign nucleic acid without disrupting the ability of the vector to replicate and/or integrate in a host cell. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. An insertional vector is capable of inserting itself into a host nucleic acid. A vector can also include one or more selectable marker genes and other genetic elements. An expression vector is a vector that contains the necessary regulatory sequences to allow transcription and translation of inserted gene or genes.

Zika virus (ZIKV): A member of the virus family *Flaviviridae* and the genus *Flavivirus*. Other members of this genus include dengue virus, yellow fever virus, Japanese encephalitis virus (JEV), West Nile virus and Spondweni virus. ZIKV is spread by the daytime-active mosquitoes *Aedes aegypti* and *A. albopictus*. This virus was first isolated from a *Rhesus* macaque from the Zika Forest of Uganda in 1947. Since the 1950s, ZIKV has been known to occur within a narrow equatorial belt from Africa to Asia. The virus spread eastward across the Pacific Ocean in 2013-2014, resulting in ZIKV outbreaks in Oceania to French Polynesia, New Caledonia, the Cook Islands, and Easter Island. In 2015, ZIKV spread to Mexico, Central America, the Caribbean and South America, where ZIKV has reached pandemic levels. Infection by ZIKV generally causes either no symptoms are mild symptoms, including mild headache, maculopapular rash, fever,

malaise, conjunctivitis and joint pain. ZIKV causes symptoms in about 20% of infected individuals, and no deaths from the virus have yet been reported. However, ZIKV infection has been linked to the birth of microcephalic infants following maternal infection, as well an increase in cases of GBS. Reports have also indicated that ZIKV has the potential for human blood-borne and sexual transmission. ZIKV has also been found in human saliva and breastmilk. There are currently no available medical countermeasures for the treatment or prevention of Zika virus infection (Malone *et al.*, *PLoS Negl Trop Dis* 10(3):e0004530, 2016).

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

Disclosed herein are chimeric flaviviruses that include non-coding regions, non-structural proteins, a capsid (C) protein and a portion of a premembrane (prM) signal sequence from a wild-type or attenuated dengue serotype 2 virus (DENV-2); and a portion of a prM signal sequence, a prM protein and at least a portion of an envelope (E) protein from a Zika virus (ZIKV). Tables 1 and 2 below provide start and stop positions of the particular genes and proteins in an exemplary Zika virus (SPH2015) and an exemplary attenuated DENV-2 vaccine strain (PDK-53). These sequences can serve as reference sequences and may be used to identify particular nucleotide or amino acid positions that correspond to positions referred to in the chimeric nucleic acids disclosed herein, or proteins encoded by the chimeric nucleic acids disclosed herein, for example by producing an alignment of a chimera and one of the virus sequences provided herein.

Table 1

Start and stop positions of noncoding regions (NCRs), structural proteins and nonstructural proteins in ZIKV strain R103451

Region	Nucleotide start/stop position (SEQ ID NO: 7)	Amino acid start/stop position (SEQ ID NO: 8)
5′ NCR	1-107	--
C	108-473	1-122
C(ss)	420-473	105-122
prM	474-977	123-290
M	753-977	216-290
E	978-2489	291-794
NS1	2490-3545	795-1146
NS2A	3546-4223	1147-1372
NS2B	4224-4613	1373-1502
NS3	4614-6464	1503-2119
NS4A	6465-6914	2120-2269
NS4B	6915-7667	2270-2520
NS5	7668-10376	2521-3423
Stop	10377-10379	--
3′ NCR	10380-10807	--

Table 2. Start and stop positions of NCRs, structural proteins and nonstructural proteins in DENV-2 vaccine strain PDK-53

Region	Nucleotide start/stop position (SEQ ID NO: 11)	Amino acid start/stop position (SEQ ID NO: 12)
5′ NCR	1-96	--
C	97-438	1-114
C(ss)	397-438	101-114
prM	439-936	115-280
M	712-936	206-280
E	937-2421	281-775
NS1	2422-3477	776-1127
NS2A	3478-4131	1128-1345

Region	Nucleotide start/stop position (SEQ ID NO: 11)	Amino acid start/stop position (SEQ ID NO: 12)
NS2B	4132-4521	1346-1475
NS3	4522-6375	1476-2093
NS4A	6376-6825	2094-2243
NS4B	6826-7569	2244-2491
NS5	7570-10269	2492-3391
3′ NCR	10270-10723	--

In the disclosed nucleic acid chimeras, the ZIKV genome can be from any strain of ZIKV, including an African genotype strain or an Asian genotype strain. In some embodiments, the ZIKV is an African genotype strain, such as MR-766. In other embodiments, the ZIKV is an Asian genotype strain, such as SPH2015, PRVABC59, R103451, P6-740 or FSS 13025. In some embodiments, the ZIKV genome is from strain R103451 (SEQ ID NO: 7; or deposited under GenBank Accession No. KX262887.1). The ZIKV genome may be a wild type strain or an attenuated (or vaccine) strain. In some examples, the ZIKV genome sequence is modified, for example to introduce restriction sites for cloning purposes. These modifications can be silent mutations (for example, nucleotide sequence changes that do not alter amino acid sequence) or they may change the amino acid sequence.

ZIKV sequences are publicly available. For example GenBank Accession Nos. KX262887.1, KU321639.1, KU501215.1, KU955595.1, KU955594.1, KU955593.1, KU955592.1, KU955591.1, KU681082.3, KU681081.3 and KX247646.1, all of which are incorporated by reference as included in GenBank on June 14, 2016. In additional examples, the ZIKV genome (or the C signal sequence, prM, and/or E protein from the ZIKV genome) are at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a publicly available ZIKV sequence.

In some embodiments, the DENV-2 strain genome is an attenuated DENV-2 strain genome. In some examples, the attenuated DENV-2 is strain PDK-53, the genome sequence of which is set forth herein as SEQ ID NO: 11.

In other embodiments, the DENV-2 strain genome is a wild-type DENV-2 strain genome. In some examples, the wild-type DENV-2 is strain 16681, the genome sequence of which is set forth herein as SEQ ID NO: 9.

In some examples, the disclosed D2/ZKV chimeras include one or more nucleic acid substitutions that result in an amino acid substitution that provides a desirable characteristic, for

example, increased stability and/or replication in vaccine virus production cell culture (such as Vero cells), or decrease virus replication in mosquito cells (such as C6/36 cells) or live mosquitoes compared to the unsubstituted virus or chimera.

The viruses containing the disclosed nucleic acid chimeras can readily be produced by replication in host cells in culture. Methods of producing viruses are well known in the art (see *e.g. Fields Virology*, Knipe and Howley, eds., Lippincott, Williams, and Wilkins, 2001; Flint *et al., Principles of Virology,* ASM Press, 2000). Host cell lines are generally selected to be easy to infect with virus or transfect with viral genomic RNA, capable of stably maintaining foreign RNA with an unarranged sequence, and have the necessary cellular components for efficient transcription, translation, post-translation modification, virus assembly, and secretion of the protein or virus particle. In addition, cells are typically those having simple media component requirements which can be adapted for growth in suspension culture. In some examples, the host cell line is a mammalian cell line that is adapted to growth in low serum or serum-free medium. Exemplary suitable host cell lines include Vero (monkey), C6/36 (mosquito), BHK21 (hamster), LLC-MK2 (monkey) SK6 (swine), L292 (mouse), HeLa (human), HEK (human), 2fTGH cells (human), HepG2 (human), and PDK (dog) cells. Suitable cell lines can be obtained from the American Type Culture Collection (ATCC), Manassas, VA.

The disclosure also provides D2/ZKV chimeras having one or more nucleic acid or amino acid substitutions, insertions, deletions, or combinations thereof, such that the resulting chimera has improved characteristics, such as improved growth in Vero cells.

Manipulation of the nucleotide sequence of the disclosed chimeric flaviviruses by standard procedures, including for instance site-directed mutagenesis or PCR and M13 primer mutagenesis, can be used to produce variants with improved characteristics (such as increased virus titer or stability in cell culture). Details of these techniques are well known. For instances, protocols are provided in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The simplest modifications involve the substitution of one or more amino acids for amino acids having similar physiochemical and/or structural properties. These so-called conservative substitutions are likely to have minimal impact on the activity and/or structure of the resultant protein. Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of conservative substitutions are shown below.

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl (or vice versa); (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or asparty (or vice versa); or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine (or vice versa).

In addition to targeted mutagenesis to produce variants of the disclosed D2/ZKV chimeras, mutations may accrue upon passage in cell culture that result in variants, some with desirable characteristics. Nucleic acid and amino acid substitutions, insertions, and/or deletions that accrue in chimeric viruses during cell culture passages are readily determined by sequence analysis of the virus amplified from isolated plaques of the virus seed, and can be engineered into infectious clones to generate D2/ZKV chimera variants that have improved characteristics (such as

replication to high titer). Consistent mutations identified from multiple seeds or isolated plaques are one indication of a desirable substitution of the chimera in the cell type. Previous studies have successfully identified substitutions which occurred in cell culture and engineered these into different chimeric virus constructs to produce chimeric viruses with improved characteristics (*e.g.,* Huang *et al., J. Virol.* 77:11436-11447, 2003; Huang *et al., J. Virol.* 12:7300-7310, 2005; U.S. Pat. No. 8,715,689; and WO 2015/196094).

Provided herein are flavivirus nucleic acid chimeras. In some embodiments, the nucleic acid chimera includes a first nucleic acid molecule comprising a 5' non-coding region, a nucleic acid encoding non-structural proteins and a C protein, and a 3' non-coding region, each from a DENV-2 strain genome, wherein the C protein comprises a portion of a prM signal sequence from the DENV-2 genome and a portion of a prM signal sequence from a ZIKV genome; and a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding a prM protein and at least a portion of an E protein from the ZIKV genome.

In some embodiments, the DENV-2 strain genome is an attenuated DENV-2 strain genome. In some examples, the attenuated DENV-2 genome includes a mutation in the 5' non-coding region at nucleotide position 57; a mutation at nucleotide position 2579 that results in the presence of an aspartate at amino acid residue 53 of the NS1 protein; and/or a mutation at nucleotide position 5270 that results in the presence of a valine at amino acid residue 250 of the NS3 protein. In particular examples, the attenuated DENV-2 is strain PDK-53. The attenuated DENV-2 may also include one or more of the mutations listed in Table 4.

In some embodiments, the DENV-2 strain genome is a wild-type DENV-2 strain genome. In some examples, the wild-type DENV-2 is strain 16681.

In some embodiments, the Zika virus is an African genotype virus, such as strain MR-766. In other embodiments, the Zika virus is an Asian genotype virus, such as strain SPH2015, PRVABC59, R103451, P6-740 or FSS 13025.

In some embodiments, the portion of the prM signal sequence from the DENV-2 genome includes the first three amino acids of the DENV-2 prM signal sequence and the portion of the prM signal sequence from the ZIKV genome includes the last 15 amino acids of the ZIKV prM signal sequence (see Strategy 1 in FIG. 1). In some examples, the first three amino acids of the DENV-2 prM signal sequence includes amino acids 101-103 of SEQ ID NO: 12 and/or the last 15 amino acids of the ZIKV prM signal sequence includes amino acids 108-122 of SEQ ID NO: 8.

In other embodiments, the portion of the prM signal sequence from the DENV-2 genome includes the first five amino acids of the DENV-2 prM signal sequence and the portion of the prM signal sequence from the ZIKV genome includes the last 13 amino acids of the ZIKV prM signal

sequence (see Strategy 2 in FIG. 1). In some examples, the first five amino acids of the DENV-2 prM signal sequence includes amino acids 101-105 of SEQ ID NO: 12 and/or the last 13 amino acids of the ZIKV prM signal sequence includes amino acids 110-122 of SEQ ID NO: 8.

In other embodiments, the C/prM junction site includes a different number of residues from the DENV-2 and the ZIKV than those listed above.

In some embodiments, a portion of the E protein is from the DENV-2 genome. In some examples, the portion of the E protein from the DENV-2 genome includes the last 14 amino acids of the modified DENV-2 E protein. In specific non-limiting examples, the last 14 amino acids of the DENV-2 E protein includes amino acids 777-790 of SEQ ID NO: 2. In other examples, the E/NS1 junction site in the D2/ZKV chimera may include an alternative number of residues from the DENV-2, such as about 0, about 2, about 4, about 6, about 8, about 10, about 12, about 13, about 14, about 16, about 18 or about 20 residues from the DENV-2.

In some embodiments, the nucleic acid chimera further includes at least one Vero cell adaptation mutation. In some examples, the Vero cell adaptation mutation results in a glutamine to arginine substitution at residue 465 of the D2/ZKV E protein (also E-465 of ZIKV); an isoleucine to threonine substitution at residue 484 of the D2/ZKV E protein (also E-484 of ZIKV); an isoleucine to phenylalanine substitution at residue 493 of the D2/ZKV E protein (as E-484 of the DENV-2); a lysine to asparagine substitution at residue 99 of the NS2A protein (NS2A protein is from DENV-2); and/or an aspartic acid to asparagine substitution at residue 23 of the NS4A protein (NS4A protein is from DENV-2) (Table 3).

In particular examples, nucleic acid chimera includes four Vero cell adaptation mutations, wherein the mutations result in a glutamine to arginine substitution at residue 465 of the D2/ZKV E protein (also E-465 of ZIKV); an isoleucine to phenylalanine substitution at residue 493 of the D2/ZKV E protein (also E-484 of DENV-2); a lysine to asparagine substitution at residue 99 of the NS2A protein (from DENV-2); and an aspartic acid to asparagine substitution at residue 23 of the NS4A protein (from DENV-2).

In other particular examples, nucleic acid chimera includes four Vero cell adaptation mutations, wherein the mutations result in a glutamine to arginine substitution at residue 465 of the D2/ZKV E (also E-465 of ZIKV) protein; an isoleucine to threonine substitution at residue 484 of the D2/ZKV E (also E-484 of ZIKV) protein; an isoleucine to phenylalanine substitution at residue 493 of the D2/ZKV E (also E-484 of DENV-2) protein; and a lysine to asparagine substitution at residue 99 of the NS2A protein (from DENV-2).

In other particular examples, nucleic acid chimera includes five Vero cell adaptation mutations, wherein the mutations result in a glutamine to arginine substitution at residue 465 of the

D2/ZIKV E protein (also E-465 of ZIKV); an isoleucine to threonine substitution at residue 484 of the D2/ZIKV E protein (also E-484 of ZIKV); an isoleucine to phenylalanine substitution at residue 493 of the D2/ZIKV E protein (also E-484 of DENV-2); a lysine to asparagine substitution at residue 99 of the NS2A protein (from DENV-2); and an aspartic acid to asparagine substitution at residue 23 of the NS4A protein (from DENV-2).

Table 3. Potential amino acid mutations for Vero-cell-fitness enhancement of chimeric DEN-2/Zika viruses

NT position on chimeric DENV-2/ZKV	Protein-AA position based on:			AA substitution (WT-Mut)
	Chimeric DENV-2/ZKV	DENV-2 AA	ZIKV AA	
2348 (A to G)	E-465	NA	E-465	Gln-Arg
2405 (T to C)	E-484	NA	E-484	Ile-Thr
2431 (A to T)	E-493	E-484*	NA	Ile-Phe
3817 (A to C)	NS2A-99	NS2A-99	NA	Lys-Asn
6487 (G to A)	NS4A-23	NS4A-23	NA	Asp-Asn

NA= not applicable; chimeric virus doesn't include the gene of the virus

*The DENV-2 E protein is shorter than the ZIKV E protein, resulting in different residue numbering between chimeric DENV-2/ZKV and DENV-2

In some embodiments, the nucleic acid chimera includes a nucleic acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23 or SEQ ID NO: 25. In some examples, the nucleic acid chimera includes the nucleic acid sequence of SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23 or SEQ ID NO: 25.

In some embodiments, the nucleic acid chimera encodes an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26. In some examples, the nucleic acid chimera encodes the amino acid sequence of SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26.

In some examples, the nucleic acid sequence is human codon optimized.

Also provided herein are chimeric flaviviruses that comprise a nucleic acid chimera disclosed herein. Compositions, such as immunogenic compositions, that include the chimeric flaviviruses are also provided by the present disclosure. In some embodiments, the immunogenic compositions further includes a pharmaceutically acceptable carrier and/or one or more adjuvants.

Further provided herein are methods of eliciting an immune response against ZIKV in a subject by administering to the subject a chimeric flavivirus or immunogenic composition disclosed herein. The immune response may include, for example, induction of ZIKV-specific antibodies (such as IgM and/or IgG antibodies) or induction of a virus-specific T cell response. In some examples, the immune response is a protective immune response.

In some embodiments, the method includes administering one to five doses (such as 1, 2, 3, 4 or 5 doses) of the immunogenic composition to the subject. In some examples, the first 1 or 2 doses is the immunogenic composition of the disclosed live-attenuated chimeric DENV-2/ZKV and the following dose(s) is/are inactivated or non-infectious ZIKV vaccine. In some examples, the method further includes administering a combination of the live-attenuated chimeric DENV-2/ZIKV vaccine disclosed herein with an inactivated or non-infectious ZIKV vaccine (such as the inactivated ZIKV or inactivated chimeric WN/ZIKV) simultaneously. In some examples, the method further includes administering one or more adjuvants to the subject.

Also provided is a method of immunizing a subject against ZIKV by administering to the subject a chimeric flavivirus or immunogenic composition disclosed herein.

In some embodiments of the methods, the subject is a human.

IV. Compositions and Methods for Eliciting an Immune Response

Provided herein are methods of eliciting an immune response in a subject by administering to the subject a chimeric dengue serotype 2/Zika virus (D2/ZKV) disclosed herein. In a particular example, the subject is a human. The chimeric D2/ZKV is used, for examples, to produce an immune response that prevents or inhibits infection with a ZIKV.

In some examples, the method further includes selecting a subject in need of enhanced immunity to ZIKV. Subjects in need of enhanced immunity to ZIKV include subjects who are at risk of ZIKV infection, subjects who have been exposed to one or more ZIKV, and subjects who have previously been vaccinated with ZIKV or other flavivirus vaccines. Residents of, or travelers to, countries or regions where ZIKV is endemic are at risk of contracting ZIKV. Additional factors that contribute to risk of infection with ZIKV include the characteristics of the location, presence of ZIKV in the area, exposure to mosquitos, and lack of preventive measures (such as insect repellant).

One or more chimeric D2/ZIKV are administered to a subject by any of the routes normally used for introducing a composition into a subject. Methods of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, parenteral, intravenous, subcutaneous, vaginal, rectal, intranasal, inhalation or oral. Parenteral administration, such as subcutaneous, intravenous or intramuscular administration, is generally achieved by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Administration can be systemic or local.

Immunogenic compositions are administered in any suitable manner, such as with pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. See, *e.g., Remington: The Science and Practice of Pharmacy*, The University of the Sciences in Philadelphia, Editor, Lippincott, Williams, & Wilkins, Philadelphia, PA, 21st Edition (2005). Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present disclosure.

The immunogenic compositions may be conveniently presented in unit dosage form and prepared using conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets commonly used by one of ordinary skill in the art.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the

like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

In some examples, the compositions disclosed herein include one or more adjuvants. In other examples, an adjuvant is not included in the composition, but is separately administered to a subject (for example, in combination with a composition disclosed herein) before, after, or substantially simultaneously with administration of one or more of the compositions disclosed herein. Adjuvants are agents that increase or enhance an immune response in a subject administered an antigen, compared to administration of the antigen in the absence of an adjuvant. One example of an adjuvant is an aluminum salt, such as aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate, or aluminum hydroxyphosphate. Other adjuvants include biological adjuvants, such as cytokines (for example, IL-2, IL-6, IL-12, RANTES, GM-CSF, TNF-α, or IFN-γ), growth factors (for example, GM-CSF or G-CSF), one or more molecules such as OX-40L or 4-1 BBL, immunostimulatory oligonucleotides (for example, CpG oligonucleotides), Toll-like receptor agonists (for example, TLR2, TLR4, TLR7/8, or TLR9 agonists), and bacterial lipopolysaccharides or their derivatives (such as 3D-MPL). Additional adjuvants include oil and water emulsions, squalene, or other agents. In one example, the adjuvant is a mixture of stabilizing detergents, micelle-forming agent, and oil available under the name PROVAX® (IDEC Pharmaceuticals, San Diego, CA). One of skill in the art can select a suitable adjuvant or combination of adjuvants to be included in the compositions disclosed herein or administered to a subject in combination with the compositions disclosed herein.

Administration is accomplished by single or multiple doses. The dose administered to a subject in the context of the present disclosure should be sufficient to induce a beneficial therapeutic response in a subject over time, or to inhibit or prevent ZIKV infection. The dose required will vary from subject to subject depending on the species, age, weight and general condition of the subject, the severity of the infection being treated, the particular immunogenic composition being used, and its mode of administration. An appropriate dose can be determined by one of ordinary skill in the art using only routine experimentation. In some examples, the dose of each chimeric virus (such as in an immunogenic composition) administered to the subject is about 100 pfu to about 1000,000 pfu. For example, a dose of the immunogenic composition can contains at least 100 pfu, at least 1000 pfu, at least 5000 pfu, at least 10,000 pfu, at least 50,000 pfu, at least 100,000 pfu, at least 500,000 pfu, or at least 1000,000 pfu of the chimeric virus.

The volume of administration will vary depending on the route of administration. By way of example, intramuscular injections may range from about 0.1 ml to about 1.0 ml. Those of ordinary skill in the art will know appropriate volumes for different routes of administration.

Repeated immunizations may be necessary to produce an immune response in a subject. When administered in multiple doses, the booster doses are administered at various time intervals, such as weeks or months to years. In other examples, the D2/ZKV chimeric viruses are used as a booster following administration of one or more ZIKV vaccines. In one example, a subject is administered a prime dose of a ZIKV vaccine followed by at least one boost dose of a D2/ZKV chimeric virus disclosed herein. In alternative examples, the D2/ZKV chimeric virus is administered first, followed by a booster administration of another ZIKV vaccine, such as a inactivated ZIKV vaccine. In some examples, the boost dose is administered about 14, 30, 60, 90, or more days after administration of the prime dose. Additional boosters can be administered at subsequent time points, if determined to be necessary or beneficial. Immunization protocols (such as amount of immunogen, number of doses and timing of administration) can be determined experimentally, for example by using animal models (such as mice or non-human primates), followed by clinical testing in humans.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1: Generation and characterization of chimeric flaviviruses

This example describes the construction of chimeric dengue-2/Zika viruses that include the prM protein and at least a portion of the E protein from a ZIKV in an attenuated or wild-type DENV-2 backbone.

Engineering and deriving chimeric D2/ZKVs

Using the infectious clones of dengue virus serotype 2 (DENV-2) engineered previously (Kinney *et al.*, *Virology* 230:300-308, 1997; Butrapet *et al.*, *J Virol* 74:3011-3019, 2000), two chimeric DENV-2/Zika viruses (D2/ZKV-P and D2/ZKV-V) were generated. D2/ZKV-P and D2/ZKV-V contain the prM and E genes of a Zika virus (ZIKV) in the genomic background of the parent (P) virus DENV-2 16681 and its vaccine (V) strain PDK-53 strain, respectively. The DENV-2 PDK-53 vaccine strain was originally developed by serial passage of the DENV-2 16681 virus 53 times in primary dog kidney cells. Table 4 provides a summary of the nucleotide and amino acid variation between the two strains (see also PCT Publication No. WO 01/060847, which is herein incorporated by reference in its entirety).

Table 4. Nucleotide and Amino Acid Sequence Differences between DENV-2 16681 and its Vaccine Derivative Strain PDK-53

Genome position	Nucleotide 16681	Nucleotide PDK-53	Amino acid 16681	Amino acid PDK-53	Protein position	Polyprotein position
57	**C**	**T**	-	-		
524	A	T	Asp	Val	prM-29	
2055	C	T	Phe	Phe	E-373	653
2579	**G**	**A**	**Gly**	**Asp**	**NS1-53**	**828**
4018	C	T	Leu	Phe	NS2A-181	1308
5270	**A**	**T**	**Glu**	**Val**	**NS3-250**	**1725**
5547	T	C	Arg	Arg	NS3-342	1817
6599	G	C	Gly	Ala	NS4A-75	2168
8571	C	T	Val	Val	NS5-334	2825

It was previously determined that the three major attenuation determinants of DENV-2 PDK-53 reside in the 5' non-coding region (NCR), NS1 protein, and NS3 protein (Butrapet *et al.*, *J Virol* 74:3011-3019, 2000), which are shown in bold in Table 4. Chimeric viruses containing the prM-E gene region from heterologous flaviviruses, within the DENV-2 PDK-53 genetic backbone, have been shown to express the appropriate heterologous virus-specific E immunogens and retain the attenuated phenotype of the DENV-2 PDK-53 vaccine virus (Huang *et al.*, *J Virol* 74:3020-3028, 2000; Huang *et al.*, *J Virol* 77: 11436-11447, 2003; Kinney *et al.*, *Intervirology* 44:176-197, 2001; Huang *et al.*, *J Virol* 79:7300-7310, 2005). DENV-2 PDK-53-based chimeric D2/D1, D2, D2/D3, D2/D4, and D2/WN viruses were previously generated for a live-attenuated tetravalent DENV vaccine and a WNV vaccine (U.S. Patent Nos. 7,094,411; 7,641,909; 8,025,887; and 8,673,316, which are herein incorporated by reference in their entirety). The tetravalent DENV vaccine containing chimeric D2/D1, D2, D2/D3, and D2/D4 has been manufactured and characterized for human clinical trials (Huang *et al.*, *PLoS Negl Trop Dis* 7(5):e2243, 2014), and is currently in phase 3 human clinical trials (George *et al.*, *J Infect Dis* 212(7):1032-1041, 2015; Osorio *et al.*, *Lancet Infect Dis* 14:830-838, 2014).

The chimeric D2/ZKV-P and D2/ZKV-V constructs were based on a similar engineering strategy used for generating chimeric D2/WNV (Strategy 1 in FIG. 1). The chimeric D2/ZKV-V, which is based on the DENV-2 PDK-53 vaccine backbone, is used for live-attenuated ZIKV vaccine development. The chimeric D2/ZKV-P, which is based on the parental DENV-2 16681

strain, is used as a parental chimeric virus for virulence and attenuation comparisons with the chimeric D2/ZKV-V vaccine, as well as for ZIKV vaccine development.

Viable chimeric D2/ZKV-P virus was recovered from C6/36 cells transfected with chimeric viral RNA which was *in vitro* transcribed from engineered chimeric cDNA. Because the DENV-2 PDK-53 vaccine virus does not replicate well in C6/36 cells (which is one of its characteristic attenuation phenotypes), initial efforts in recovering chimeric D2/ZKV-V from C6/36 cells did not yield detectable infectious virus. Also, the first generation of the D2/ZK-P and D2/ZK-V constructs did not generate viable chimeric viruses from transfected Vero cells. The D2/ZK-P virus seed recovered from C6/36 cells also did not infect Vero cells efficiently, suggesting incompatible chimeric genes of the virus for Vero cell infection. Because Vero cells are essential for the manufacture of live-attenuated vaccine viruses, it is necessary to engineer a chimeric virus that replicates well and is stable in Vero cells. D2/ZK-P obtained from C6/36 cells was adapted to grow in Vero cells by serial passage of the virus in Vero cells at a high multiplicity of infection (MOI).

After just one passage in Vero cells at high MOI, successful Vero-adapted D2/ZK-P virus was recovered and sequenced to identify the genetic mutations involved in Vero cell adaption. Further Vero cell passages of the Vero-adapted D2/ZK-P virus resulted in significantly higher titers of D2/ZK-P virus seeds which are also sequenced to identify more mutations that may enhance fitness of the chimera in Vero cells. The necessary Vero-adapted mutation(s) are incorporated into the vaccine D2/ZK-V constructs. In previous studies, various chimeric viruses were modified with mutations for Vero cell adaption to enhance and/or stabilize the chimeric viruses for Vero cell culture, and such modification resulted in successful live-attenuated chimeric dengue viruses and chimeric D2/WN vaccine candidates using the same DENV-2 PDK-53 vaccine backbone.

Chimeric D2/Zika viruses

Provided below is a list of chimeric D2/ZK viruses for generating live-attenuated ZIKV vaccine candidates:

D2/ZK-P (also referred to as D2/ZK-PS): This chimeric virus includes the parental (P) backbone of D2 16681 and the prM-E genes of Zika virus strain SPH2015 (S). The virus was constructed using Strategy 1 illustrated in FIG. 1. Viable virus was recovered from transfected C6/36 cells, but not from Vero cells, and could not be plaque titrated in Vero cells. Evidence of virus recovery was based on >90% IFA positive C6/36 cells by 4G2, and a strong RT-PCR band from nucleic acid amplified from C6/36 culture fluid.

D2/ZK-P Vero: A Vero cell adapted version of D2/ZK-P. To develop a Vero cell adapted virus, Vero cells were infected with a high MOI of D2/ZK-P virus recovered from C6/36 cells. The

first round of adaptation resulted in $7x10^6$ PFU/ml of chimeric virus replicated from Vero cells. The adapted virus was plaque titrated in Vero cells and exhibited clear plaques with mixed sizes. Sequence of the virus identified 7 amino acid mutations (Table 5). Further Vero passages are expected to increase the most adapted chimeric virus in the seed preparation. Plaque purification of larger plaques of the chimera from infected Vero cells is also conducted to further determine the most useful mutation(s) for Vero cell adaption. Four of the identified mutation(s) are engineered into the chimeric constructs to improve D2/ZKV growth in Vero cells (Table 3), and further confirmed as critical mutations for chimeric D2/ZKV adaption and stability in Vero cells (see Example 2). The other 3 mutations at E-191, NS4B-24, and NS4B-245 were found not required for Vero cell adaption or stability.

Table 5. Mutations identified from D2/ZK-P Vero cell adapted virus

NT position on chimeric DENV-2/ZKV	Protein-AA position based on			AA substitution (WT-Mut)
	Chimeric **DENV-2/ZKV**	DENV-2 AA position	ZIKV AA position	
1610 (A to T)	E-191	NA	E-191	His-Leu
2348 (A to G)	E-465	NA	E-465	Gln-Arg
2405 (T to C)	E-484	NA	E-484	Ile-Thr
2431 (A to T)	E-493	E-484	NA	Ile-Phe
3817 (A to C)	NS2A-99	NS2A-99	NA	Lys-Asn
6941 (G to A)	NS4B-24	NS4B-24	NA	Glu-Gly
7603 (A to T)	NS4B-245	NS4B-245	NA	Asn-Leu

D2/ZK-V (also referred to as D2/ZK-VS): This chimeric virus includes the vaccine (V) strain PDK-53 backbone and the prM-E genes of Zika virus strain SPH2015 (S). The nucleotide and amino acid sequences of this chimeric virus are set forth herein as SEQ ID NO: 1 and SEQ ID NO: 2, respectively. No virus was recovered from C6/36 cells or Vero cells. The vaccine strain backbone is attenuated in C6/36 cells so virus recovery was not expected. The chimera was also not viable in Vero cells (similar to the D2/ZK-P described above). Vero-adaptation mutation(s) identified from D2/ZK-P Vero adapted virus are incorporated into the construct to make viable D2/ZK-V as a live-attenuated vaccine candidate.

D2/ZK-P2A: This chimeric virus includes the parental (P) D2 16681 backbone, but with a mutation that results in a methionine to valine substitution at residue 22 of the NS2A protein, and

includes the prM-E gene from Zika virus strain SPH2015. The M22V mutation was identified as a strong Vero cell adaption mutation for the previous D2/WNV chimera. Vero cells were transfected with D2/ZK-P2A to evaluate virus growth. Transfected Vero were cultured at 37°C or 28°C to evaluate the temperature sensitivity of the chimeric construct. The results showed the NS2A-22 mutation did not significantly improve the virus stability in Vero cells.

D2/ZK-V2A: This chimeric virus includes the vaccine (V) D2 PDK-53 backbone, but with a mutation that results in a methionine to valine substitution at residue 22 of the NS2A protein, and includes the prM-E gene from Zika virus strain SPH2015. The nucleotide and amino acid sequences of this chimeric virus are set forth herein as SEQ ID NO: 5 and SEQ ID NO: 6, respectively. Vero cells were transfected with D2/ZK-V2A to evaluate virus growth. Transfected Vero cells were cultured at 37°C or 28°C to evaluate the temperature sensitivity of the chimeric virus. On day 11 post-transfection, RT-PCR analysis of the Vero-28°C culture showed a strong positive result, but the Vero-37°C culture was negative. This result suggested that the NS2A-22 mutation by itself is not sufficient for efficient Vero cell adaption of the chimeric virus.

D2/ZK-5V: This chimeric virus is generated using prM/E junction Strategy 2 shown in FIG. 1. The nucleotide and amino acid sequences of this chimeric virus are set forth herein as SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

The Zika prM-E gene differences in chimeric D2/ZKV variants

The chimeric D2/ZKV constructs were made with the sequence of the ZIKV SPH2015 strain obtained from Genbank (Accession No. KU321639.1), before the PRVABC59 and R103451 strains were isolated from travelers acquiring ZIKV infection during the 2015 outbreak at CDC's diagnostic lab. The R103451 and PRVABC59 strains are now available as wild-type (wt) ZIKV controls, but the SPH2015 strain is not. There is only 1 amino acid (AA) sequence difference between SPH2015 and PRVABC59 (or R103451) within the prM-E gene region included in the D2/ZIKV chimeric constructs. The difference is at E protein amino acid position 23 position (E-23), with an isoleucine (Ile) in the SPH2015 strain and a valine (Val) in the PRVABC59 and R103451 strains. For the nucleotide sequences of the prM-E, there is one nucleotide difference (silent) between the chimeras and strain R103451, and six silent differences between the chimeras and the PRVABC59 strain.

Attenuation characterization and vaccine development

The D2/ZKV-V vaccine candidate is evaluated for the previously established attenuation phenotypes of DENV-2 PDK-53 based vaccine candidates, which include small plaques in Vero or

LLC-MK2 cells, temperature sensitivity in Vero or LLC-MK2 cells, poor growth in mosquito C6/36 cells, mouse neuro-attenuation, and diminished mosquito midgut infection/salivary gland dissemination/transmission (Huang *et al., J. Virol.* 77:11436-11447, 2003; Huang *et al., J. Virol.* 12:7300-7310, 2005; Huang *et al., PLoS Negl Trop Dis* 7(5):e2243, 2014). Vaccine candidates are also tested in a small animal model (mouse) and non-human primate for immunogenicity and protective efficacy.

Vaccine Applications

Based on previous success in DENV and WNV vaccine development using the DENV-2 PDK-53 based chimeric virus platform, it is expected that this platform can be used successfully for the development of a live-attenuated ZIKV vaccine. A chimeric D2/ZKV can be used alone (univalent) or in combination with a live-attenuated tetravalent DENV vaccine based on the same PDK-53 backbone (pentavalent vaccine). Combined vaccination strategies using both live-attenuated and inactivated ZIKV vaccine candidates can be evaluated.

Additional Chimeric Constructs

Additional chimeric D2/ZKV are generated using alternative junction site strategies, incorporating Vero cell adaptation mutations and/or introducing additional mutations to adjust the attenuation level or enhance the fitness/genetic stability of the chimeric D2/ZKV-V.

In one example, a chimeric D2/ZKV is generated using Strategy 2 shown in FIG. 1. In Strategy 2, the prM signal sequence includes the first five amino acids from DENV-2 PDK-53 and the last 13 amino acids from a ZIKV (such as SPH2015).

In other examples, a chimeric D2/ZIKV is generated by incorporating one or more Vero adaption mutations identified from Vero serial passages of D2/ZK-P seeds. In one example, any one of the mutations listed in Table 3 is incorporated into the D2/ZK vaccine virus to improve Vero cell adaption. In other examples, any combinations of the mutations listed in Table 3 are incorporated into the D2/ZKV vaccine to improve growth in Vero cells.

In another example, the chimeric D2/ZKV includes an Ile to Val substitution at E23 to match the sequence of ZIKV strains PRVABC59 and R103451.

Example 2: Chimeric D2/ZK viruses for vaccine development

This example describes the generation and characterization of 10 additional chimeric DENV-2/ZKV (D2/ZK) constructs referred to as D2/ZKV-V2, P3.1, V3.1, V3.2, V3, P4, V4, P5 and V5 (see Table 6).

Based on the mutations identified from Vero cell adapted D2/ZKV-P described in Example 1 (Table 5), some of the mutations were incorporated into the chimeric construct to determine important mutations for Vero cell adaption and stability. Among the 7 mutations, 4 of them (E-465, E-484, E-493, and NS22A-99) were identified for Vero-cell-fitness enhancement of the chimeric D2/ZK viruses (Table 3). Interestingly, all three E mutations were within the transmembrane domain of the E protein, which would not affect the antigenic property of the E protein. In addition, two of them (E-484 and E-493) were near the chimeric E/NS1 junction site, suggesting the mutations might compensate the defect caused by the chimerization between the E proteins of ZIKV and DENV-2 (FIG. 1 shows an example with E-484T and E-493F in D2/ZKV). Table 3 also includes the NS4A-23 mutation that was identified during study of D2/ZKV-V2 construct. Without any Vero-cell-adapted mutations, both D2/ZK-P and D2/ZK-V are incompetent to replicate in Vero cells. It is possible to recover viable D2/ZK-P virus (on D2 16681 backbone) from C6/36 cells, but not D2/ZK-V virus (on D2 PDK-53 backbone).

Ten additional chimeric viruses that are viable in Vero cells were generated. However, each chimeric virus has a different plaque phenotype, growth efficiency and genetic stability. A brief summary of each chimeric virus is provided below:

D2/ZKV-V2: This chimeric virus construct contains E-465R and E-484T mutations, but is still not stable in Vero cells; after 2 passages in Vero cells it acquired multiple mutations. After plaque purification of five clonal V2 viruses, it was determined that three of them had acquired a NS4A-23 N mutation, including one that also acquired a NS2A-99 N mutation, and another clone acquired the E-493 F mutation. Based on these results, the NS4A-23 mutations was included in later constructs. The NS2A-99 and E-493 F mutations were previously identified in the original D2/ZKV-P adapted to Vero cells, which suggests these two mutations play important roles in Vero cell adaption.

D2/ZKV-P3.1 and V3.1: Both of these chimeric viruses were engineered with 3 of the 5 mutations described in Table 3 above – E-493, NS2A-99, and NS4A-23. Both viruses replicated well and produced uniform and clear plaques in Vero cells, however upon further sequencing analysis, it was determined that both recovered viruses acquired the E-465 R mutation. Although the P3.1 and V3.1 viruses were not sufficiently stable for vaccine development, based on the plaque phenotypes and the consistency of the E-465 mutation in both chimeras, it was hypothesized that chimeras containing the E-465R, E-493F, NS2A-99N, and NS4A-23N (RFNN) mutations would be stable for Vero cell amplification and would be potential live-attenuated ZIKV vaccine candidates. The nucleotide and amino acid sequences of the P-RFNN and V-RFNN chimeric viruses are set forth herein as SEQ ID NOs: 15-18.

D2/ZKV-V3.2: This chimeric virus, which has the E-465, E-484, and NS4A-23 mutations, was not stable enough for Vero cell growth. The virus grew poorly in Vero cells, and produced fuzzy pinpoint size plaques.

D2/ZKV-V3: This virus contains one additional NS2A-99 mutation relative to the V3.2 virus, but is still not stable enough for Vero cell growth. After 2-3 passages in Vero cells, the chimera acquired an additional E-493 F mutation (as with V2 described above).

D2/ZKV-P4 and V4: These chimeric viruses were constructed with the E-465, E-484, E-493, and NS2A-99 mutations. They grew well and produced uniform plaques and are therefore candidates for live-attenuated vaccine development. The nucleotide and amino acid sequences of the P4 and V4 chimeric viruses are set forth herein as SEQ ID NOs: 19-22.

D2/ZKV-P5 and V5: These two chimeras, on either the 16681 backbone (P5) or PDK-53 backbone (V5), grew very well in Vero cells and produced plaques that were larger than all other constructs described above. Growth kinetics studies of these two viruses were performed in Vero cells, which confirmed that both viruses replicate efficiently in Vero cells. The V5 virus replicated somewhat slower than the P5 virus, which is consistent with the slower growth of the D2 PDK-53 backbone virus than the D2 16681 virus. Plaque size of both P5 and V5 were smaller than that of the wt ZIKV, suggesting both replicate less efficiently than the wt ZIKV in Vero cells. However, the plaques of V5 virus were slightly smaller than those of P5 virus, which agrees with previous observation that chimeric virus based on the PDK-53 produces smaller plaques than chimeric viruses based on wt D2 16681 backbone. The V5 virus also retained the attenuation phenotype as its D2 PDK-53 backbone in C6/36 cells. In addition, a mouse study was conducted to measure the neurovirulence levels of the D2/ZK-P5 and -V5 viruses (see below). The results indicated both P5 and V5 are fully attenuated for newborn mice. Based on these results, these two viruses are good candidates for live-attenuated vaccine development. The nucleotide and amino acid sequences of the P5 and V5 chimeric viruses are set forth herein as SEQ ID NOs: 23-26.

Table 6. Chimeric DEN-2/Zika viruses

Virus Names		**Nt and AA position based on chimeric D2/ZIKV genome**						**Growth in Vero cells**
(D2/ZIKV-backbone)*		Nt	2348 (A-G)	2405 (T-C)	2431 (A-T)	3819 (G-T)	6487 (G-A)	
16681	PDK-53	AA**	E465 (Q-R)	E484 (I-T)	E493 (I-F)	NS2A-99 (K-N)	NS4A-23 (D-N)	
P	V	wt*	Q	I	I	K	D	Not viable in Vero

Virus Names		Nt and AA position based on chimeric D2/ZIKV genome					Growth in Vero cells
(D2/ZIKV-backbone)*		Nt	2348 (A-G)	2405 (T-C)	2431 (A-T)	3819 (G-T)	6487 (G-A)	
16681	PDK-53	AA**	E465 (Q-R)	E484 (I-T)	E493 (I-F)	NS2A-99 (K-N)	NS4A-23 (D-N)	
	V2		**R**	**T**	I	K	D	Not stable, acquired multiple mutations
P3.1	V3.1		Q	I	**F**	**N**	**N**	Not stable acquired E465R
P-RFNN	V-RFNN		**R**	I	**F**	**N**	**N**	Evolved from 3.1 viruses; potential candidates
-	V3.2		**R**	**T**	I	K	**N**	Poor growth, pinpoint plaques
P3	V3		**R**	**T**	I	**N**	**N**	Not stable, evolved to P5 or V5 like viruses
P4	V4		**R**	**T**	**F**	**N**	D	Uniform plaques; potential candidates
P5	V5		**R**	**T**	**F**	**N**	**N**	Uniform plaques; potential candidates

Underline indicates potential live-attenuated ZIKV vaccine candidates

*Chimeric viruses based on D2 16681 parental virus named as D2/ZIKV-P#; Chimeras based on D2 PDK-53 vaccine virus named as D2/ZIKV-V#

**Mutations in bold

Attenuation characterization

Vaccine candidate viruses were characterized for the previously established attenuation phenotypes of DENV-2 PDK-53. These include small plaques in Vero or LLC-MK2 cells, temperature sensitivity in Vero or LLC-MK2 cells, poor growth in mosquito C6/36 cells, mouse neuro-attenuation, and diminished mosquito midgut infection/salivary gland dissemination/transmission.

Plaque size in Vero cells: The four chimeras V4, V5, P4 and P5 all exhibited smaller plaques than the wt ZIKV. Plaques of the chimeras in the P backbone were somewhat larger than

plaques produced from their counterpart V chimeras. In addition, plaques of P5 and V5 were larger than the P4 and V4 versions. The Vero-adapted P-FRNN and V-RFNN (evolved from P3.1 and V3.1) also showed similarly small plaques as P4 and V4 viruses.

Growth kinetics in Vero cells: Viral growth of P5 and V5 viruses was tested in Vero cells. As shown in FIG. 2A, both viruses replicated efficiently in Vero cells, but V5 was somewhat slower than P5, and both were slower than wt ZIKV.

Growth Kinetics in C6/36 cells: Growth kinetics of the P5 and V5 viruses was evaluated in C6/36 cells. The results are shown in FIG. 2B. Although P5 still grew efficiently in C6/36 cells, the growth was significantly lower than the wt ZIKV PRVABC59, and somewhat lower than its wt DENV-2 16681 backbone virus. The V5 virus retained the attenuation phenotype as its DENV-2 PDK-53 vaccine backbone virus in the C6/36 cells. It is believed that most V viruses will retain the crippled-replication attenuation phenotype of their backbone DENV-2 PDK-53 vaccine, while most P viruses may still replicate well in C6/36 cells. Although the P viruses may not have the *in vitro* attenuation phenotype in C6/36 cells, they may still be attenuated in whole live mosquitoes due to the chimerization between DENV-2 and ZIKV.

Mouse neurovirulence: P5 and V5 viruses were compared with the wt ZIKA and D2 16681 viruses in newborn ICR mice. An initial test in 7-day old ICR (CD-1) mice showed that the newborn mice at 7-days old already developed resistance to wt ZIKV and D2 16681 intracranial challenge. A neurovirulence challenge ICR mouse model for D2 16681 virus using 0-1 day old mice was previously established. Therefore, in the second experiment, 0-2 day old newborn ICR mice were used. Litters of newborn mice (day 0-2 after birth) were grouped in 9-10 newborns/litter inoculated intracranially with 10^4 pfu of virus. All 29 baby mice (3 litters) inoculated with wt ZIKV died with 8.4 ± 0.49 average survival days (ASD ±SD), and all 20 mice (2 litters) inoculated with D2 16681 also died with ASD of 9.05±0.59 (FIG. 3). Strikingly, none of the 30 mice inoculated with either P5 or V5 viruses became ill, and there was no weight loss throughout the experiment.

Immunogenicity and protective efficacy of the vaccine candidates:

Studies are conducted to evaluate the immunogenicity and protective efficacy of the vaccine candidates. Mice are administered either single or double dose vaccine schedules, and are challenged with a lethal dose of wt ZIKV PRVABC59 a month after final immunization. It is expected that the chimeric viruses will induce strong anti-ZIKV neutralization antibodies and protect mice from lethal challenge.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A nucleic acid chimera comprising:

a first nucleic acid molecule comprising a 5' non-coding region, a nucleic acid encoding non-structural proteins and a capsid (C) protein, and a 3' non-coding region, each from a dengue serotype 2 virus (DENV-2) strain genome, wherein the C protein comprises a portion of a premembrane (prM) signal sequence from the DENV-2 genome and a portion of a prM signal sequence from a Zika virus (ZIKV) genome; and

a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding a prM protein and at least a portion of an envelope (E) protein from the ZIKV genome.

2. The nucleic acid chimera of claim 1, wherein the DENV-2 strain genome is an attenuated DENV-2 strain genome.

3. The nucleic acid chimera of claim 2, wherein the attenuated DENV-2 genome comprises a mutation in the 5' non-coding region at nucleotide position 57.

4. The nucleic acid chimera of claim 2 or claim 3, wherein the attenuated DENV-2 genome comprises a mutation at nucleotide position 2579 that results in the presence of an aspartate at amino acid residue 53 of the NS1 protein.

5. The nucleic acid chimera of any one of claims 2-4, wherein the attenuated DENV-2 genome comprises a mutation at nucleotide position 5270 that results in the presence of a valine at amino acid residue 250 of the NS3 protein.

6. The nucleic acid chimera of any one of claims 2-5, wherein the attenuated DENV-2 is strain PDK-53.

7. The nucleic acid chimera of claim 1, wherein the DENV-2 strain genome is a wild-type DENV-2 strain genome.

8. The nucleic acid chimera of claim 7, wherein the wild-type DENV-2 is strain 16681.

9. The nucleic acid chimera of any one of claims 1-8, wherein the ZIKV is strain SPH2015, PRVABC59 or R103451.

10. The nucleic acid chimera of any one of claims 1-9, wherein the portion of the prM signal sequence from the DENV-2 genome comprises the first three amino acids of the DENV-2 prM signal sequence and the portion of the prM signal sequence from the ZIKV genome comprises the last 15 amino acids of the ZIKV prM signal sequence.

11. The nucleic acid chimera of claim 10, wherein the first three amino acids of the DENV-2 prM signal sequence comprises SAG (amino acids 101-103 of SEQ ID NO: 12).

12. The nucleic acid chimera of claim 10 or claim 11, wherein the last 15 amino acids of the ZIKV prM signal sequence comprises amino acids 108-122 of SEQ ID NO: 8.

13. The nucleic acid chimera of any one of claims 1-9, wherein the portion of the prM signal sequence from the DENV-2 genome comprises the first five amino acids of the DENV-2 prM signal sequence and the portion of the prM signal sequence from the ZIKV genome comprises the last 13 amino acids of the ZIKV prM signal sequence.

14. The nucleic acid chimera of claim 13, wherein the first five amino acids of the DENV-2 prM signal sequence comprises amino acids 101-105 of SEQ ID NO: 12.

15. The nucleic acid chimera of claim 13 or claim 14, wherein the last 13 amino acids of the ZIKV prM signal sequence comprises amino acids 110-122 of SEQ ID NO: 8.

16. The nucleic acid chimera of any one of claims 1-15, wherein a portion of the E protein is from the DENV-2 genome.

17. The nucleic acid chimera of claim 16, wherein the portion of the E protein from the DENV-2 genome comprises the last 14 amino acids of the DENV-2 E protein.

18. The nucleic acid chimera of claim 17, wherein the last 14 amino acids of the DENV-2 E protein comprises amino acids 777-790 of SEQ ID NO: 2.

19. The nucleic acid chimera of any one of claims 1-18, further comprising at least one Vero cell adaptation mutation.

20. The nucleic acid chimera of claim 19, wherein the at least one mutation results in:
a glutamine to arginine substitution at residue 465 of the E protein;
an isoleucine to threonine substitution at residue 484 of the E protein;
an isoleucine to phenylalanine substitution at residue 493 of the E protein;
a lysine to asparagine substitution at residue 99 of the NS2A protein; or
an aspartic acid to asparagine substitution at residue 23 of the NS4A protein.

21. The nucleic acid chimera of claim 19 or claim 20, comprising four Vero cell adaptation mutations, wherein the mutations result in:
a glutamine to arginine substitution at residue 465 of the E protein;
an isoleucine to phenylalanine substitution at residue 493 of the E protein;
a lysine to asparagine substitution at residue 99 of the NS2A protein; and
an aspartic acid to asparagine substitution at residue 23 of the NS4A protein.

22. The nucleic acid chimera of claim 19 or claim 20, comprising four Vero cell adaptation mutations, wherein the mutations result in:
a glutamine to arginine substitution at residue 465 of the E protein;
an isoleucine to threonine substitution at residue 484 of the E protein;
an isoleucine to phenylalanine substitution at residue 493 of the E protein; and
a lysine to asparagine substitution at residue 99 of the NS2A protein.

23. The nucleic acid chimera of claim 19 or claim 20, comprising five Vero cell adaptation mutations, wherein the mutations result in:
a glutamine to arginine substitution at residue 465 of the E protein;
an isoleucine to threonine substitution at residue 484 of the E protein;
an isoleucine to phenylalanine substitution at residue 493 of the E protein;
a lysine to asparagine substitution at residue 99 of the NS2A protein; and
an aspartic acid to asparagine substitution at residue 23 of the NS4A protein.

24. The nucleic acid chimera of any one of claims 1-23, comprising a nucleic acid sequence at least 95% identical to SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23 or SEQ ID NO: 25.

25. The nucleic acid chimera of claim 19, comprising the nucleic acid sequence of SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23 or SEQ ID NO: 25.

26. The nucleic acid chimera of any one of claims 1-25, wherein the nucleic acid chimera encodes an amino acid sequence at least 95% identical to SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26.

27. The nucleic acid chimera of claim 26, wherein the nucleic acid chimera encodes the amino acid sequence of SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26.

28. A chimeric flavivirus comprising the nucleic acid chimera of any one of claims 1-27.

29. An immunogenic composition comprising the chimeric flavivirus of claim 28 and a pharmaceutically acceptable carrier.

30. The immunogenic composition of claim 29, further comprising one or more adjuvants.

31. A method of eliciting an immune response against Zika virus (ZIKV) in a subject, comprising administering to the subject the chimeric flavivirus of claim 28, or the immunogenic composition of claim 29 or claim 30.

32. The method of claim 31, comprising administering to the subject one to five doses of the chimeric flavivirus or the immunogenic composition.

33. The method of claim 31 or claim 32, further comprising administering one or more adjuvants to the subject.

34. The method of any one of claims 31-33, further comprising administering an inactivated Zika virus vaccine.

35. The method of any one of claims 31-34, wherein the subject is a human.

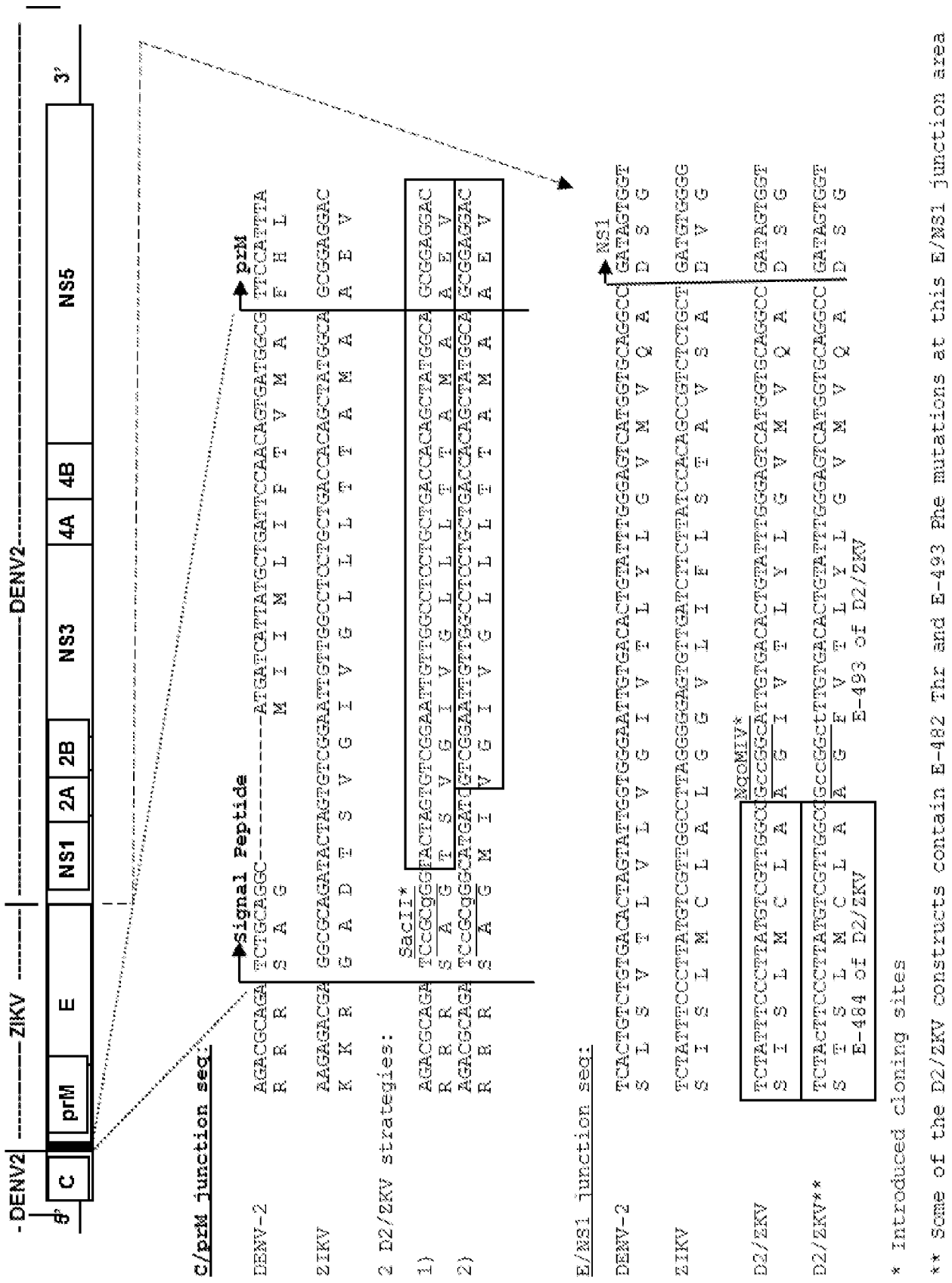

-DENV2
ZIKV
DENV2
5'
C
prM
E
NS1
2A
2B
NS3
4A
4B
NS5
3'
C/prM junction seq:
Signal Peptide
prM
DENV-2
ZIKV
2 D2/ZKV strategies:
1)
2)
SacII*
E/NS1 junction seq:
NS1
DENV-2
ZIKV
D2/ZKV
D2/ZKV**
MgoMIV*
* Introduced cloning sites
** Some of the D2/ZKV constructs contain E-482 Thr and E-493 Phe mutations at this E/NS1 junction area

FIG. 1

FIG. 2B

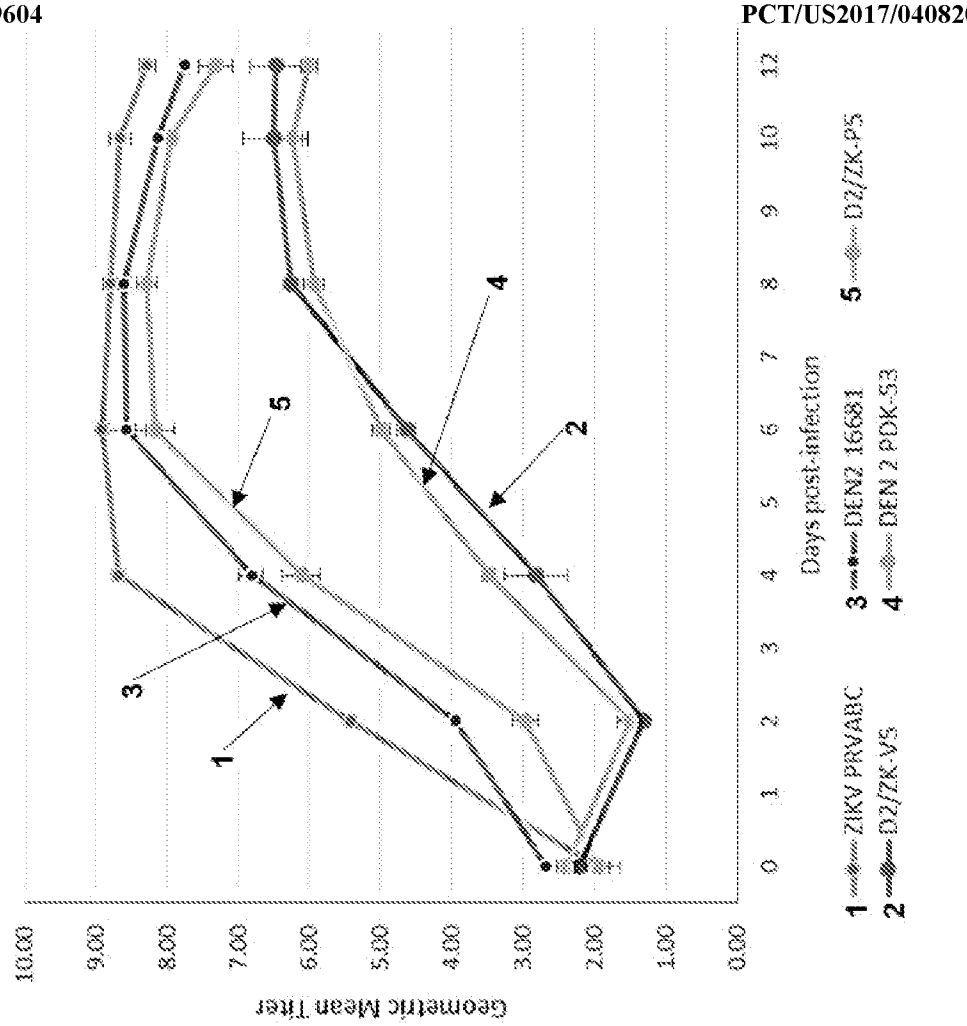

C6/36 Growth Curve
Geometric Mean Titer
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0 1 2 3 4 5 6 7 8 9 10 12
Days post-infection
1 ZIKV PRVABC
2 D2/ZK-V5
3 DEN2 16681
4 DEN 2 PDK-53
5 D2/ZK-P5
1
2
3
4
5

FIG. 2A

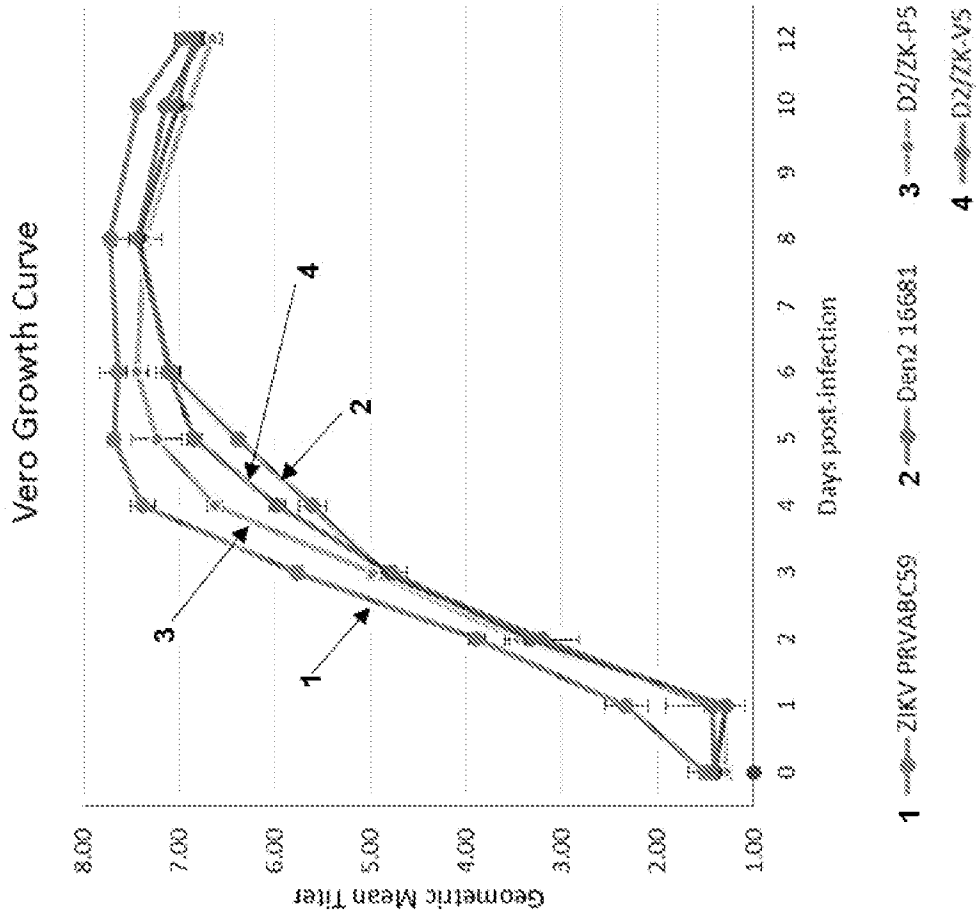
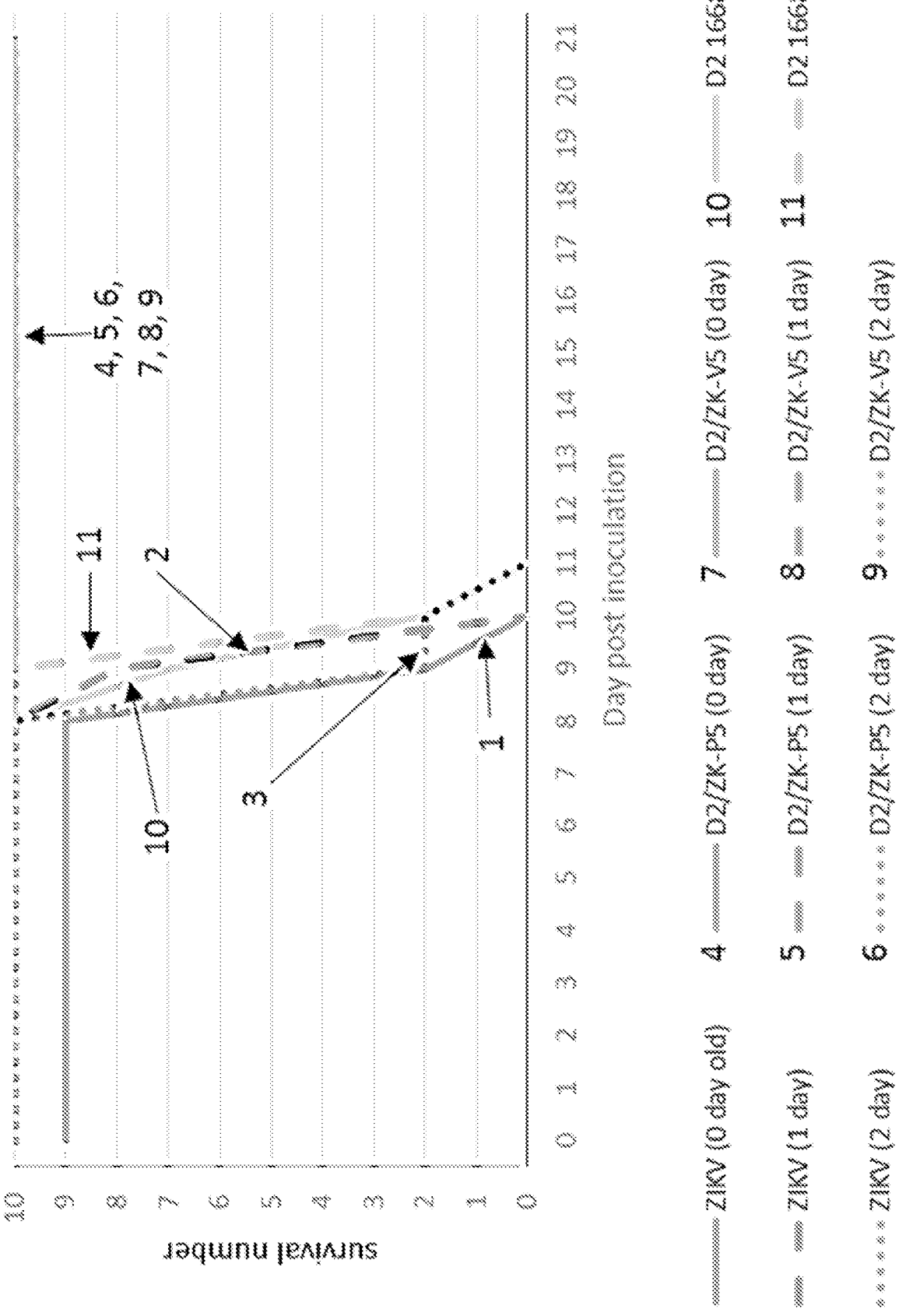

Vero Growth Curve
Geometric Mean Titer
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0 1 2 3 4 5 6 7 8 9 10 12
Days post-infection
1 ZIKV PRVA8C59
2 Den2 16681
3 D2/ZK-P5
4 D2/ZK-V5
1
2
3
4

FIG. 3


survival number
Day post inoculation
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
10 9 8 7 6 5 4 3 2 1 0
4, 5, 6, 7, 8, 9
11
2
10
3
1
1 ZIKV (0 day old)
2 ZIKV (1 day)
3 ZIKV (2 day)
4 D2/ZK-P5 (0 day)
5 D2/ZK-P5 (1 day)
6 D2/ZK-P5 (2 day)
7 D2/ZK-V5 (0 day)
8 D2/ZK-V5 (1 day)
9 D2/ZK-V5 (2 day)
10 D2 16681 (0 day)
11 D2 16681 (1 day)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/040820

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/005 C12N7/00 A61K39/12
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, IBM-TDB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUANG C Y-H ET AL: "Chimeric dengue 2 PDK-53/West Nile NY99 viruses retain the phenotypic attenuation markers of the candidate PDK-53 vaccine virus and protect mice against lethal challenge with West Nile virus", JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 79, no. 12, 1 June 2005 (2005-06-01), pages 7300-7310, XP002544716, ISSN: 0022-538X, DOI: 10.1128/JVI.79.12.7300-7310.2005 the whole document in particular abstract; page 7300 col. 2 and page 7301 col. 1; Fig. 1;page 7302 col. 1 first full paragraph and page 7308 col. 1 par. 1 ----- -/--	1-35

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2017	02/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dumont, Elisabeth

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/040820

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/60847 A2 (US HEALTH [US]; KINNEY RICHARD M [US]; KINNEY CLAIRE Y H [US]; BUTRAPE) 23 August 2001 (2001-08-23) the whole document page 4, line 15 - page 8, line 30; figure 1; example 6 page 70; table 1	1-35
Y	CHAO SHAN ET AL: "Zika Virus: Diagnosis, Therapeutics, and Vaccine", ACS INFECTIOUS DISEASES, vol. 2, no. 3, 11 March 2016 (2016-03-11), pages 170-172, XP055276341, ISSN: 2373-8227, DOI: 10.1021/acsinfecdis.6b00030 the whole document in particular page 171, col. 2, "vaccines"	1-35
Y	J. COHEN: "The race for a Zika vaccine is on", SCIENCE, vol. 351, no. 6273, 5 February 2016 (2016-02-05), pages 543-544, XP055360676, ISSN: 0036-8075, DOI: 10.1126/science.351.6273.543 the whole document in particular page 543, col. 2 par. 1	1-35
A	BARRETT P NOEL ET AL: "Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines", EXPERT REVIEW OF VACC, EXPERT REVIEWS LTD, GB, vol. 8, no. 5, 1 May 2009 (2009-05-01), pages 607-618, XP009128183, ISSN: 1744-8395, DOI: 10.1586/ERV.09.19 the whole document in particular abstract	1-35
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-/--

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/040820

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	CHAO SHAN ET AL: "A live-attenuated Zika virus vaccine candidate induces sterilizing immunity in mouse models", NATURE MEDICINE, vol. 23, no. 6, 10 April 2017 (2017-04-10), pages 763-767, XP055407797, ISSN: 1078-8956, DOI: 10.1038/nm.4322 the whole document in particular abstract and page 4, col. 2, last full paragraph -----	1-35

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/040820

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